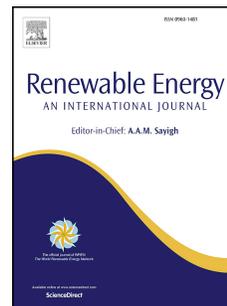


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1 **Process optimization for biodiesel production from waste cooking oil using**
2 **multi-enzyme systems through response surface methodology**

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8

9 **Abstract:**

10 Lipase from *Rhizomucor miehei* (RML) and lipase B from *Candida antarctica* (CALB) were
11 covalently immobilized onto epoxy-functionalized silica. In this study, we developed a multi-
12 enzyme system to produce biodiesel with waste cooking oil and methanol. To increase the
13 biodiesel production yield, a mixture of 1,3-specific lipase (RML) and nonspecific lipase
14 (CALB) was used. Response Surface Methodology (RSM) and a central composite rotatable
15 design (CCRD) was used to study the effects of four factors, CALB:RML ratio, ratio of *t*-butanol
16 to oil (wt.%), water adsorbent content (wt.%) and reaction time on the fatty acid methyl esters
17 (FAME) yield. A quadratic polynomial equation was obtained for methanolysis reaction by
18 multiple regression analysis. The optimum combinations for the reaction were CALB:RML ratio
19 (3:1), *t*-butanol to oil (10 wt.%), water adsorbent content (22.5 wt.%) at the reaction time of 10
20 h. FAME yield of 91.5%, which was very close to the predicted value of 95.6%, was obtained.
21 Verification experiment confirmed the validity of the predicted model.

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

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24 1. Introduction

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

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

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

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

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

66 The chemical properties of waste oil is different compared with pure vegetable oils so the
67 required process of converting them into biodiesel, is therefore different. Optimizing the process
68 could improve the catalytic performance of the biocatalyst. The process in this study was
69 optimized by the response surface methodology (RSM). RSM is one of the most commonly used
70 methods for the optimization process and has been widely applied to the synthesis of biodiesel
71 [12-14]. The prediction of the optimum operating conditions to obtain the higher yield of desired
72 product was studied using suitable modeling techniques from response surface methodology and
73 design of experiments.

74

75 **2. Materials and Methods**

76 *2.1. Materials*

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

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

90 *2.2. Preparation of biocatalyst*

91 One gram of dry silica gel was mixed in a dry toluene solution (30 ml) containing 3-GPTMS (1
92 ml) and Et₃N (0.15 ml). The resulting mixture was refluxed under argon atmosphere and
93 constant stirring for 4 h. The silica gel was then washed thoroughly with CHCl₃ and dried at
94 60°C for 2h. Epoxy functionalized silica (1 g) was mixed with 10 mg lipase in 10 ml phosphate
95 buffer 25 mM (pH 7) followed by incubation at 25°C for 24 h. Immobilized lipase was recovered
96 by filtration, washed thoroughly with distilled water, and then dried overnight at room
97 temperature. The amount of dissolved protein was determined by the Bradford method [15]. The
98 amount of lipase bound to the carrier was determined as the difference between the initial and
99 residual protein concentration. The yield of bound enzyme was calculated as the ratio of the
100 amount bound on silica gels to the initial amount.

101 *2.3. Enzymatic transesterification of waste cooking oil*

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

113 *2.4. Analysis of fatty acid methyl esters*

114 Fatty acid methyl ester contents were analyzed based on EN standard 14103 using a Thermo-
115 Quest-Finnigan (Plymouth, Minnesota, USA) GC instrument equipped with an RTX-1 column
116 and a flame ionization detector (FID). Nitrogen was used as the carrier gas at a constant flow of
117 1.2 mL/min. A specified amount of methyl laurate as the internal standard and 0.5 ml hexane
118 were added to the accurately weighted sample from the upper layer of the reaction mixture.
119 Then, 2.0 μ l of the diluted sample was injected into the GC gas chromatograph. The column
120 temperature was kept at 150 °C for 0.5 min, raised to 270 °C at 10 °C/min, and then maintained
121 at this temperature for 3 min. The injector and detector temperatures were set at 220°C and
122 250°C, respectively. By comparing the retention times and peak areas of standard fatty acid
123 methyl ester peaks, the total quantities of biodiesel in the reaction mixtures were calculated.
124 Methyl laurate as an internal standard material was used for quantification of FAME, which was
125 calculated by the following equations:

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

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

129 *2.5.Experimental design*

130 The biodiesel synthesis from waste oil was developed and optimized using response surface
131 methodology (RSM) provided by Design-Expert software version 7.0.0 (Stat-Ease Inc.,
132 Minneapolis, USA). The 5-level-4-factor Central Composite Design CCD has been employed in
133 this study, requiring 30 experiments, consisting of 16 factorial points, 8 axial points and 6
134 replicates at the center points. The center points are usually repeated 4–6 times to determine the
135 experimental error (pure error) and the reproducibility of the data. Four identified independent
136 variables are A: t-butanol concentration (0-40 % w/w); B: CALB-epoxy:RML epoxy ratio (0:1-
137 4:1); C: water adsorbent (blue silica gel) (15-35 % w/w); D: reaction time (5-25 h). The levels of
138 each independent variable were chosen based on our previous investigations. The independent
139 variables are coded to two levels namely: low (-1) and high (+1), the α value was fixed at 2
140 which is the distance of the axial point from center and makes the design rotatable, the axial

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

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

145 2.6. Statistical analysis

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

$$149 \quad Y = \beta_0 + \sum_{i=1}^4 \beta_i X_i + \sum_{i=1}^4 \beta_{ii} X_i^2 + \sum_{i=1}^3 \sum_{j=i+1}^4 \beta_{ij} X_i X_j + \sum_{i=1}^4 \beta_{iii} X_i^3 \quad (2)$$

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

155 3. Results and discussion

156 3.1. Optimization of biodiesel production parameters

157 Central composite design model was used to optimize biodiesel production parameters. The
 158 waste cooking oil methyl ester yield ranged from 30.2% to 98.7%. Design Expert 7.0.0 program
 159 was used to calculate the effect of each factor and its interactions. Among the models that fitted
 160 to the response (linear, two factor interaction (2FI), quadratic and cubic polynomial), the
 161 quadratic model was selected as a best model due to its highest order polynomial with
 162 significance of additional terms and the model was not aliased. This quadratic model was
 163 suggested by the RSM software as shown in Table 2. The model expressed by Eq. (3) represents
 164 methyl ester yield (Y) as a function of t -butanol concentration (wt%) (A), CALB:RML ratio (B),
 165 water adsorbent (C) and reaction time (D). Positive sign in front of the terms indicates
 166 synergistic effect in increase FAME yield, whereas negative sign indicates antagonistic effect
 167 [14]. The results at each point based on the central composite design (CCD) and their
 168 corresponding predicted values are presented in Table 3.

169 $Y = +37.10 - 9.11 A + 6.07 B + 6.02 C + 0.28 D - 10.08 AB + 6.79 AC + 6.97 AD - 3.39 BC -$
170 $1.28 BD - 1.46 CD + 7.89 A^2 + 6.48 B^2 + 1.06 C^2 + 9.31 D^2$ (3)

171
172 The result of statistical analysis of variance (ANOVA) which was carried out to determine the
173 significance (by F-test) and fitness of the quadratic model as well as the effect of significant
174 individual terms and their interaction on the selected responses are presented in Table 4.

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

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

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

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

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

200

201 *3.2. Parameter study and interaction between independent variables*

202

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

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

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

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

235 Fig. 3a shows the interaction effect between *t*-butanol and CALB:RML ratio toward biodiesel
236 yield. The 3D response surface revealed that increment of CALB to RML ratio from low level
237 (1:1) to high level (3:1) leads to the increase of FAME content at low level *t*-butanol content (10
238 wt%) on the contrary, the increase of CALB does not improve the biodiesel yield at high level of
239 *t*-butanol (30 wt%). *t*-Butanol as an organic solvent reduces the viscosity of the reaction mixture
240 and can improve solubility of methanol and/or glycerol.

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

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

256

257 3.3. Validation of the model

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

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

269 3.4. economical advantage of immobilized preparations

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

279

280 **Conclusion**

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

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

293

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297

298

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300

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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.

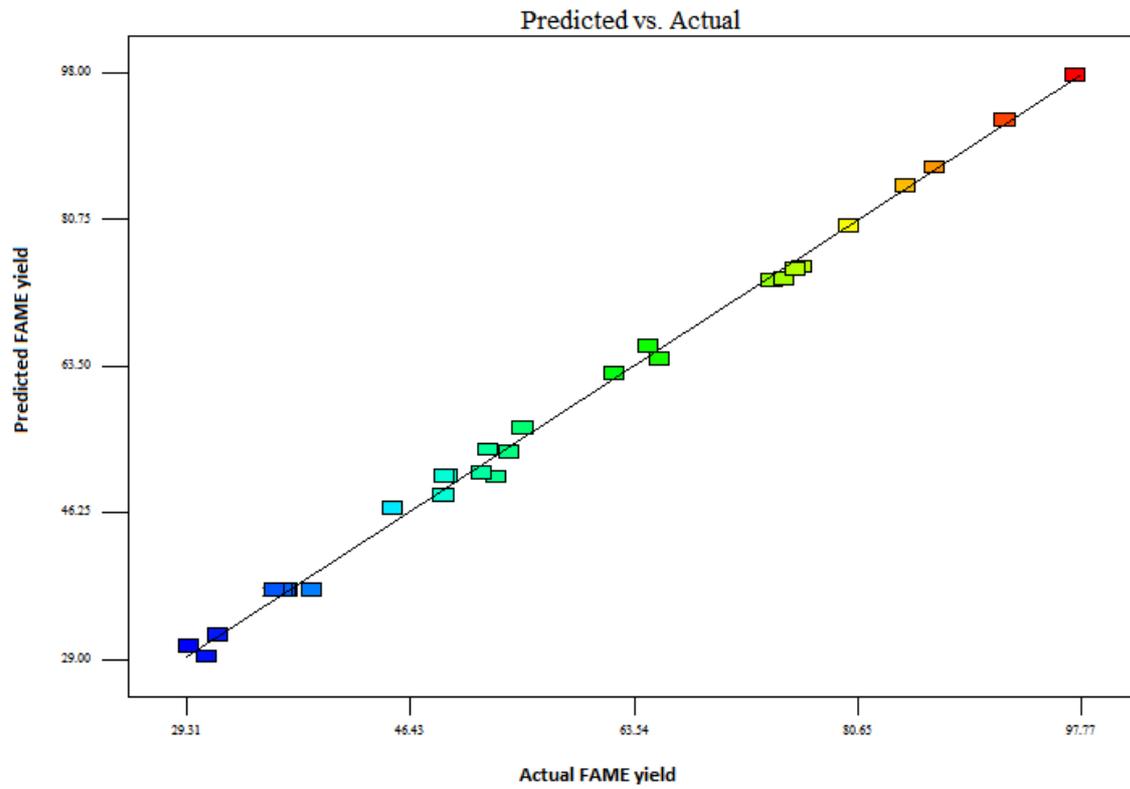
variables	Goal	Lower limit	Upper limit
<i>t</i> -butanol concentration (wt %)	In range	10	30
CALB:RML ratio	In range	1	3
Water adsorbent (wt%)	In range	20	30
Reaction time (h)	In range	10	20
FAME yield (%)	Maximize	29.5	97.3

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

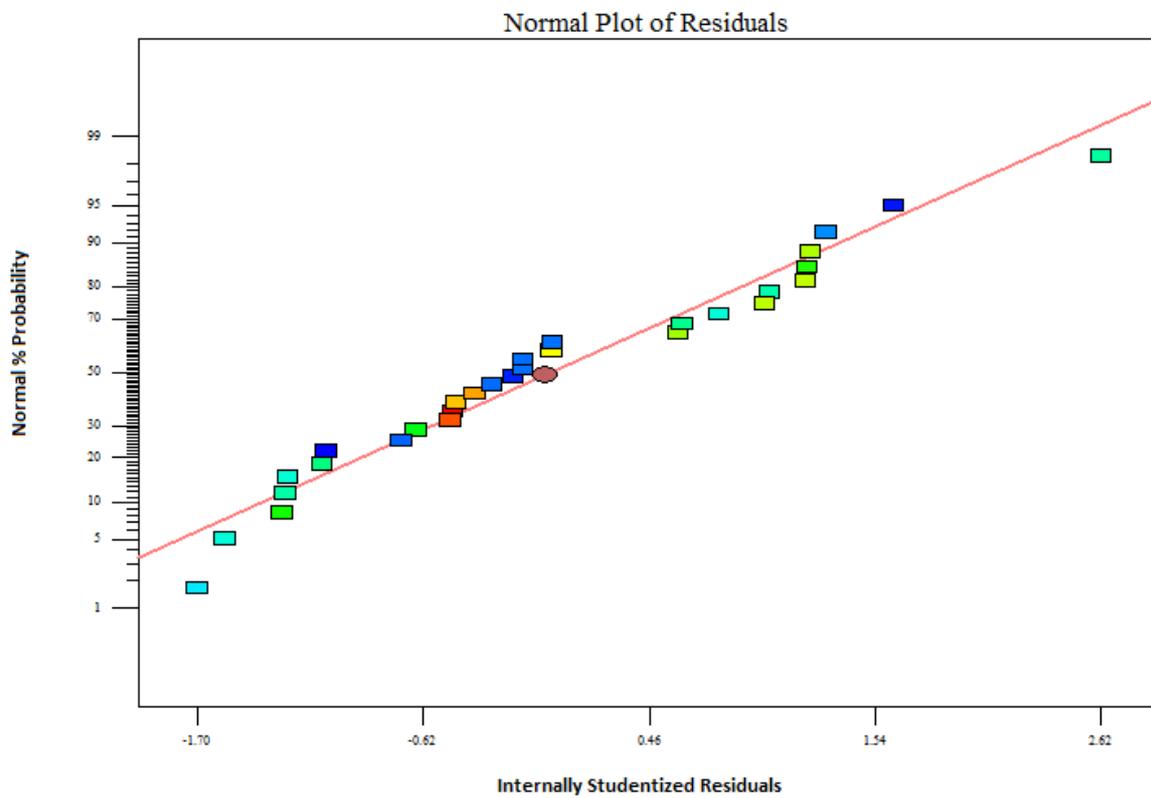
Table 6. Specific cost (€ /unit) of immobilized lipase compared with commercial lipases [10].

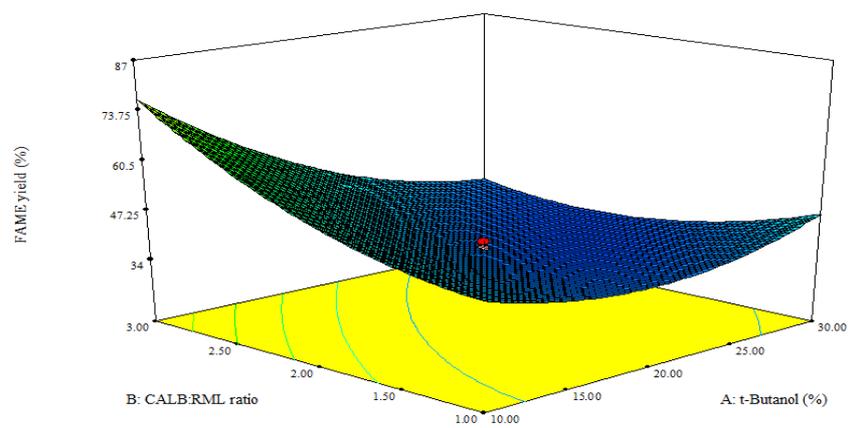
Immobilized enzyme	Cost €/g	catalytic activity U/g ^a	specific cost €/unit
Novozyme 435 (Lipase immobilized on acrylic resin from <i>Candida antarctica</i>)	22.1	5000	0.004
Lipase, immobilized on Immobead 150 from <i>Rhizomucor miehei</i>	10.9	300	0.04
CALB-epoxy	2.9	1510	0.002
RML-epoxy	3	1700	0.002

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

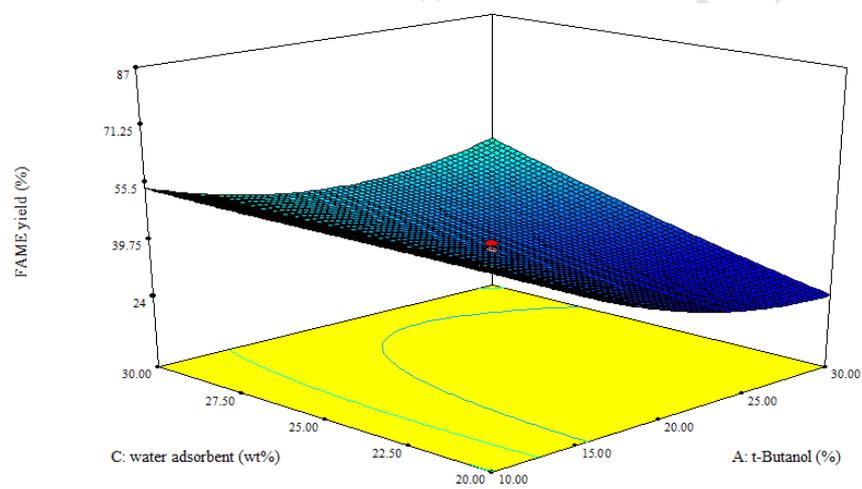


(a)

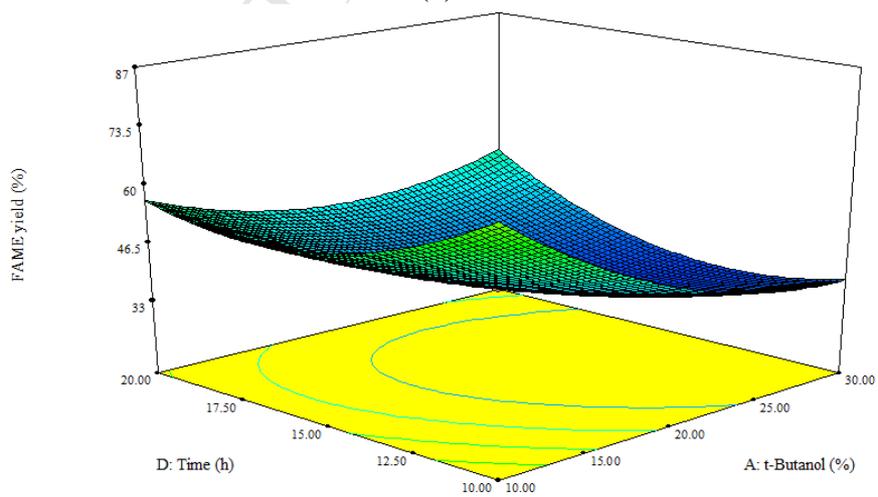




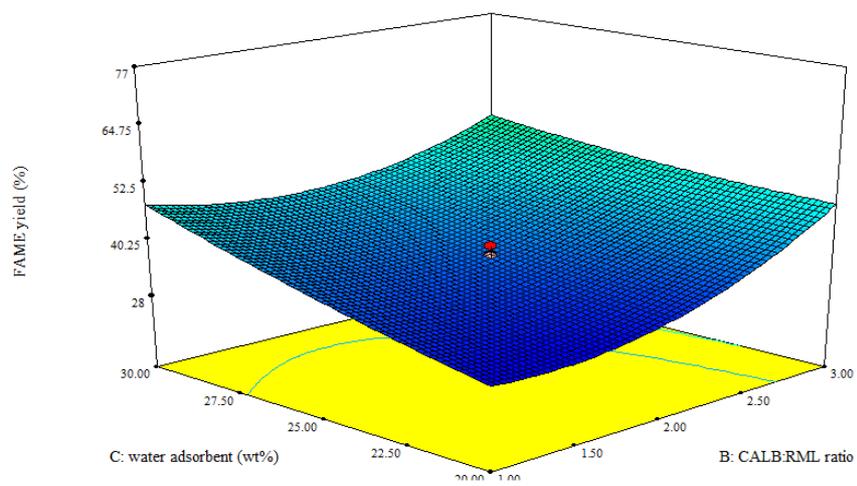
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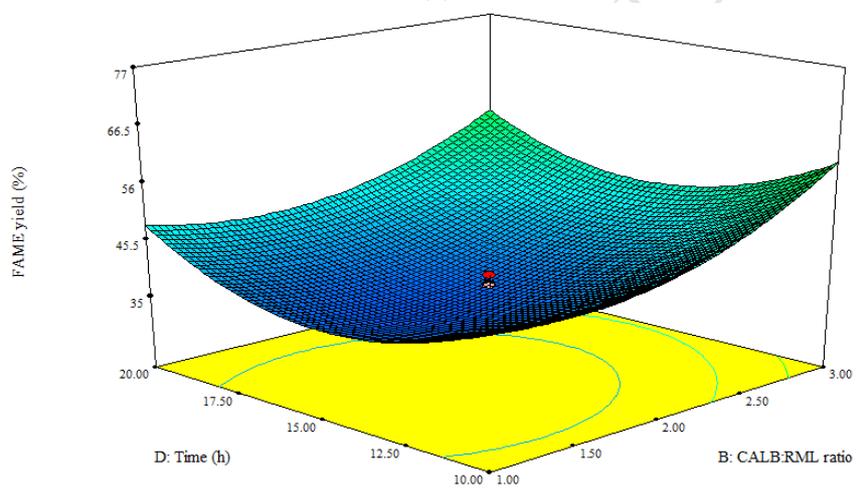
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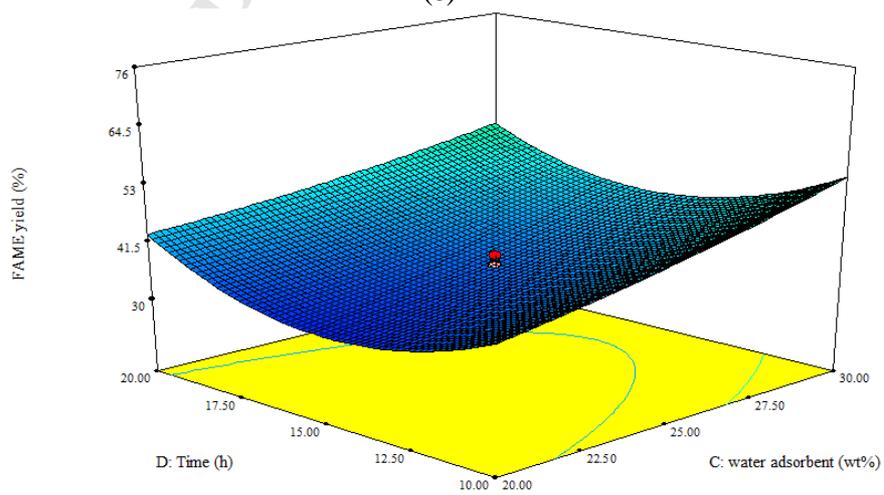
(c)



(d)



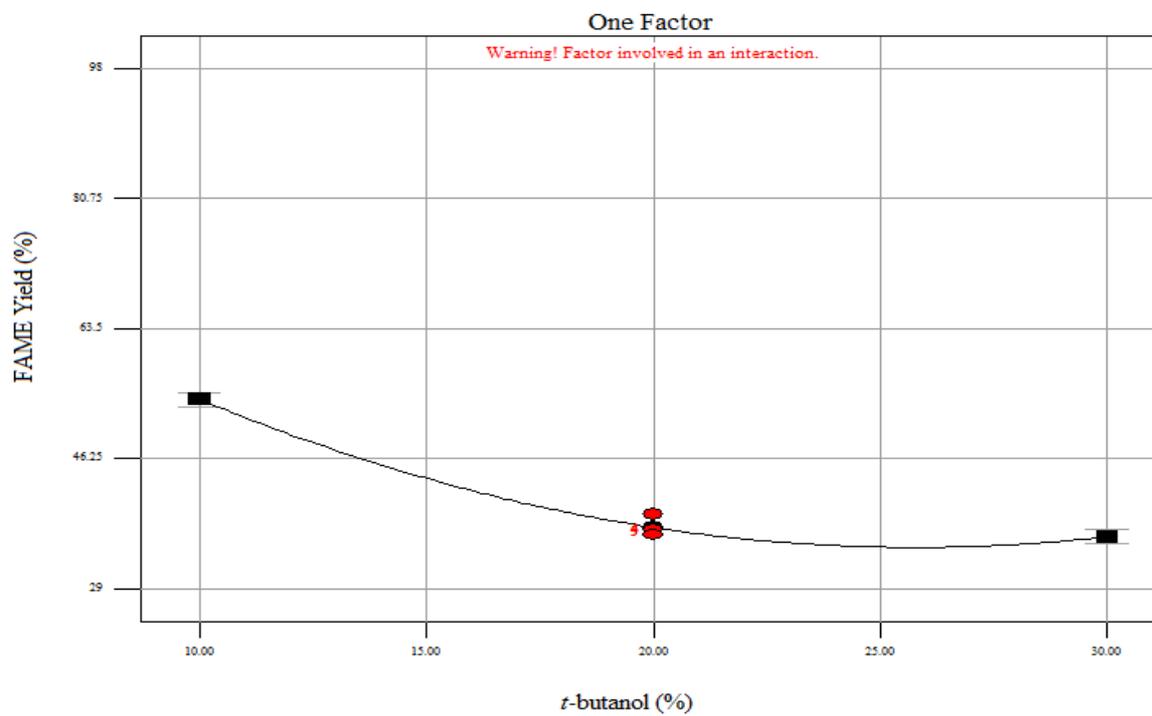
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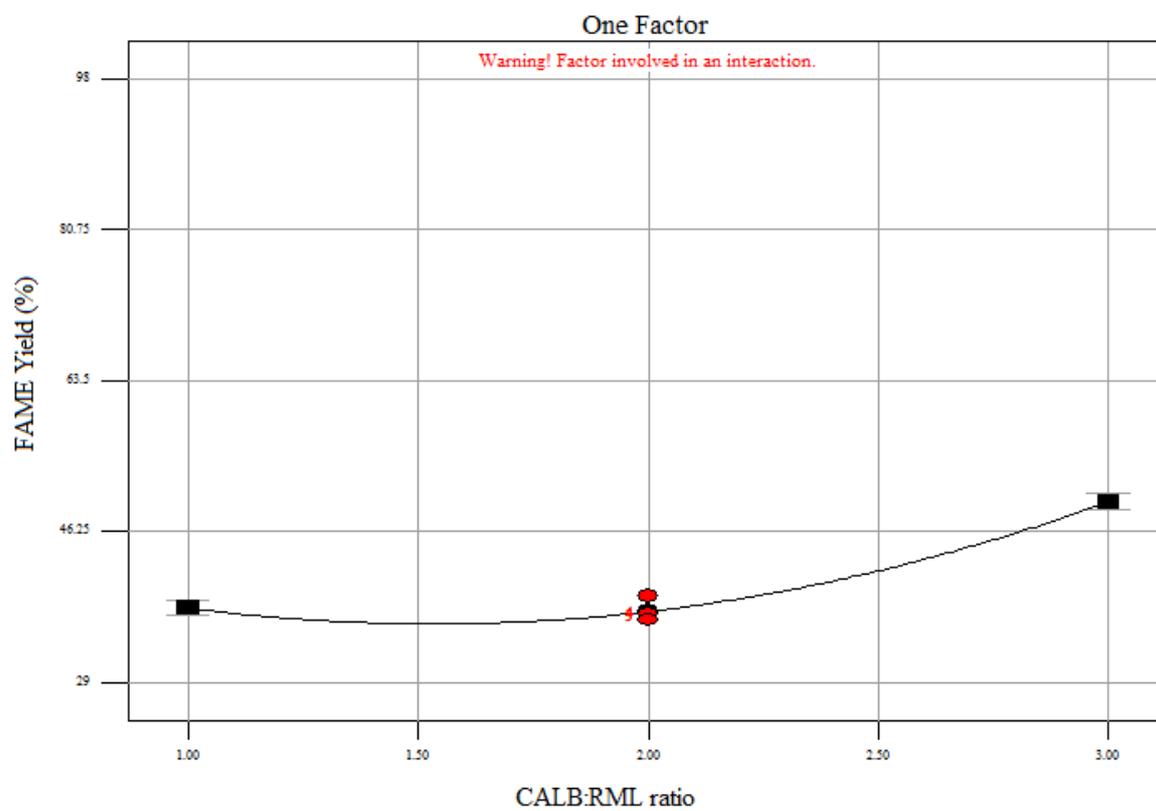
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Fig. 3. Response surface plot and contour plot of FAME conversion; (a) CALB:RML ratio vs. *t*-butanol, (b) water adsorbent vs. *t*-butanol, (c) time vs. *t*-butanol, (d) water adsorbent vs. CALB:RML ratio, (e) time vs. CALB:RML ratio, (f) time vs.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

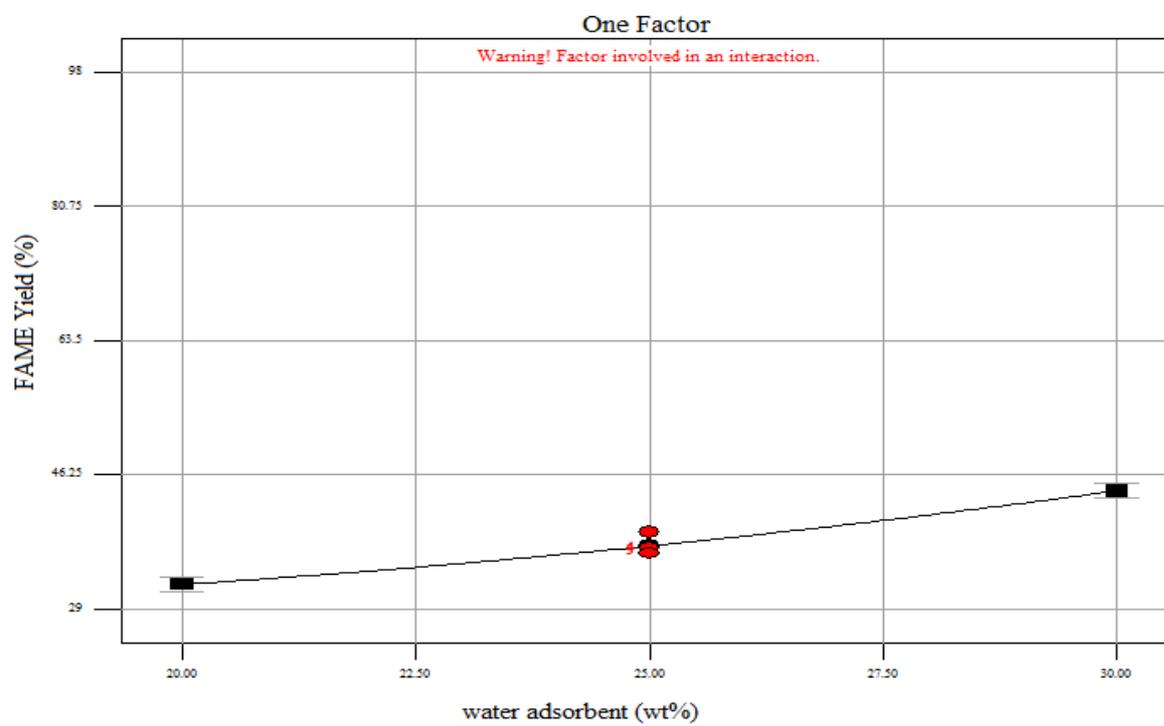
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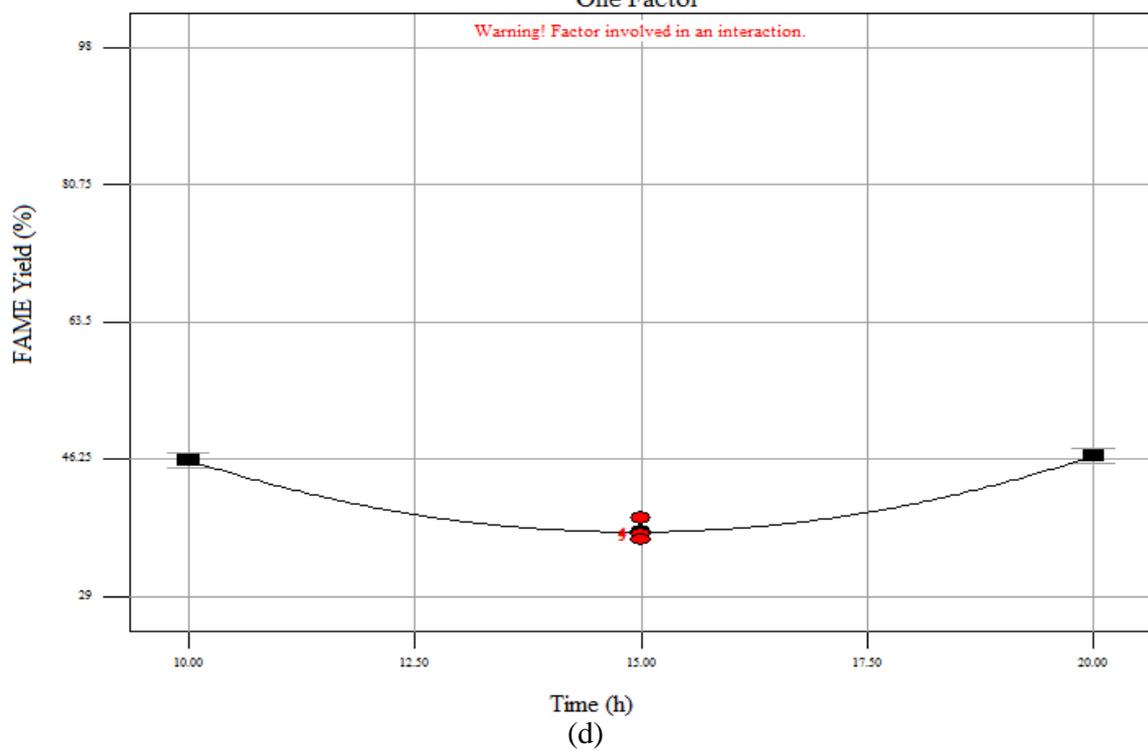
(a)



(b)



(c)



(d)

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

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- 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.