Bima Journal of Science and Technology, Vol. 7 (2) June, 2023 ISSN: 2536-6041

DOI: 10.56892/bima.v7i2.437

DIAGNOSTIC MODELS OF 2-LEVEL FRACTIONAL FACTORIAL DESIGN OF IMMOBILIZED *Candida rugosa* LIPASE BIODIESEL PRODUCTION

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ABSTRACT

This study examined the strength of the 2 level fractional factorial design in screening optimum biodiesel production parameters by examining the significant variables and variables interactions as displayed in the diagnostic models generated by the design expert software. The displayed experimental runs models (ANOVA and Diagnostic plots) and variables interaction of the models is and indication that all the biodiesel parameters investigated via 2-level fractional factorial design are fit and their significant factors were screened accordingly. This study supports the success of the previously published screening of significant factors of biodiesel production.

Keywords: Diagnostic Models, 2-Level Fractional Factorial Design, Immobilized *Candida rugosa* Lipase, Biodiesel Production

INTRODUCTION

Biodiesel also known as fatty acid methyl ester has become a new paradigm of clean burning fuel that can be adopted in place of the conventional diesel fuel. This is due to its biodegradability nature, non-tooxicity and low emission profile (Marchetti *et a*l., 2007), thus making it fit as a fuel for the modern times perpetually challenged by global and environmental energy emission reduction. Biodiesel is famed as a carbon neutral fuel, this is so because the carbon in the exhaust was originally fixed from the atmosphere (Dizge and Keskinler, 2008). The major challenge in biodiesel production is the price of the raw materials (Shao *et al*., 2008), thus gearing researchers to look for suitable materials and methods to arrive at larger scale biodiesel production.

The traditional methods of performing experiments to optimize a biodiesel production is by varying a factor at a time. One factor at a time (OFAT) experiment is hectic and laborious in practice (Rao *et al*., 2008). Above all it overlook the interactions between the factors (Rao *et al*., 2008) and does not reveal some statistical index needed for acceptance and rejection of variables. OFAT also do not provide models of variables interactions. This limitations my undermine biodiesel optimum outcome. Nowadays the introduction of experimental design via fractional factorial design and ultimately to RSM has prove easier and more reliable in screening and optimization of the factors (Rodrigues *et al.*, 2009). The duo revels statistical index needed for acceptance and rejection of variables. Hence the need to support our previous research (Deba *et al*., 2018) with the statistical index for fitness of models, and the diagnostics models of variables interactions.

In our previous work, we presented only the result of 2 level fractional factorial screening of biodiesel parameters, regression equations

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and the productions obtained. In this work we are supporting our previous data with diagnostic models and how it agrees with the design expert statistical software to allow further factors optimization.

MATERIALS AND METHODS

The waste coking oil (WCO) sample was collected from "KB Delight" restaurant, at Taman Universiti, Johor, Johor Bahru, Malaysia and characterized according to American Oil Chemist Society (AOCS method Ca 5a-40, 2009). Lipase (3.1.1.3) (Type 1176 U/mg) from *Candida rugosa* was a commercial enzyme purchased from Sigma Aldrich. Lipase preparation as well as immobilization, and enzymatic transeterification process were reported in our previous study (Zain *et al*., 2010; Deba *et al*. 2018).

Phosphate Buffer and *Candida rugosa* Lipase Preparation

A 10 mL of Potassium Phosphate Buffer (PPB) was prepared to dissolve lipase obtained from *Candida rugosa* lipase. 34.015 g of KH_2PO4 was dissolved in 500 mL of distilled water and stirred using a magnetic stirrer. 43.545 g of K_2HPO4 was dissolved in 500 mL of distilled water and stirred using magnetic stirrer until completely dissolved. 0.4 mL and 1.6 mL from KH_2PO4 and K_2HPO4 respectively were mixed together, then 8.0 mL distilled water was added to obtain 10 mL PPB solution with pH of 7.5 and the solution was sterilized. 0.5 g of lipase was added into the PPB solution. The solution of lipase enzyme was filtered using 0.45 µm nylon syringe filter and kept at 4 °C.

Lipase Immobilization Procedure

A 10 mL of prepared lipase solution was added to 90 mL PVA (12% w/v) and sodium alginate (1% w/v) solution. The mixture was dropped inside a solution of boric acid (5% w/v) and calcium chloride (2% w/v) by using a syringe (by using a rotary pump) in order to form beads and then stirred for 30 mins. The beads were allowed to stay in the latter solution for 24 hours at 4 °C. The beads were stirred in 10 % boric acid solution for 30 mins and then with 0.5 M sodium sulfate solution for another 30 mins and kept at 4 °C in sterile distilled water. Figure 2.1 shows the PVA-alginate sulfate bead production

Figure 1: Beads production by rotary pump

Enzymatic Transesterification

The stepwise methanolysis of WCO was conducted. A ratio of WCO to methanol used involving; 1:1 and 1:6, enzyme concentration of 20U and 60U and water content of 0.5 and 2.5w/v. 3molar of methanol was added to WCO oil in 3 steps and stirred at 100 and 200 rpm for 24 hours at 30 °C and 50 °C. The mixture was centrifuged at 13000 rpm for 30 minutes. The supanatant was used for of fatty acid methyl ester analysis in Gas Chromatography (Perkin Elmer Autosystem) (Charuchinda *et al.,* 2011).

Experimental Design

A Design expert software, version 6.0.4 using 2-level fractional factorial design was used. A total of 32 treatment combinations were obtained using the variables in Table 1 as generated by the design matrix (See Deba *et al.*, 2018). The Experiment was carried out as per the design matrix in a 250 ml Erlenmeyer flasks with varying amount of the variables.

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Table 1: Variables in the Experimental Design

Variables	Alphabetical codes	Symbols	Coded levels		
			-1	0	+1
Oil: Methanol ratio	A	X_1	1:1	-	1:6
Temperature (°C)	B	X_2	30	-	50
H_2O content (v/v)	C	X_3	0.5	-	2.5
Agitation (rpm)	D	X_4	100	-	200
Enzyme concentration (U)	E	X_5	20	-	60

RESULTS AND DISCUSSION

A recap of the biodiesel (palmitic acid methyl ester, linoleic acid methyl esters and oleic acid methyl esters) produced from our previous study (Deba *et al.*, 2018) is in Table 2. The production obtained would guide the discussion in this study.

Table 2: Fatty acid methyl ester obtained from 2 level fractional factorial design

Methyl ester	Parameters	Conformation Experimental production	Predicted Production by the Design expert	Actual experiment production
Palmitic acid	Oil to methanol ratio of 1:6, Temperature 48.79 °C, H_2O content 2.50 (v/v), Agitation 200 rpm and Enzyme concentration of 59.95 (U)	1.201 g/L	1.12915 g/L with 84.8 % desirability index	1.32 g/L
Linoleic acid	Oil to methanol ratio of 1:6, Temperature 49.62 °C, H_2O content 2.50 (v/v), Agitation 200 rpm and Enzyme concentration of 60 (U)	1.2911 g/L	1.20514 g/L with a desirability of 85.2 %	1.41 g/L
Oleic acid	Oil to methanol ratio of 1:6, Temperature 50 °C, H_2O content 2.42 (v/v), Agitation 199.81 rpm and Enzyme concentration of 60 (U)	4.1912 g/L	4.11033 g/L desirability of 97.5 %	4.16 g/L

Analysis of Variance (ANOVA) for Biodiesel Production

The Analysis of Variance (ANOVA) results for Biodiesel (palmitic, linoleic and oleic acid methyl esters) from the response surface linear model generated by the design expert software were reported in Table 3. The p-value symbolizes the significance of the model because they are essential in understanding variables interactions (Haaland, 1989). The probability values less than 0.0500 is indicating that the model is significant (Haaland, 1989), thus all the variables in the ANOVA table with less than 0.0500 p-values are significant in biodiesel production and vice versa.

Bima Journal of Science and Technology, Vol. 7 (2) June, 2023 ISSN: 2536-6041

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Table 3: ANOVA

Palmitic acid methyl ester			**Linoleic acid methyl ester**			**Oleic acid methyl ester**		
Model	F value	Prob <F	Model	F value	Prob <F	Model	F value	Prob <F
A	50.9	<0.0001	A	35.36	<0.0001	A	1067.77	<0.0001
B	81.26	<0.0001	B	45.19	<0.0001	B	1169.34	<0.0001
D	87.11	<0.0001	D	26.88	<0.0001	D	1295.67	<0.0001
AB	63.9	<0.0001	E	5.07	0.0379	E	24.03	0.0001
AD	97.09	<0.0001	AB	31.26	<0.0001	AB	1136.21	<0.0001
BD	59.75	<0.0001	AD	47.19	<0.0001	AD	1297.00	<0.0001
CE	77.79	<0.0001	BD	41.39	<0.0001	BC	20.18	<0.0001
-	-	-	CE	55.94	<0.0001	BD	1170.29	<0.0001
			-	-	-	CE	1040.57	<0.0001
Lack of fit	1.31	0.2691	Lack of fit	0.41	0.5299	Lack of fit	4.07	0.0617
R^2 = 0.9692,			R^2= 0.9455			R^2= 0.9979		
Adj R^2=0.9438			Adj R^2=0.9006			Adj R^2=0.9962		
Pred R^2=0.8908			Pred R^2=0.8069			Pred R^2=0.9927		
STD=0.078			STD=0.12			STD=0.077		
Mean=0.24			Mean=0.23			Mean=0.74		

Oil to methanol ratio (A), temperature (B) and agitation (D) were identified as the significant parameters that influence the production of palmitic acid methyl ester (Table 2). Some other significant factors include the interaction between A & B, A & D, B &D and C &E. This interaction implies that oil to methanol molar ratio (A) is a significant factor relative to temperature (B) and agitation (D). While B & D interaction implies that temperature is a significant factor in association with agitation (D) while C & E interaction implies that the water content factor is significant in association with enzyme concentration. R^2 value of 0.9692 implies that the palmitic acid model shows the effect of the variables towards the response. Since R^2 presents a determinant of variability from the response observed values to be described by the experimental factors and interactions. Therefore, the nearer the value of R^2 to one, the more the model is better in predicting response. Thus, the predicted R^2 value of 0.8908 gives the amount of disparity in the recent model provided by the software. The obtained 0.8908 value shows a correlation linking the predicted and the experimental values of methyl palmitic production. Shao *et al*. (2008) conducted a study in producing biodiesel by immobilized *Candida rugosa* lipase in chitosan. The suggested model showed that the coefficient of determination (R^2) for the model was 92.86%. The value of R^2 thus obtained suggests a high degree of correlation between the experimental and the predicted values. In the present investigation the calculated coefficient of determination value indicates that the model could explain 89.08% of the variability in the response.

A, B, D & E were determined as significant parameters that influence the production of linoleic acid. Other interactive significant factors were AB, AD, BD and CE. The AB combination indicates that oil to methanol volume ratio (A) is significant relative to other parameters; temperature (B) and agitation (D). BD implies that temperature is a significant parameter in association with agitation (D) while CE implies that water content is only significant in association with other factors.The insignificant lack of fits indicates the fitness of the model. R^2 with value of 0.9455 implies the model ability to accurately predict the effect of the variable on the production of methyl linoleic. The predicted R^2 value of 0.8069 gives the amount of variation in the recent model provided by the software. The obtained predicted R^2 0.9006 value shows a relationship existing between the predicted and the experimental values of methyl linoleic production.

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A, B, D and E were highlighted as significant parameters that influence the production of methyl oleic (Table 3). Other significant interactive variables were AB, AD, BC, BD and CE. The AB combination indicates that oil to methanol volume ratio (A) is significant when combined with other parameters; temperature (B) and agitation (D). BD implies that temperature is a significant parameter in association with agitation (D) while CE implies that water content is significant in association with other factors. The insignificance of lack of fits revealed the fitness of the model. R^2 of 0.9979 implies that the model can show the effect of the variables towards the response. The predicted R^2 figure of 0.9927 depicts the quantity of variation in the recent model generated by the design. The obtained value shows a correlation existing between the predicted and the experimental values of methyl oleic production.

Diagnostic Plots Models

Palmitic acid methyl ester

The normal plot of residual examines the pattern of residual that follow the normal distribution. The residual plots provide information on whether the assumption had been met with the response (palmitic acid methyl ester). The normality of every data can be verified by plotting the normal probability plot of the residuals as in this case. If the data points on the plot fall in nearly straight line, the data is said to be normally distributed (Antony, 2003). Figure 2 (a) shows the points of residual disperse along the length of the straight line which is approximately linear. While the graph explains good relationship existing between the actual and the predicted data, small errors that result to deviation from the straight line could be attributed to measurement error. The outlier plots depicted in Figure 2 (b) shows the standard deviation of the actual data from the predicted as generated by the software. It clearly illustrates all the runs obtained from the experiment scattered in the border limit of ±3.5 as generated by the software. Thus, no incidence of irregular runs in the experiment which results in response departing farther than the predicted value.

The Cook's Distance plot is shown in Figure 2 (c). This figure displays the effect of every distinct experiment on the generated design. From Cook's distance figure plot it shows run 4, 7, 11, 16, 17, 18, 25, 27 and 30 situated close to the border of the plot, meanwhile run 13 and 19 positioned remote from the rest of the runs. Nevertheless, considering the outlier plot in Figure 2 (b), all the runs had not departed out of the border of ±2.5 and recognized in the model. Thus, the runs could be considered as accepted. Figure 2 (d) shows the graph of the predicted versus the actual. The straight line illustrates the predicted value from the model. The actual values are observed scattered in close proximity to the predicted line, thus confirming good prediction. Hence the model fits the experiment data accordingly.

The Leverage Plot in Figure 2 (e) illustrates the result of every run in the suitability of the model. The distant the position of each runs far away from the borderline of 1 and 0 implies none of the particular run controls fitness of the model. Studying both Leverage plot and Cook’s Distance, it is evidently shown that the model was obtained by taking into account of all the experiment's response.

Bima Journal of Science and Technology, Vol. 7 (2) June, 2023 ISSN: 2536-6041

DOI: 10.56892/bima.v7i2.437

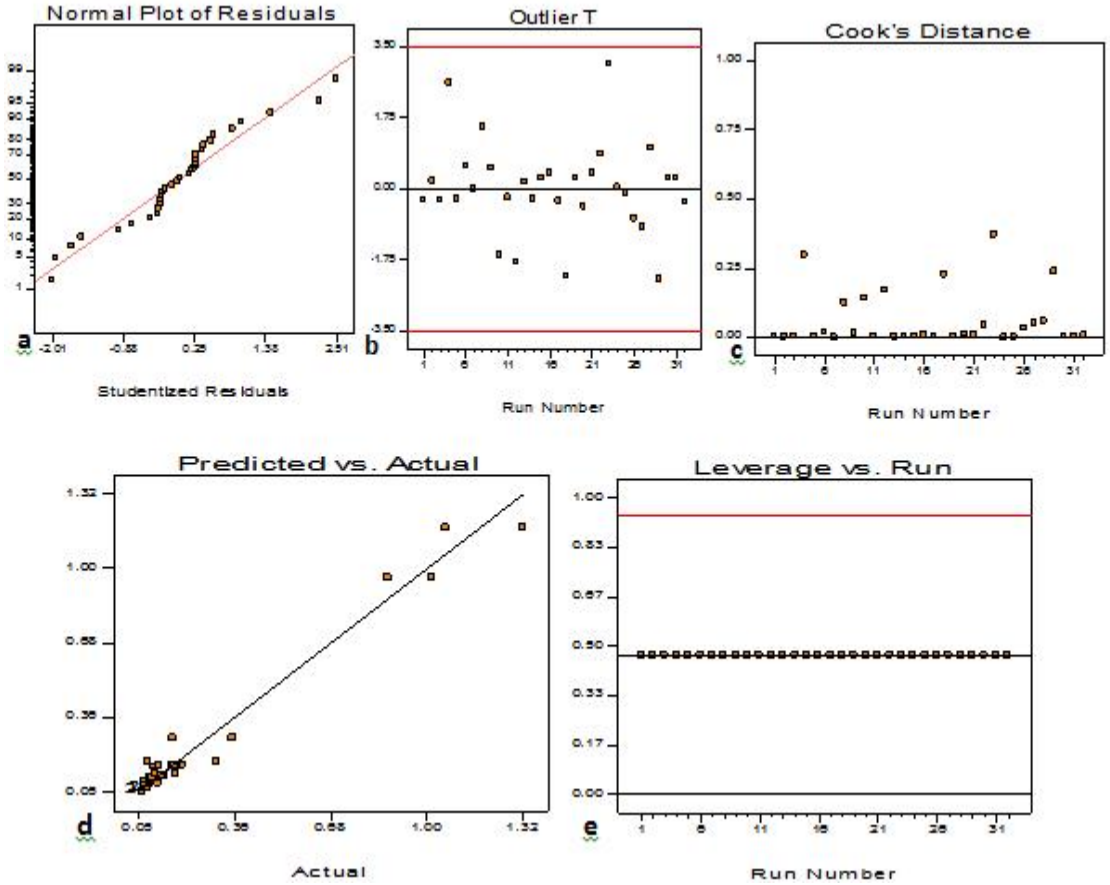

Figure 2: Diagnostic plots for Palmitic acid methyl ester

Optimum value for each parameter for methyl palmitic acid production as suggested by the software are oil to methanol volume ratio of 1:6, temperature 48.79 °C, water content 2.50 (v/v), agitation 200 rpm and enzyme concentration of 59.95 (U). These values yield predicted value of 1.12915 g/L with a desirability of 84.8 % and experimental value of 1.201 g/L (Table 2). This value is approaching the actual value however higher than the predicted value. The percentage difference between the actual 1.32 g/L in run 16 and the predicted 1.12915 g/L is 0.0944%. Moreover, low discrepancy between the actual and predicted from 0.01% to 0.10% signifies good precision and consistency of the experiments (Kuehl, 2000).

Linoleic acid methyl ester

Normal plot of residual investigates the pattern of residual that follow the normal distribution. Figure 3 (a) shows the points of residual from conducted experiments along the path of an approximately linear line. The outlier plot in Figure 3 (b) shows the standard deviation of actual and the predicted values as created by the software. It clearly shows all the runs from the experiment scattered within the limit of ±2.5. This result suggests no incidence of anomalous runs in the experiment resulting in response departing farther than the predicted.

Figure 3 (c) shows the Cook's Distance. This figure shows clearly the effect of each particular experiment on the generated design. The farther the distance of a point from other points shows that the run has less effect on the model and could be regarded as an outlier. From the Cook's distance figure plot, run 7, 13, 31, 9, 25, 4, 10, 24, 20, 5, 6, 30, 21, 15, 28 situated close to the border of the plot, meanwhile run 8 and 19 located away from the other runs. Nevertheless, the

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outlier plot in Figure 3 (b) indicates all the runs had not departed across the border of ±2.5 and was accepted in the model. As a result of that, the runs remained accepted. Figure 3 (d) shows the graph of predicted vs. actual. The straight line demonstrates the predicted value of the model. The actual point values are scattered in close proximity to the predicted line indicating a good prediction. The Leverage Plot in Figure 3 (e) reveals the effect of each one run in the suitability of the model. The position of every run is far from the borderline of 1 and 0 shows none of the single solitary run control the fitness of the model.

The optimum value for each parameter in determining linoleic was suggested by the design. Oil to methanol ratio of 6, temperature 49.62 °C, water content 2.50 (v/v), agitation of 200 rpm and enzyme concentration of 60 (U) gave predicted value of 1.20514 g/L with a desirability of 85.2 % (Table 2). The experiment conducted based on the suggested values gave a production of 1.2911 g/L. The percentage difference between the actual 1.41 g/L in run 16 and the predicted 1.20514 g/L is 0.088%. Obviously, low difference between the actual and predicted from 0.01% to 0.10% signifies good precision (Kuehl, 2000).

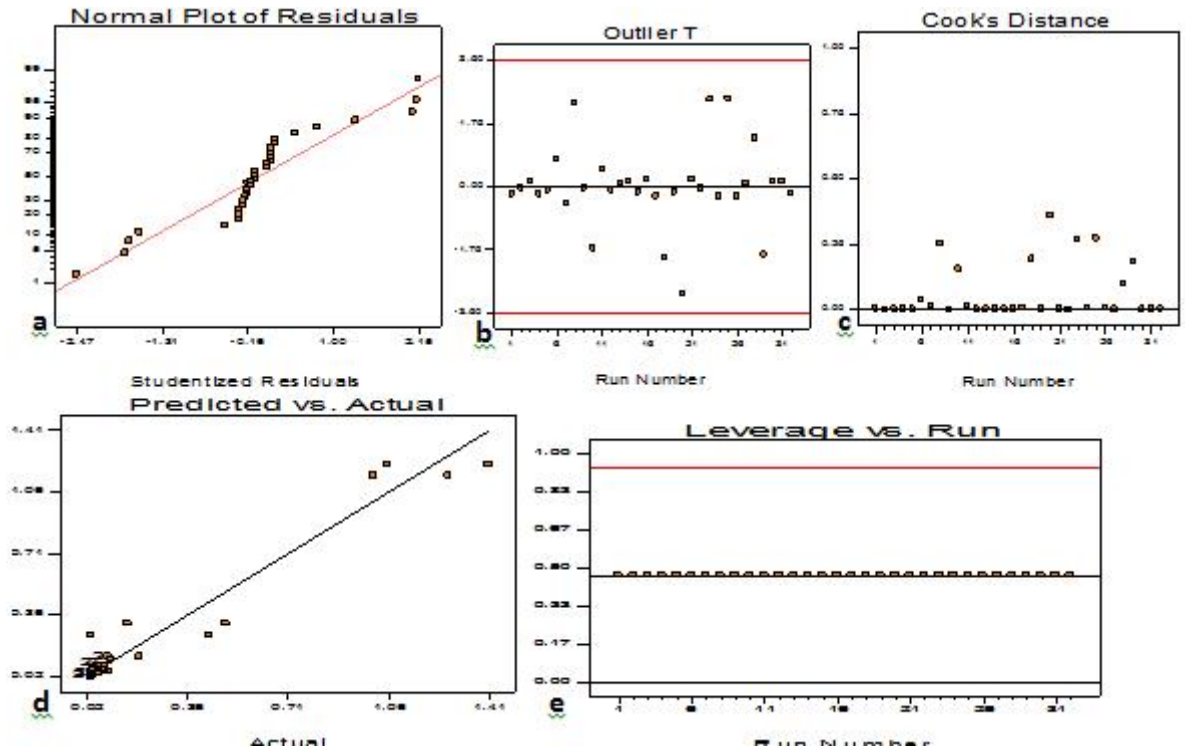

Figure 3: Diagnostic plot for Linoleac acid methyl ester

Oleic acid methyl ester

The normal plot of residual shows the outline of residual that follow the normal distribution. Figure 4 (a) point out the residual points from the perform experiments scattered along length path of the straight line which is virtually linear. Although the graph illustrates good relationship between the two values, actual and the predicted, a postulation made is that minute errors that cause points deviating from the straight line may be attributed to human errors. The outlier plot shown in figure 4 (b) shows the standard deviation of actual and the predicted values as obtained by the software. Evidently all the runs of the experiment scattered within the range of ±3.5, suggesting no incidence of unusual runs in the experiment.

Bima Journal of Science and Technology, Vol. 7 (2) June, 2023 ISSN: 2536-6041

DOI: 10.56892/bima.v7i2.437

The Cook's Distance plot can be view in the Figure 4 (c) shows the effect of every single experiment on to the design generated. The farther the distance of any point away from others shows that, the very distinct run has reduced effect on the model and could be an outlier. From the Cook's distance figure plot it is shown that run 26, 4, 20, 19 and 3 are situated close to the border of the plot, meanwhile run 8, 14 and 29 positioned far from the rest of the runs. Nevertheless, from the outlier plot in Figure 4 (b), all the runs had not departed across the limit of ±2.5 and were acknowledged in the model. Thus, the runs remained accepted. Figure 4 (d) shows the graph of predicted vs. actual value. Straight line in the graph illustrates the predicted data from the model. Values from the actual are scattered in close proximity to the predicted line, this indicates an excellent prediction. Therefore the model fits the experiment accordingly. The Leverage Plot in Figure 4 (e) illustrates the result of every run in the suitability of the model. The distant the position of every runs far away from the borderline of 1 and 0 show none of the sole particular run dictates the fitness of the model. By studying both Cook's Distance figure and Leverage plot, it is shown that the model was generated by considering of all the experiment's response.

The optimum value for each parameter in determining methyl oleic was suggested by the design. Oil to methanol volume ratio of 1:6, temperature 50 °C, water content 2.42 (v/v), agitation 199.81 rpm and enzyme concentration of 60 (U) gave predicted value of 4.11033 g/L with a desirability of 97.5 % (Table 2). The experiment conducted based on the suggested values gave a production of 4.1912 g/L. This value is approaching the actual value however higher than the predicted value. The percentage difference between the actual 4.16 g/L in run 16 and the predicted 4.11033 g/L is 0.0746 %. Low value discrepancy between the actual and predicted from 0.01% to 0.10% signified good precision and consistency of the experiments (Kuehl, 2000).

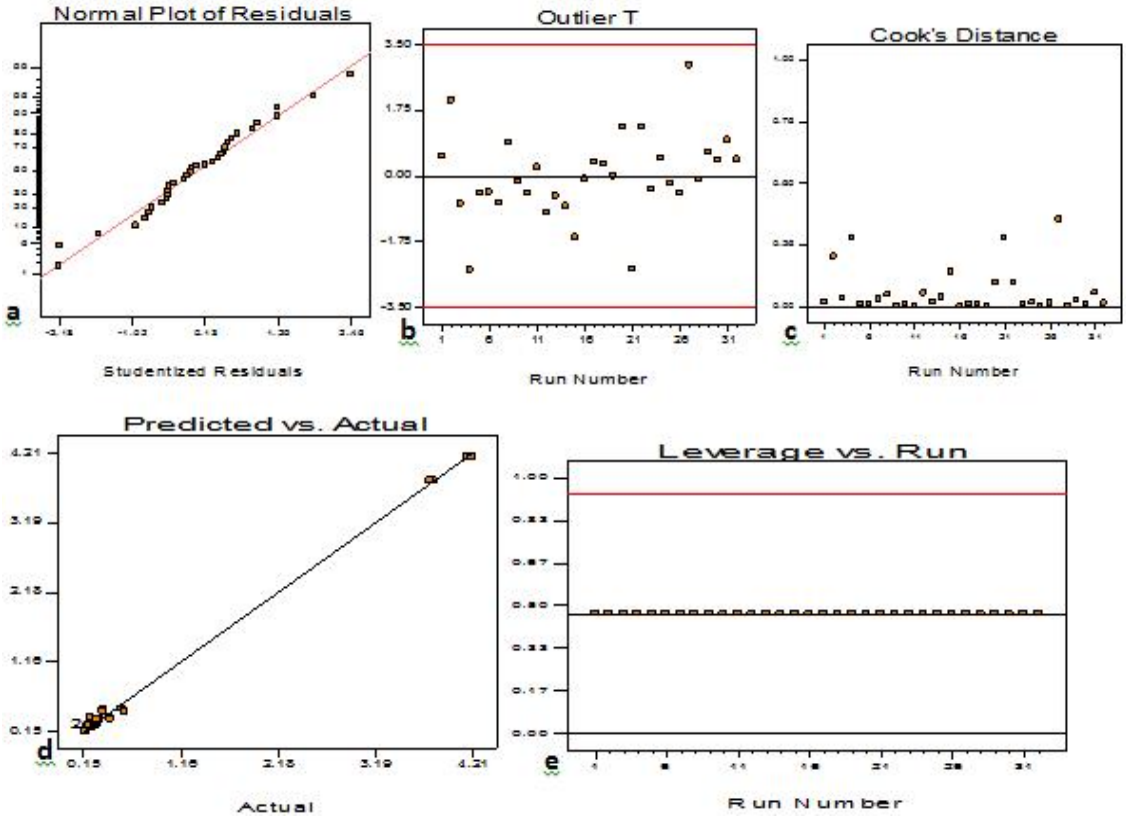

Figure 4: Diagnostic plots of Oleic acid methyl ester.

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Revisiting Figure 2-4, the plots indicated that the actual residuals ikon have scattered in closely to predicted line. This according to Li *et al.* (2007) has good predictions on the response (biodiesel productions). Therefore, all the plots in this study made respective suppositions, of adequate abilities to describe the responses (biodiesel).

CONCLUSION

The application of lipase immobilized in PVA-alginate-sulfate beads is a promising method for biodiesel production. Having provided the result of the study in our previous work we established the fitness of the design using diagnostic models in this work. Thus, this work really supported the strength of the 2 level fractional factorial design in screening optimum biodiesel production variables using the design expert software, since all the diagnostic models examined fit.

Acknowledgement

The Authors wish to express their gratitude to Universiti Teknologi Malaysia (UTM), Johor, Johor Bahru Malaysia for providing an enabling environment for the conduct of this research.

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