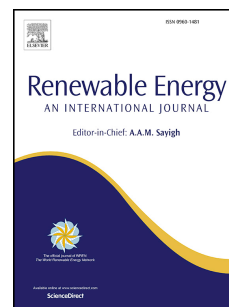


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Process optimization for biodiesel production from waste cooking oil using multi-enzyme systems through response surface methodology

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Abstract:

Lipase from *Rhizomucor miehei* (RML) and lipase B from *Candida antarctica* (CALB) were covalently immobilized onto epoxy-functionalized silica. In this study, we developed a multi-enzyme system to produce biodiesel with waste cooking oil and methanol. To increase the biodiesel production yield, a mixture of 1,3-specific lipase (RML) and nonspecific lipase (CALB) was used. Response Surface Methodology (RSM) and a central composite rotatable design (CCRD) was used to study the effects of four factors, CALB:RML ratio, ratio of *t*-butanol to oil (wt.%), water adsorbent content (wt.%) and reaction time on the fatty acid methyl esters (FAME) yield. A quadratic polynomial equation was obtained for methanolysis reaction by multiple regression analysis. The optimum combinations for the reaction were CALB:RML ratio (3:1), *t*-butanol to oil (10 wt.%), water adsorbent content (22.5 wt.%) at the reaction time of 10 h. FAME yield of 91.5%, which was very close to the predicted value of 95.6%, was obtained. Verification experiment confirmed the validity of the predicted model.

Keywords: Biodiesel, Lipase, Immobilization, Waste cooking oil, Response surface methodology

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1. Introduction

Serious depletion of fossil fuels and the increasing environmental pressure on green-house gases coming from the fossil fuels have led to search for biotechnological transformation of oils and fats, seeking the use of these raw materials in the production of high value added products such as renewable fuels. Biodiesel is a renewable, clean-burning fuel produced from grease, vegetable oils, recycled restaurant grease or animal fats. According to the US Standard Specification for Biodiesel (ASTM 6751-02), biodiesel is defined as a fuel comprised of mono-alkyl esters of long-chain fatty acids derived from vegetable oils or animal fats [1]. Biodiesel has been reported as a possible substitute for conventional petrol-based diesel. Biodiesel burns up to 75% cleaner than petroleum diesel fuel. Biodiesel can be mixed with petro-diesel in any proportion, with no need for a mixing additive.

Biodiesel is most commonly produced by transesterification of vegetable oils and animal fats by short chain alcohol (e.g., methanol) with the assistance of catalyst and results in the production of biodiesel and glycerine. Conventionally the synthesis of alkyl esters is accomplished by chemical transesterification but alkali or acid catalyzed processes have some drawbacks, such as, difficulties in the recovery of glycerol, difficulties in the recovery of catalyst, and high energy requirements of the processes. Enzymatic approaches serve as a promising technology for biodiesel production due to mild reaction conditions, compatibility with variations in the quality of the raw material, fewer process steps, higher quality of glycerol, improved phase separation (no emulsification from soaps) and reduced energy consumption and wastewater volumes [2].

Enzymatic reactions involving lipases as catalyst in transesterification can be an excellent alternative to produce biodiesel as it produces high purity product and the operating temperature of the process is low compared to other techniques [3]. Also oils containing high free fatty acids such as waste cooking oils are incompletely transesterified using chemical methods but the high cost of enzyme catalysed biodiesel synthesis limits its industrial scale production. The development of an enzyme immobilization method is strongly needed to decrease the production cost of biodiesel using this approach. Among all the immobilization methods, covalent binding to the heterogeneous solid carriers makes the enzyme very stable [4-7]. In our previous works silica gel as a low cost carrier was selected and various lipases were immobilized on epoxy-functionalized silica. Biodiesel production using this low cost biocatalyst was performed by

transesterification of canola oil and the effect of various parameters such as temperature, oil to methanol ratio, water content and etc. were evaluated [8-11]. Waste cooking oil is much less expensive than pure refined vegetable oils, disposal of waste oil may cause the environment and human health risk. In addition utilization of waste cooking oil as a low cost feedstock for biodiesel production is a remarkable advantage compared with those derived from refined vegetable oils and fossil diesel [12].

In this work, we evaluated the potential of waste cooking oil as a suitable feedstock for the synthesis of fatty acid methyl esters (FAME) using two lipases from *Candida antarctica* (CALB) and *Rhizomucor miehei* (RML); immobilized on epoxy functionalized silica as a catalyst. The main objective of the study was investigation of the enzymatic esterification and transesterification of waste oil to biodiesel using a mixture of different lipases which each has specific characters that can act upon several substrates.

The chemical properties of waste oil is different compared with pure vegetable oils so the required process of converting them into biodiesel, is therefore different. Optimizing the process could improve the catalytic performance of the biocatalyst. The process in this study was optimized by the response surface methodology (RSM). RSM is one of the most commonly used methods for the optimization process and has been widely applied to the synthesis of biodiesel [12-14]. The prediction of the optimum operating conditions to obtain the higher yield of desired product was studied using suitable modeling techniques from response surface methodology and design of experiments.

2. Materials and Methods

2.1. Materials

Lipase from *Rhizomucor miehei* (RML), methyl ester standards (methyl laurate, methyl stearate, methyl linoleate, methyl oleate, methyl palmitate and methyl myristate), Silica gel 70-230 mesh (distribution particle size of 0.063-0.2 mm) were purchased from Sigma-Aldrich. Lipase from *Candida antarctica* was a kind gift from Novozymes A/S (Bagsvaerd, Denmark). Waste cooking oil (with an initial saponification number of 196.2 mg KOH/g, acid value of 76 mg KOH/g corresponding to a free fatty acid (FFA) level of 38%) was obtained from local restaurant with the following composition in fatty acids (w/v): 40.6% oleic acid, 17.6% linoleic acid, 32.2% palmitic acid, 5.2% stearic acid. The molecular weight of the oil which was calculated from its

saponification value was determined to be 856.3 g/mol. Water content in the oil measured by Karl Fischer titration method was determined to be 0.01% (w/w). Methanol, *t*-butanol, triethylamine (Et₃N) and blue silica gel were purchased from Merck. 3-Glycidyloxypropyl trimethoxysilane (3-GPTMS) was purchased from Acros. All other chemicals were obtained commercially and were of analytical reagent grade.

2.2. Preparation of biocatalyst

One gram of dry silica gel was mixed in a dry toluene solution (30 ml) containing 3-GPTMS (1 ml) and Et₃N (0.15 ml). The resulting mixture was refluxed under argon atmosphere and constant stirring for 4 h. The silica gel was then washed thoroughly with CHCl₃ and dried at 60°C for 2h. Epoxy functionalized silica (1 g) was mixed with 10 mg lipase in 10 ml phosphate buffer 25 mM (pH 7) followed by incubation at 25°C for 24 h. Immobilized lipase was recovered by filtration, washed thoroughly with distilled water, and then dried overnight at room temperature. The amount of dissolved protein was determined by the Bradford method [15]. The amount of lipase bound to the carrier was determined as the difference between the initial and residual protein concentration. The yield of bound enzyme was calculated as the ratio of the amount bound on silica gels to the initial amount.

2.3. Enzymatic transesterification of waste cooking oil

The enzymatic transesterification reactions were carried out according to each design points and the results of FAME yield were used as the response values in order to optimize the reaction conditions. In a typical experiment, the reaction was performed in 10 mL screw-capped vessel under continuous stirring containing waste cooking oil and anhydrous methanol, at oil-to-methanol molar ratio of 1:3. Methanol was added by a two-step procedure and each one molar equivalent of methanol was added at the reaction time of 0, and in half time of reaction time, respectively. The mixtures were incubated with the immobilized lipases on silica-epoxy at 50 °C under constant magnetic agitation of 250 rpm C. Methanolysis reactions were carried out with varying amount of biocatalyst, *t*-buthanol and water adsorbent. After the reactions were completed, an aliquot of reaction medium was taken centrifuged (12000 rpm, 10 min), mixed with methyl laurate (as an internal standard) and analyzed by the GC method as described below.

2.4. Analysis of fatty acid methyl esters

Fatty acid methyl ester contents were analyzed based on EN standard 14103 using a Thermo-Quest-Finnigan (Plymouth, Minnesota, USA) GC instrument equipped with an RTX-1 column and a flame ionization detector (FID). Nitrogen was used as the carrier gas at a constant flow of 1.2 mL/min. A specified amount of methyl laurate as the internal standard and 0.5 ml hexane were added to the accurately weighted sample from the upper layer of the reaction mixture. Then, 2.0 µl of the diluted sample was injected into the GC gas chromatograph. The column temperature was kept at 150 °C for 0.5 min, raised to 270 °C at 10 °C/min, and then maintained at this temperature for 3 min. The injector and detector temperatures were set at 220°C and 250°C, respectively. By comparing the retention times and peak areas of standard fatty acid methyl ester peaks, the total quantities of biodiesel in the reaction mixtures were calculated. Methyl laurate as an internal standard material was used for quantification of FAME, which was calculated by the following equations:

$$C = \frac{\sum A - A_{IS}}{A_{IS}} \times \frac{C_{IS} \times V_{IS}}{m} \times 100\% \quad (1)$$

Where $\sum A$ =total peak area; A_{IS} = internal standard (methyl laurate) peak area; C_{IS} =concentration of the internal standard solution in mg/mL; V_{IS} = volume of the internal standard solution used in mL; m = mass of the sample, in mg.

2.5. Experimental design

The biodiesel synthesis from waste oil was developed and optimized using response surface methodology (RSM) provided by Design-Expert software version 7.0.0 (Stat-Ease Inc., Minneapolis, USA). The 5-level-4-factor Central Composite Design CCD has been employed in this study, requiring 30 experiments, consisting of 16 factorial points, 8 axial points and 6 replicates at the center points. The center points are usually repeated 4–6 times to determine the experimental error (pure error) and the reproducibility of the data. Four identified independent variables are A: t-butanol concentration (0-40 % w/w); B: CALB-epoxy:RML epoxy ratio (0:1-4:1); C: water adsorbent (blue silica gel) (15-35 % w/w); D: reaction time (5-25 h). The levels of each independent variable were chosen based on our previous investigations. The independent variables are coded to two levels namely: low (−1) and high (+1), the α value was fixed at 2 which is the distance of the axial point from center and makes the design rotatable, the axial

points are coded as -2 ($-\alpha$) and $+2$ ($+\alpha$). The complete CCD design matrix in terms of independent variable is presented in Table 1.

The experiments were run at random in order to minimize errors from the systematic trends in the variables.

2.6. Statistical analysis

The experimental data obtained from central composite design were analyzed by response surface methodology. A mathematical model, following a second-order polynomial Eq. (2) which includes all interaction terms was used to calculate the predicted response:

$$Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^4 \beta_{ij} X_i X_j + \sum_{i=1}^4 \beta_{iii} X_i^3 \quad (2)$$

where Y is the yield of biodiesel from waste cooking oil, β_0 is the offset term, β_i is the linear effect, β_{ii} is the squared effect, β_{ij} is the interaction effect, x_i is the i th independent variable and x_j is the j th independent variable. The data were analyzed using Design Expert program and the coefficients were interpreted using F-test. Analysis of variance (ANOVA), regression analysis and plotting of contour plot were used to establish the optimum conditions for the FAME yield.

3. Results and discussion

3.1. Optimization of biodiesel production parameters

Central composite design model was used to optimize biodiesel production parameters. The waste cooking oil methyl ester yield ranged from 30.2% to 98.7%. Design Expert 7.0.0 program was used to calculate the effect of each factor and its interactions. Among the models that fitted to the response (linear, two factor interaction (2FI), quadratic and cubic polynomial), the quadratic model was selected as a best model due to its highest order polynomial with significance of additional terms and the model was not aliased. This quadratic model was suggested by the RSM software as shown in Table 2. The model expressed by Eq. (3) represents methyl ester yield (Y) as a function of t -butanol concentration (wt%) (A), CALB:RML ratio (B), water adsorbent (C) and reaction time (D). Positive sign in front of the terms indicates synergistic effect in increase FAME yield, whereas negative sign indicates antagonistic effect [14]. The results at each point based on the central composite design (CCD) and their corresponding predicted values are presented in Table 3.

$$Y = +37.10 - 9.11 A + 6.07 B + 6.02 C + 0.28 D - 10.08 AB + 6.79 AC + 6.97 AD - 3.39 BC - 1.28 BD - 1.46 CD + 7.89 A^2 + 6.48 B^2 + 1.06 C^2 + 9.31 D^2 \quad (3)$$

The result of statistical analysis of variance (ANOVA) which was carried out to determine the significance (by F-test) and fitness of the quadratic model as well as the effect of significant individual terms and their interaction on the selected responses are presented in Table 4.

The statistical significance of Eq. (3) was controlled by F-test. Values of probability (P) > F less than 0.05 indicate that model terms are significant. Values greater than 0.05 indicate that the model terms are not significant. The smaller the p-value, the more significant the corresponding coefficient is [16].

From the ANOVA results, the main model terms suggested that variables with significant influence on FAME yield response were *t*-butanol concentration (wt%) (A), CALB:RML ratio (B), water adsorbent (C) and the interaction terms were found to exist between the main factors (AB, AC, AD and BC), while the significant quadratic terms were A^2 , B^2 and D^2 .

The terms incorporated in the model F-value of 352.71 with p-value < 0.0001 implies that the model is significant at 95% confidence level. The smaller the p-value, the bigger the significance of the corresponding coefficient is.

The goodness of fit of regression equation developed could be measured by adjusted determination coefficient. The R^2 value of 0.9970 and adjusted R^2 of 0.9941 shows that the model could be significant predicting the response. The Predicted R^2 of 0.9843 is in reasonable agreement with the “Adjusted R^2 of 0.9941. The model also depicted the statistically non significant lack of fit (p 0.0978), indicating that the responses are adequate for employing in this model showed that the model satisfactorily fitted to experimental data (Fig 1a). Insignificant lack of fit is most wanted as significant lack of fit indicates that there might be contribution in the regressor-response relationship that is not accounted for by the model [17].

This analysis was examined using the normal probability plot of the residuals (Fig. 1b) and the plot of the residuals versus predicted response (Fig. 1c). The normal probability plot of the residuals indicates that the errors are distributed normally in a straight line and insignificant. On the other hand, the plot of residuals versus predicted response showed a structureless plot suggesting that the model is adequate and that the model does not show any violation of the independence or constant variance assumption [14].

3.2. Parameter study and interaction between independent variables

In our previous reports we investigated methanolysis of canola oil by three covalently immobilized lipases *Candida antarctica* (CALB), *Thermomyces lanuginosus* (TLL) and *Rhizomucor miehei* (RML) on silica-epoxy support and in optimization studies, the effect of water, *t*-butanol and blue silica gel as water adsorbent on the yield of FAME was considered. In this study our aim is investigating of interaction between parameters which might influence significantly the response of the process by response surface methodology (RSM). CALB is a non-specific lipase from *Candida antarctica* B and lipase from *Rhizomucor miehei* is a 1,3-specific lipase, so we used both of these enzymes to evaluate the effect of these combination on the biodiesel yield and also understanding the relationship between important reaction parameters. In some reports a new process for biodiesel production using a mixture of immobilized *Rhizopus oryzae* and *Candida rugosa* lipases were developed successfully and optimal conditions were obtained [18-20]. Mixture of 1, 3-specific lipase and non-specific lipase removed the acyl-migration step, which is the rate-determining step of biodiesel production, and enzyme activity was notably enhanced [21].

Fig. 2 shows the effect of four factors; *t*-butanol, CALB:RML ratio, water adsorbent and time of reaction as one factor on biodiesel yield. Tertiary alcohols such as *t*-amyl alcohol and *t*-butyl alcohol have been shown to be good solvents for immobilized lipase-mediated conversion of oil to biodiesel [22] but in this study *t*-butanol showed a negative effect at high level on the reaction yield (Fig. 2a) these lower conversions may be due to dilution effect of solvent in higher *t*-butanol percentages. As can be seen in Fig. 2b the influence of increasing the amount of CALB to RML ratio is evaluated by increasing the ratio from 1:1 to 3:1 and as a result an increase in the reaction conversion is observed. RML is a typical lipase with a 1,3-positional specificity so when its combination with CALB as a non-specific enzyme is used, high methyl ester yield could be possible. Fig. 2c shows the effect of blue silica gel as water adsorbent on FAME yield. The optimum water content is a compromise between minimizing hydrolysis and maximizing enzyme activity for the transesterification reaction [23]. In enzymatic production of biodiesel, water would be produced during the esterification of free fatty acids with methanol. Since 38% of waste cooking oil which used in this investigation is composed of free fatty acids, the effect of

water adsorbent quantity on the reaction was investigated. As Fig. 2c show, the increment of blue silica gel cause significant increase in FAME yield.

Fig. 3 shows the surface and contour plots of fatty acid methyl ester yield from transesterification of waste cooking oil by methanol.

Fig. 3a shows the interaction effect between *t*-butanol and CALB:RML ratio toward biodiesel yield. The 3D response surface revealed that increment of CALB to RML ratio from low level (1:1) to high level (3:1) leads to the increase of FAME content at low level *t*-butanol content (10 wt%) on the contrary, the increase of CALB does not improve the biodiesel yield at high level of *t*-butanol (30 wt%). *t*-Butanol as an organic solvent reduces the viscosity of the reaction mixture and can improve solubility of methanol and/or glycerol.

The dependence of FAME yield on the *t*-butanol content and water adsorbent is shown in Fig. 3b. The biodiesel yield remained <50% with the *t*-butanol increased from 10 to 30 wt% at the water adsorbent of 20-30 wt%. In the low level water adsorbent content (20 wt%), the 3D surface plot show the increment of organic solvent caused significant decrease in FAME yield (49 to 23%). However, the FAME yield was slightly influenced by the raise of water adsorbent at all organic solvent contents.

The simultaneous dependence of FAME yield on the reaction time and *t*-butanol content is shown in Fig. 3c in the reaction time of 10 h, the 3D surface plot show the increment of organic solvent caused decrease in FAME yield (75 to 35%) at low level of reaction time. However, the FAME yield was slightly influenced by the raise in *t*-butanol amount at higher reaction time (20 h). Fig. 3d and 3e show again this fact that increasing CALB to RML ratio at both low and high level of water adsorbent and reaction time resulted in increasing the FAME yield. Figure 3f represents dependence of FAME yield on the reaction time and water adsorbent, from the figure, it is obvious that an increase in methyl ester yield was observed with the increase of water adsorbent and increasing the time of reaction has no effect on the yield.

3.3. Validation of the model

The composition of optimum waste oil methyl ester yield was determined from the model (Eq. (3)). To validate the proposed model, a series of three experiments were carried out, whose reaction conditions was selected among the range of variables. In this way, reaction variables and corresponding yields for each reaction are shown in Table 5. As can be seen in the table,

achieved experimental values are close to theoretical calculated data provided by the proposed models. The overall average optimized conditions for biodiesel yield were obtained as follows: *t*-butanol (wt%) 10%, CALB:RML ratio 3:1, water adsorbent 22.5%, reaction time 10 h with biodiesel yield of 91.5%. The predicted biodiesel yield was 95.6%. This means that the experimental value obtained was reasonably close to the predicted value calculated from the model. It can be concluded that the model from central composite design was accurate and reliable for predicting the methyl ester yield for lipase-catalyzed transesterification of waste oil.

3.4. economical advantage of immobilized preparations

In one of our previous studies we compared the cost of immobilized lipase preparations with corresponding commercial immobilized lipases [10]. The laboratory preparation of CALB-epoxy had a specific cost of 0.002 €/unit of lipase activity on the immobilized preparation which was half of Novozyme 435. The corresponding cost for commercially available *Rhizomucor miehei* lipase is considerably (twenty times) higher than RML-epoxy. As can be seen from the results (Table 6), our preparations had reasonable costs compared with commercial lipases. With regard to reusability of CALB-epoxy (up to 14 cycles; keeping 93% of its initial activity) and RML-epoxy (94% activity after 11 runs) [10], it can be concluded that biodiesel production by these enzyme derivatives from an economical point of view is feasible.

Conclusion

A central composite design and response surface methodology were conducted to study the effects and optimization of reaction time, CALB:RML ratio, water adsorbent and *t*-butanol content as organic solvent as variables on the esterification and transesterification of waste cooking oil. The biocatalysts used were mixed immobilized biocatalysts consisting of immobilized CALB and immobilized RML. The conversion of FAME reached 91.5% under optimal reaction conditions. The study indicated that increasing in the amount of nonspecific enzyme CALB relative to 1,3-specific enzyme resulted to improving the FAME yield. Previous experiments investigating reusability of biocatalyst indicated that immobilized lipases on epoxy-functionalized silica show good capability to be used repeatedly. The proposed process may lower the production cost of biodiesel and facilitate the disposal of waste cooking oil also this

investigation indicated that the optimization process improve and enhance the process of converting waste cooking oil to biodiesel.

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Table 1. Independent variables and levels used for response surface design.

Independent variables	Symbols	Levels				
		-2	-1	0	1	2
<i>t</i> -butanol concentration (wt.%)	A	0	10	20	30	40
CALB:RML ratio	B	0	1	2	3	4
Water adsorbent (wt.%)	C	15	20	25	30	35
Reaction time (h)	D	5	10	15	20	25

Table 2. Sequential model sum of squares.

Source	Sum of squares	df	Mean square	F value	Prob>F	
Mean	97127.16	1	97127.16			
Linear	3746.92	4	936.73	3.08	0.0343	
2FI	3383.22	6	563.87	2.54	0.0564	
Quadratic	4188.47	4	1047.12	456.82	<0.0001	Suggested
Cubic	13.96	8	1.74	0.60	0.7568	Aliased
Residual	20.42	7	2.92			
Total	0.0000108	30	3616.00			

Table 3. Experimental design for five-level four-factor surface response design on transesterification and esterification of waste cooking oil using immobilized lipases.

Experimental	Type	Actual values of variables				Fatty acid methyl ester yield (%)
		A: <i>t</i> -butanol concentration (wt %)	B: CALB:RML ratio	C: Water adsorbent (wt%)	D: Reaction time (h)	Experimental
1	Fact	10	1:1	20	10	55.1
2	Fact	30	1:1	20	10	29.5
3	Fact	10	3:1	20	10	97.3
4	Fact	30	3:1	20	10	31.7
5	Fact	10	1:1	30	10	65.5
6	Fact	30	1:1	30	10	64.6
7	Fact	10	3:1	30	10	91.9
8	Fact	30	3:1	30	10	52.4
9	Fact	10	1:1	20	20	49.0
10	Fact	30	1:1	20	20	49.3
11	Fact	10	3:1	20	20	84.3
12	Fact	30	3:1	20	20	45.1
13	Fact	10	1:1	30	20	49.0
14	Fact	30	1:1	30	20	80.0
15	Fact	10	3:1	30	20	74.1
16	Fact	30	3:1	30	20	62.0
17	Axial	0	2:1	25	15	86.5
18	Axial	40	2:1	25	15	53.0
19	Axial	20	0:1	25	15	51.9
20	Axial	20	4:1	25	15	76.4
21	Axial	20	2:1	15	15	30.9
22	Axial	20	2:1	35	15	54.0
23	Axial	20	2:1	25	5	75.0

24	Axial	20	2:1	25	25	75.9
25	Center	20	2:1	25	15	36.9
26	Center	20	2:1	25	15	37.1
27	Center	20	2:1	25	15	36.9
28	Center	20	2:1	25	15	36.7
29	Center	20	2:1	25	15	36.1
30	Center	20	2:1	25	15	38.9

Table 4. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of transesterification parameters

Source	Sum of squares	df	Mean square	F value	p-value Prob>F	
Model	11318.60	14	808.47	352.71	<0.0001	significant
A- <i>t</i> -butanol	1989.81	1	1989.81	868.08	<0.0001	
B-CALB:RML ratio	885.13	1	885.13	386.15	<0.0001	
C-water adsorbent	870.13	1	870.13	379.61	<0.0001	
D-reaction time	1.85	1	1.85	0.81	0.3827	
AB	1624.29	1	1624.29	708.62	<0.0001	
AC	736.99	1	736.99	321.52	<0.0001	
AD	777.71	1	777.71	339.29	<0.0001	
BC	183.94	1	183.94	80.25	<0.0001	
BD	26.04	1	26.04	11.36	0.0042	
CD	34.25	1	34.25	14.94	0.0015	
A ²	1706.90	1	1706.90	744.66	<0.0001	
B ²	1153.03	1	1153.03	503.03	<0.0001	
C ²	31.03	1	31.03	13.54	0.0022	
D ²	2379.26	1	2379.26	1037.99	<0.0001	
Residual	34.38	15	2.29			
Lack of fit	29.90	10	2.99	3.34	0.0978	Not significant
Pure error	4.48	5	0.90			
Cor Total	11352.99	29				
			R-squared	0.9970		
Standard of deviation	1.51		Adj R-squared ^b	0.9941		
Mean	56.90		Pred R-squared ^c	0.9843		
C.V. ^a	2.66		Adeq Precision ^d	63.942		

^a Coefficient of variation

^b Adjusted R²

^c Predicted R²

^d Adequate precision

Table 5. (a) Optimization criteria for maximum FAME yield and (b) results of model validation at the optimum condition.

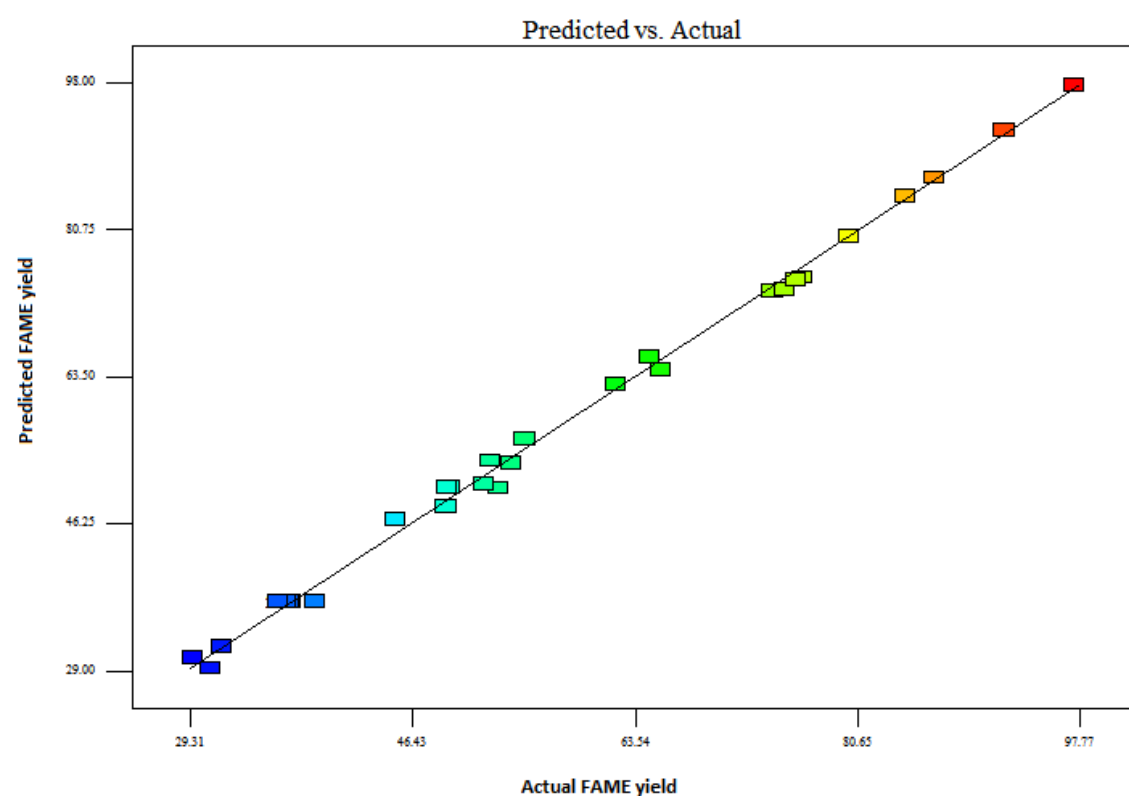
variables	Goal	Lower limit	Upper limit
<i>t</i> -butanol concentration (wt %)	In range	10	30
CALB:RML ratio	In range	1	3
Water adsorbent (wt%)	In range	20	30
Reaction time (h)	In range	10	20
FAME yield (%)	Maximize	29.5	97.3

Entry	<i>t</i> -butanol concentration (wt %)	CALB:RML ratio	Water adsorbent (wt%)	Reaction time (h)	Predicted FAME yield (%)	Experimental FAME yield (%)
1	10.0	3.0	22.5	10.0	95.6	91.5
2	10.0	3.0	20.0	10.7	94.2	90.2
3	10.0	3.0	25.0	10.0	93.9	91.1

Table 6. Specific cost (€ /unit) of immobilized lipase compared with commercial lipases [10].

Immobilized enzyme	Cost €/g	catalytic activity U/g ^a	specific cost €/unit
Novozyme 435 (Lipase immobilized on acrylic resin from <i>Candida antarctica</i>)	22.1	5000	0.004
Lipase, immobilized on Immobead 150 from <i>Rhizomucor miehei</i>	10.9	300	0.04
CALB-epoxy	2.9	1510	0.002
RML-epoxy	3	1700	0.002

^a Specific activity (U/g lipase) is expressed as micromole of *p*-nitrophenyl butyrate hydrolyzed per minute per gram of immobilized protein.



(a)

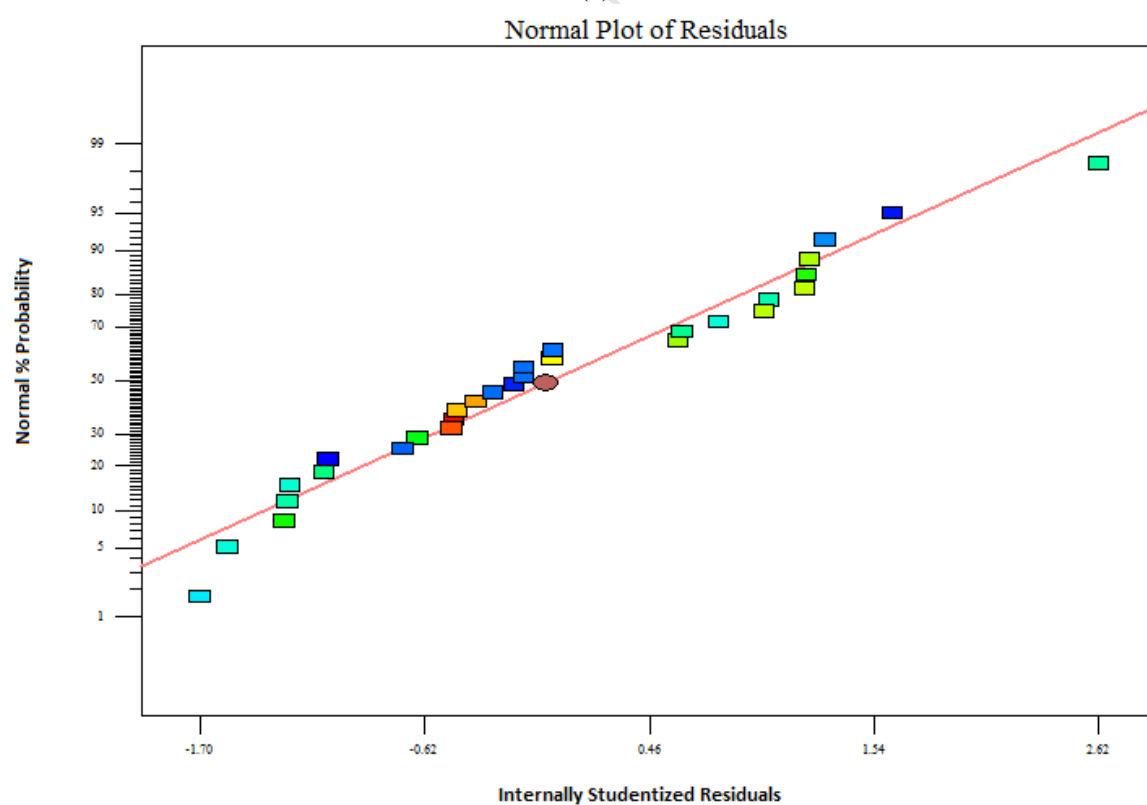
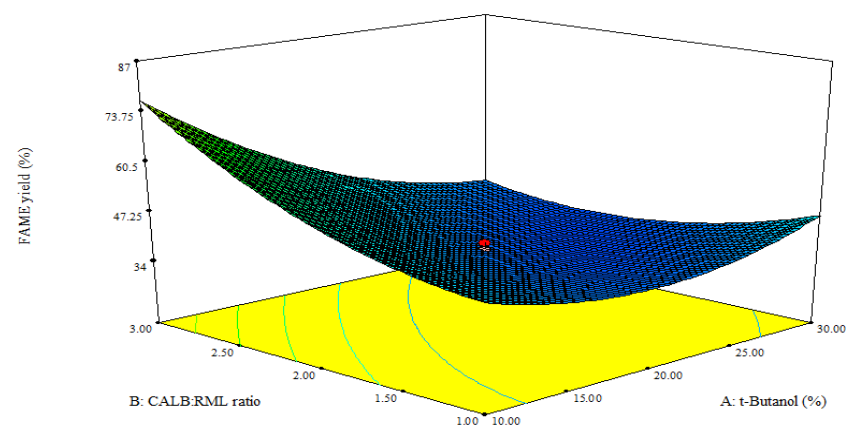
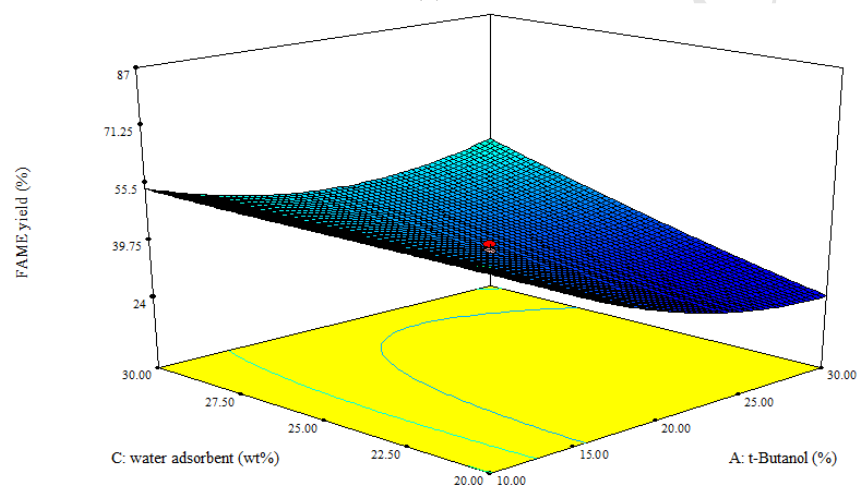




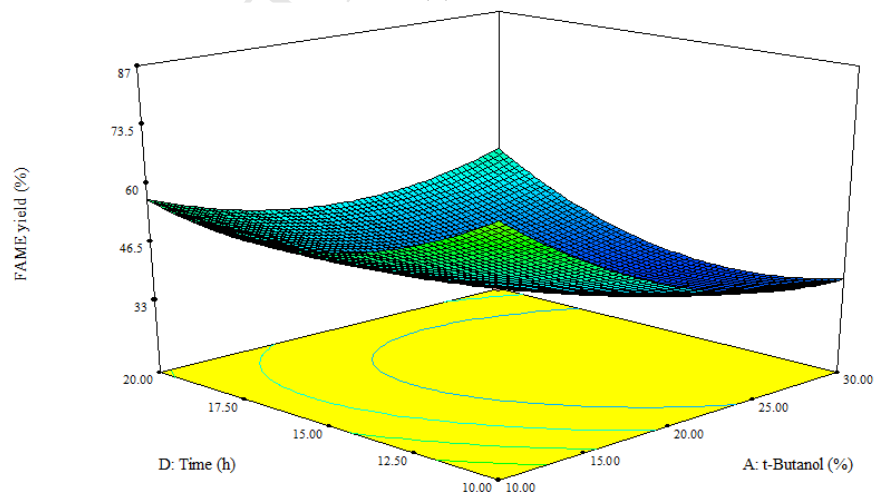
Fig.1. Predicted fatty acid methyl ester yield versus experimental fatty acid methyl ester yield (a), Normal probability plots of residuals (b) and plot of the residuals versus the predicted response (c).



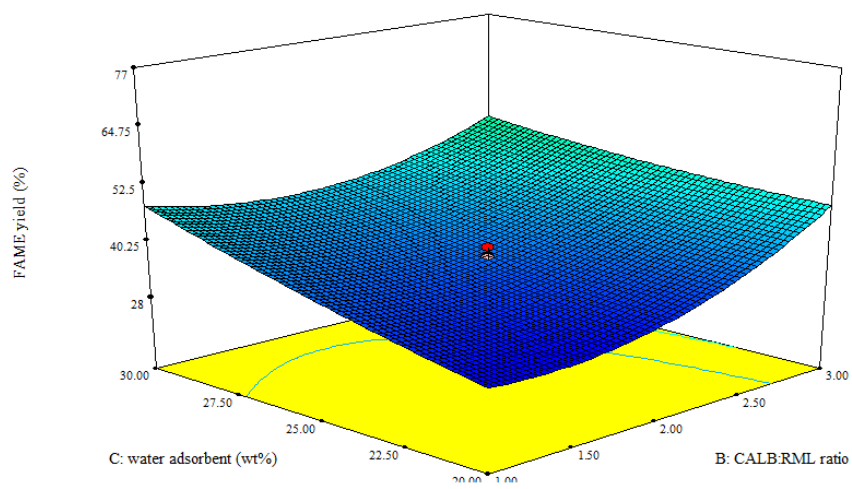
(a)



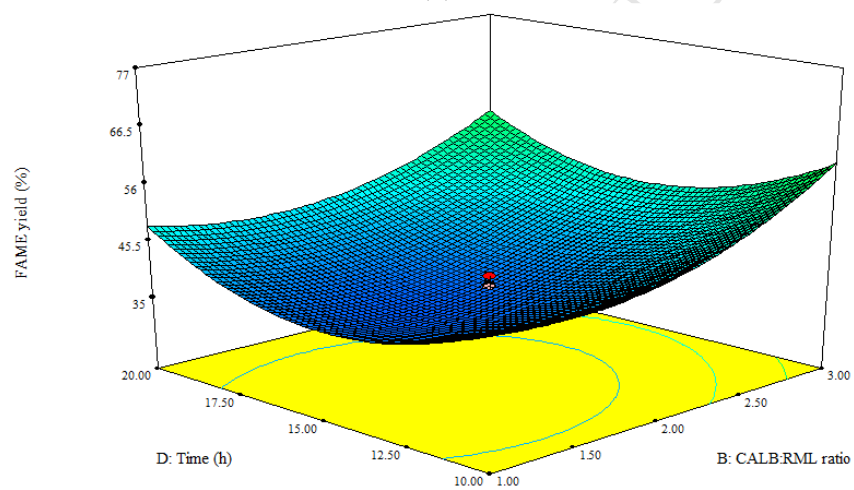
(b)



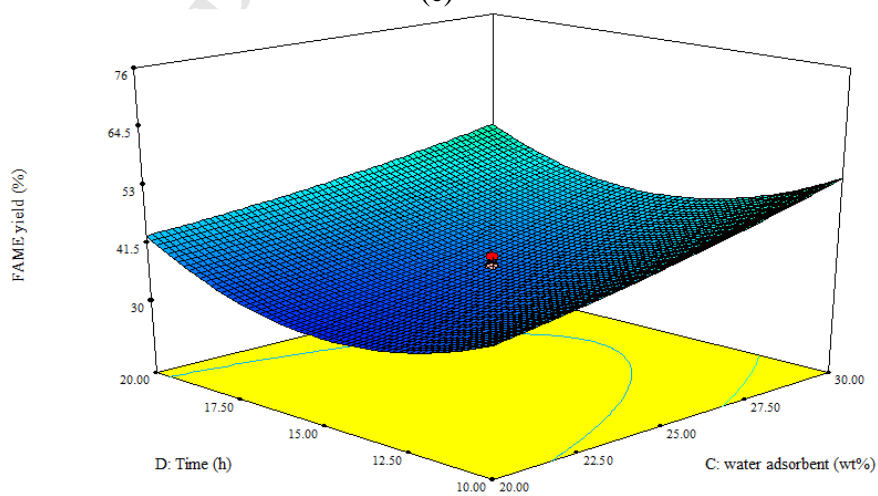
(c)



(d)

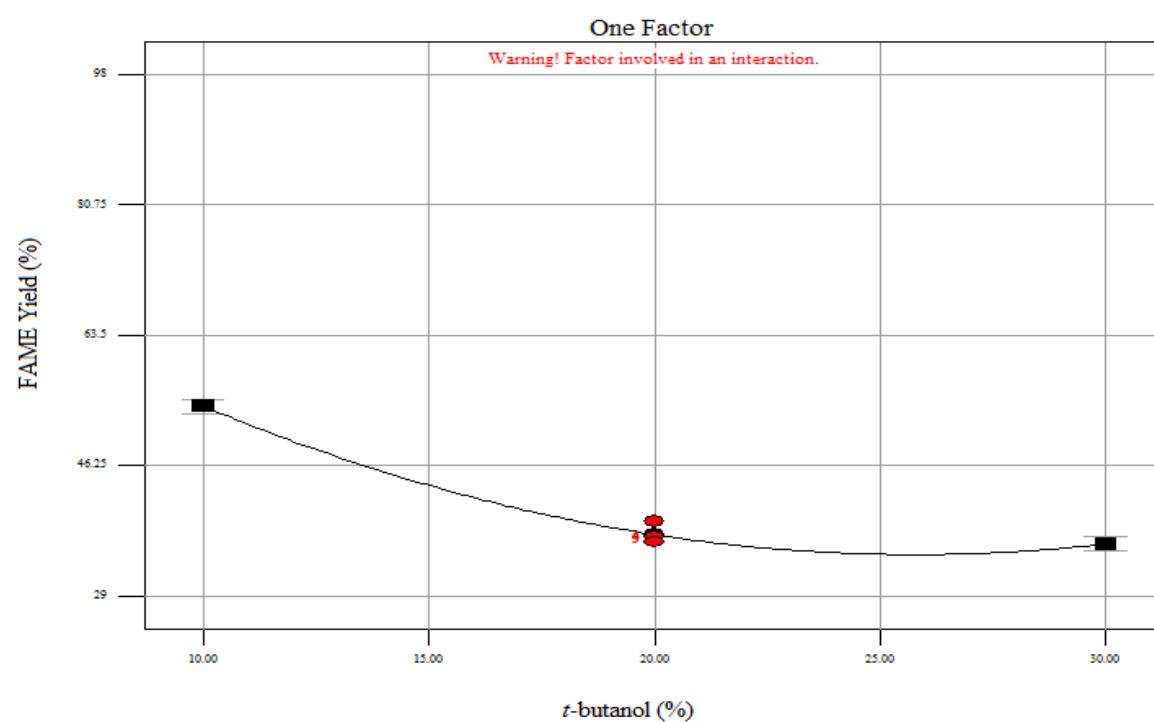


(e)

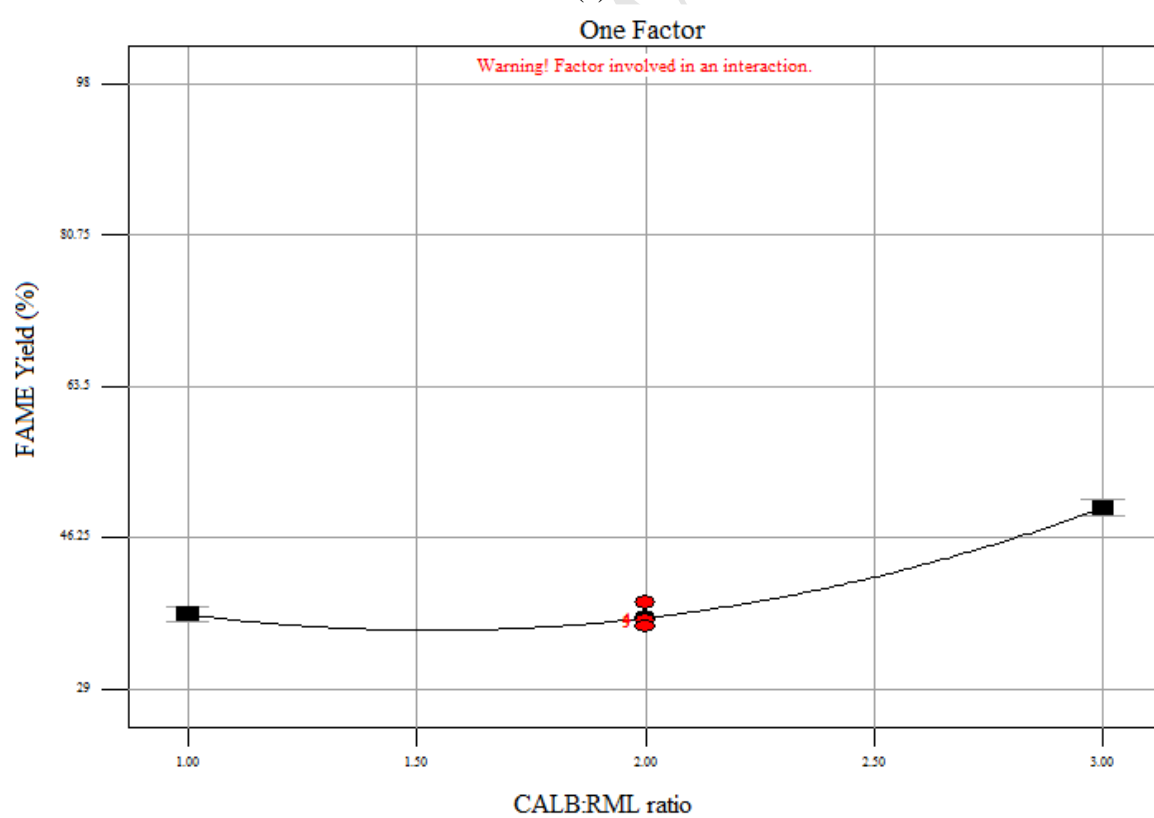


(d)

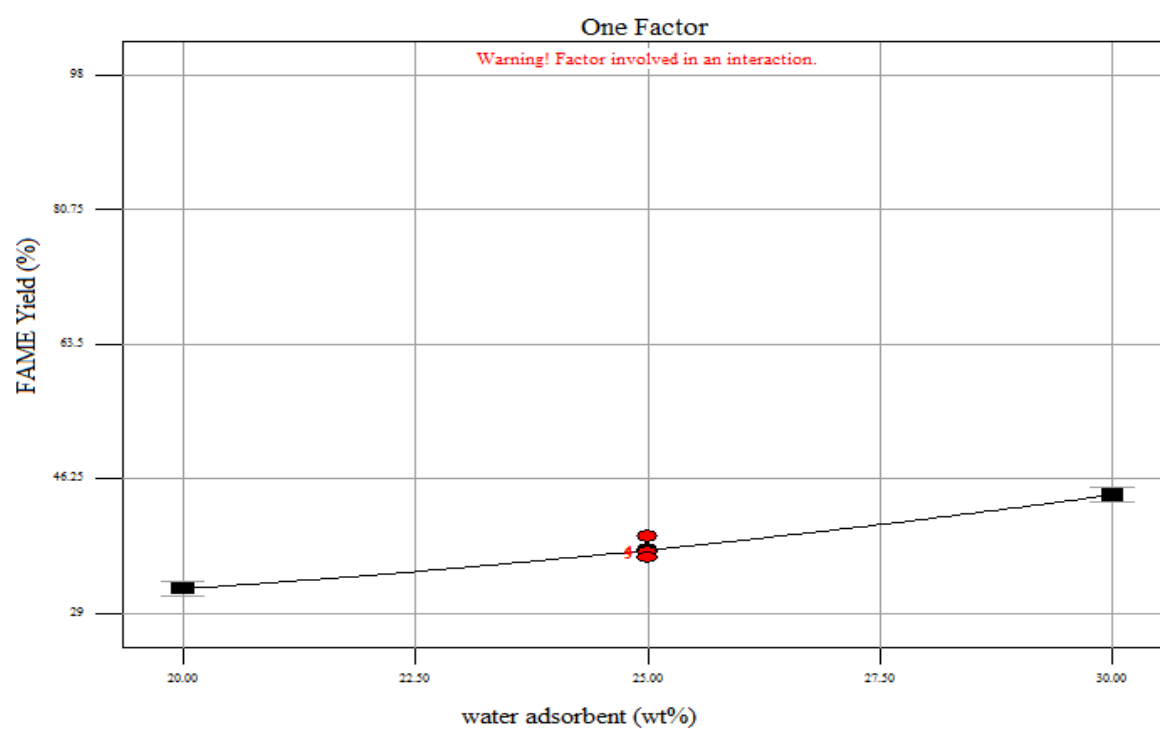
Fig. 3. Response surface plot and contour plot of FAME conversion; (a) CALB:RML ratio vs. *t*-butanol, (b) water adsorbent vs. *t*-butanol, (c) time vs. *t*-butanol, (d) water adsorbent vs. CALB:RML ratio, (e) time vs. CALB:RML ratio, (f) time vs. water adsorbent



(a)



(b)



(c)

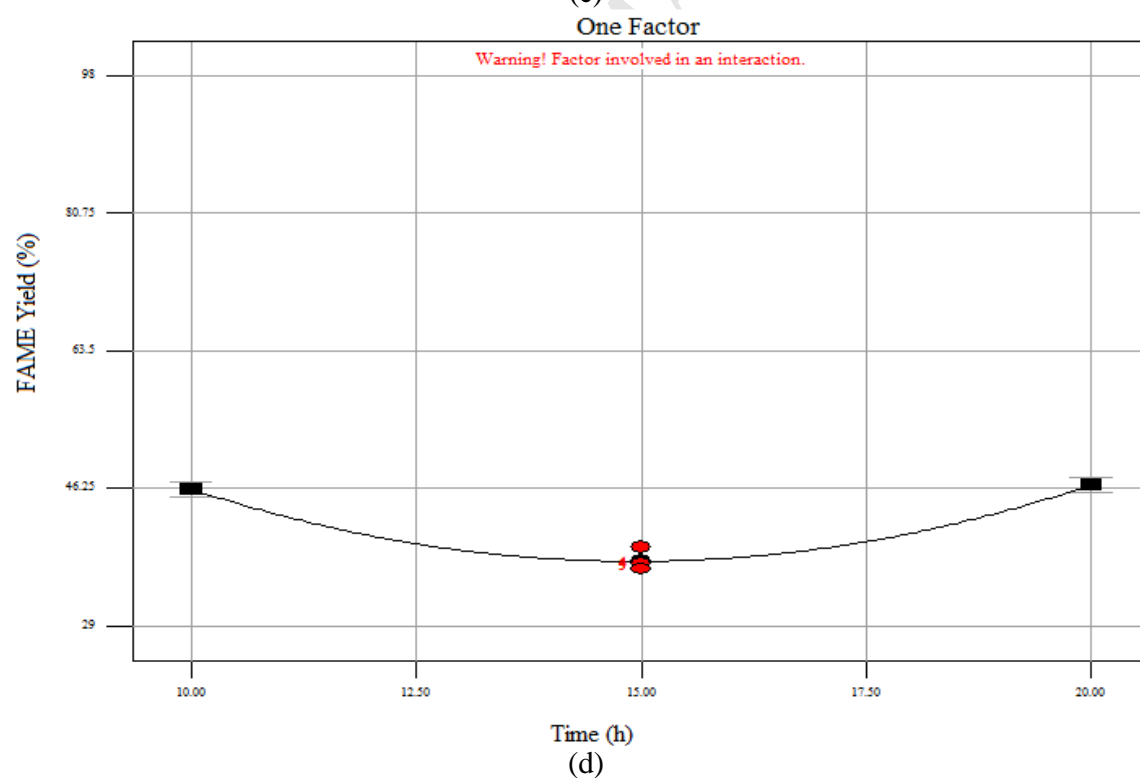


Figure 2. Effect of independent variable (a) *t*-butanol (%), (b) CALB:RML ratio, (c) water adsorbent (wt%), (d) Time (h) on FAME yield

- Lipases were immobilized by covalent binding onto epoxy-functionalized silica.
- The immobilized lipases were studied for production of biodiesel from waste cooking oil.
- A mixture of 1,3-specific lipase (RML) and nonspecific lipase (CALB) was used.
- The effect of water, *t*-butanol and blue silica gel on the FAME% was considered.
- Epoxy-functionalized silica resulted to high catalytic activity.