

## RESPONSE SURFACE METHODOLOGY OPTIMIZATION OF LIPASE-CATALYZED TRANSESTERIFICATION OF *JATROPHA CURCAS* L. SEED OIL FOR BIODIESEL PRODUCTION

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### ABSTRACT

The immobilized lipase-catalyzed transesterification of *Jatropha curcas* L. seed oil and methanol for biodiesel production in tert-butanol was investigated. The effects of different tert-butanol volume, methanol molar ratio, reaction temperature, reaction time and immobilized lipase amount on the total conversion were systematically analyzed by response surface methodology (RSM). RSM analysis showed good correspondence between experimental and predicted values. The optimal conditions for the transesterification were a reaction time of 17.355 h, a reaction temperature of 34.868 °C, an immobilized lipase amount of 12.435 %, a methanol molar ratio of 5.282:1, a tert-butanol volume ratio of 0.577:1. The optimal predicted yield of fatty acid methyl esters (FAME) was 88.5 % and the actual value was 88.1 %. The predicted yield of fatty acid esters and the real one was very close, indicating that the RSM based on central composite design (CCD) was adaptable for a FAME study for the present transesterification system. Moreover, the infrared spectrum of biodiesel showed the characteristic bands of C=O, O–C–O, C=C and  $-(CH_2)_n-$ . Furthermore, GC-linked mass spectrometry showed that biodiesel was mainly composed of the methyl esters of hexadecanoic, 9,12-octadecadienoic and 9-octadecadienoic acid.

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**Keywords:** *Jatropha curcas* L. seed oil, response surface methodology, infrared spectrum, GC-linked mass spectrometry

### Introduction

The rising costs of petroleum-derived fuels, together with environmental pollution and global warming, have encouraged research on alternative fuels and low-energy design. Biodiesel (fatty acid esters) is expected as a substitute for conventional fossil fuel (3, 9). Biodiesel synthesis from vegetable oils or animal fats and short-chain alcohols (such as methanol or ethanol) has attracted considerable attention as a renewable biodegradable and non-toxic fuel. In China, edible oils are not encouraged to be used for production of fatty acid esters because China imports more than 400 million tons of edible oils annually to satisfy its consumption need (4). There are several non-edible oil seed species which could be utilized as a source for biodiesel production. Among these, *Jatropha curcas* is considered to be a potential source for biodiesel primarily due to its relatively high seed oil content and non-competing demand with edible oil supplies (12). In China, the plantation area is more than 2 000 000 ha and is being expanded quickly along the Yangtze River, as promoted by an environment protection act (10). The seed kernel contains 40 % to 60 % (w/w) of oil and the oil from *J. curcas* seeds is also promising with its good oxidation stability as compared to soybean oil. Therefore, *J. curcas* seed oil is a prospective candidate for biodiesel in terms of availability and cost.

Biodiesel production mostly relies on base-catalyzed transesterification, which has drawbacks such as high energy and methanol consumption, difficulty in glycerol recovery, and a large amount of alkaline wastewater from the catalyst (7). Furthermore, oils containing free fatty acids and/or water are incompletely transesterified using a chemical catalyst (8). Enzymatic approaches can overcome these problems, since lipases can operate in moderate environments. More and more attention has focused on production of biodiesel via lipase (5). In order to reduce the operational costs, immobilized lipases have generally been used because immobilization can improve the operational stability of lipases and give reusable enzyme derivatives enabling the recycling of the enzyme (13).

Optimization of the reaction conditions involved in lipase-catalyzed biodiesel synthesis is usually done by varying one factor at a time and keeping the others constant. However, with this method it is difficult to explain the relationships between the variables and the response when there is interaction between the variables (10). The transesterification reaction is actually affected simultaneously by many factors such as methanol quantity, reaction temperature, reaction time and the content of lipases. Response surface methodology (RSM) is a popular and effective statistical technique and commonly used for the investigation of these types of complex processes (2). The optimal reaction conditions for biodiesel synthesis catalyzed by free lipases can also be obtained by this method.

In the present study, the enzymatic transesterification of *J. curcas* seed oil with a methanol system was studied in order to obtain high degrees of transesterification and high

contents of methyl esters. To determine the best operational conditions, a response surface analysis of several parameters including reaction time, temperature, lipase amount, methanol-to-oil molar ratio and *tert*-butanol-to-oil volume ratio, was performed. In addition, the properties of biodiesel were also analyzed by infrared spectroscopy and GC-linked mass spectrometry (GC-MS).

## Materials and Methods

Novozym435 (Nov435, immobilized *Candida antarctica* lipase) was purchased from Novo Nordisk (Bagsvaerd, Denmark). The specific activities of the lipases for hydrolysis of tributyrin were 3 000 U/g. *Jatropha curcas* L. seed oil was obtained locally with an average molecular weight 878.90 g/mol. All other purchased chemicals and reagents were of analytical grade.

### General procedure for enzymatic transesterification

The transesterification reactions were carried out in covered shaking flasks and heated to reaction temperature on a reciprocal shaker. The standard reaction mixture consisted of oil, *tert*-butanol, methanol, and immobilized lipases. Samples (100  $\mu$ L) were taken after 48 h and centrifuged to obtain the upper layer. Five-microliter samples were mixed with 95  $\mu$ L of hexane and 300  $\mu$ L of internal standard solution (heptadecanoic acid methyl ester hexane solution) for gas chromatographic analysis.

### Fatty acid methyl esters (FAME)

The fatty acid methyl ester content in the reaction mixture was analyzed on a GC-14B gas chromatograph equipped with an FFAP capillary column (0.32 mm $\times$ 25 m) and an FID detector. The column temperature was kept at 150  $^{\circ}$ C for 0.5 min, raised to 250  $^{\circ}$ C at 15  $^{\circ}$ C/min and maintained at this temperature for 10 min. The temperatures of the injector and detector were set at 245  $^{\circ}$ C and 250  $^{\circ}$ C, respectively. Nitrogen at 70 mL/min was used as the carrier gas. Pentadecanoic acid (C15:0, Sigma) methyl ester at 2 mg/mL was used as the internal standard. The conversion of biodiesel was calculated as the percentage by weight of fatty acid methyl esters formed divided by the weight of feed stock initially taken for the reaction.

### RSM experimental design

A five-level five-factor D-Optimal design and RSM were employed in this study, totally requiring 29 experiments. A Box–Behnken design was employed to study the response  $Y$ , namely, methyl conversion. The parameters of five independent variables were  $X_1$ ,  $X_2$ ,  $X_3$ ,  $X_4$  and  $X_5$  representing the amount of lipase to oil (w/w), *tert*-butanol-to-oil ratio (v/v), methanol-to-oil ratio (mol/mol), reaction temperature ( $^{\circ}$ C) and reaction time (h), respectively.

The settings for the independent variables were as follows (low/high value): methanol molar ratio (3:1 to 7:1), enzyme concentration (5 % to 15 %), *tert*-butanol-to-oil ratio (0.2 to 0.8), reaction time (4 h to 24 h) and reaction temperature (25  $^{\circ}$ C to 65  $^{\circ}$ C). The measured response was the methyl ester

yield (ME yield, %). The independent factors ( $X_i$ ), levels and experimental design are presented in **Table 1** and **Table 2**. Twenty-nine experiments were performed.

### Statistical analysis

The experimental data were fitted to the multiple linear regression (MLR) procedure. The following second-order polynomial equation was obtained by SPSS 16.0 and Matlab R2009b:

$$Y = \beta_0 + \sum_{i=1}^5 \beta_i X_i + \sum_{i=1}^5 \beta_i X_i^2 + \sum_{i=1}^4 \sum_{j=i+1}^5 \beta_{ij} X_i X_j$$

where  $Y$  is the response (fatty acid esters yield, wt%);  $X_i$  and  $X_j$  are the uncoded independent variables and  $\beta_0$ ,  $\beta_i$ ,  $\beta_i^2$ ,  $\beta_{ij}$  are intercept, linear, quadratic, and interaction coefficients, respectively. The fitness of the model was evaluated by the coefficient of determination ( $R$ ) and the analysis of variance (ANOVA). Quadratic polynomial equations were attained by holding one of the independent variances at a constant value and changing the level of the other variables.

### Fourier transform infrared analysis

The infrared absorption spectra of the samples were obtained in a Fourier transform infrared spectrometer (NICOLET 5700, Thermo Electron Corporation) using KBr tablets in the range of 4000  $\text{cm}^{-1}$  to 400  $\text{cm}^{-1}$ .

### Composition analysis of biodiesel

The composition of *J. curcas* L. seed oil was analysed by Thermo trace GC-MS (DSQII) equipped with a Varian VF-5ms column. The temperature of the ion source was 250  $^{\circ}$ C, and the scanning range was from 45 to 450.

### Fuel properties

The fuel properties, namely, density at 15  $^{\circ}$ C, viscosity at 40  $^{\circ}$ C, flash point, water content, ash content, carbon residue, acid value, free glycerol and total glycerol content of the produced biodiesel were determined by the prescribed methods and compared with the latest American and European standards (ASTM D6751-02, DINEN14214).

## Results and Discussion

### Response surface methodology for the optimization of the process variables

In this study, the applicability of *J. curcas* L. seed oil as a fatty acid esters feedstock and the optimization of the reaction parameters were investigated. In order to optimize the reaction condition of biodiesel synthesis, central composite design (CCD) with five-level-five-factors: i.e. reaction time, reaction temperature, lipase amount, methanol molar ratio and *tert*-butanol volume ratio were selected and presented in **Table 1**. Twenty-nine experimental values are shown in **Table 2**. The methyl ester yield ranged from 22.1 % to 88.5 %, and the design points of no.6 and no.19 gave the minimum and maximum yields, respectively. The fatty acid esters yield was significantly changed in all the combinations, indicating

that these parameters clearly affected the transesterification reaction.

**TABLE 1**

Independent variables and their levels for central composite design

Independent variables	Codes	Variable levels				
		-2	-1	0	+1	+2
Enzyme amount (%)	$X_1$	5	7.5	10	12.5	15
<i>Tert</i> -butanol to oil (v/v)	$X_2$	0.2	0.35	0.5	0.65	0.8
Methanol molar ratio	$X_3$	3	4	5	6	7
Reaction temperature (°C)	$X_4$	25	35	45	55	65
Reaction time (h)	$X_5$	4	8	12	16	20

**TABLE 2**

Central composite design and experimental data

Run	$X_1$	$X_2$	$X_3$	$X_4$	$X_5$	True model (%)	RSM model (%)
1	-1	0	-2	-2	-2	38.1	39
2	2	2	-2	-2	-2	50.2	50
3	2	-2	2	-2	-2	45.3	44.5
4	-2	2	2	-2	-2	46.3	45.9
5	-2	-2	-2	-1	-2	22.1	21.2
6	0	2	-1	1	-2	64.2	64.7
7	2	-2	-2	2	-2	43.1	41.5
8	-2	2	-2	2	-2	25.4	23.8
9	-2	-2	2	2	-2	23.2	22.4
10	2	2	2	2	-2	47.6	46.1
11	2	1	-2	1	-1	60.6	59.2
12	1	-1	-1	2	-1	64.1	64.3
13	-1	-2	0	-2	0	64.8	65.5
14	-2	2	-2	-2	1	49.2	48.9
15	0	1	2	-1	1	78.6	79.8
16	2	-1	1	0	1	81.7	85
17	2	-2	-2	-2	2	66.1	65.5
18	1	1	1	-2	2	88.5	88.1
19	-2	-2	2	-2	2	50.6	50.1
20	2	2	2	-2	2	76.5	75.7
21	-1	2	-2	-1	2	61.2	61.3
22	-2	1	-1	-1	2	61.4	61
23	-2	-2	-2	2	2	28.3	27.6
24	2	2	-2	2	2	46.7	45.5
25	2	-2	2	2	2	62	59.3
26	-2	2	2	2	2	28.3	27.3
27	0	0	0	0	0	87.8	84.9
28	0	0	0	0	0	86	84.9
29	0	0	0	0	0	88.5	84.9

**TABLE 3**

Estimated regression coefficient for experimental yield (%)

Term	Coefficient	Standard error	T value	P value
Constant	-182.721	10.994	-16.621	0.000
$X_1$	10.871	1.337	8.128	0.000
$X_2$	84.512	19.866	4.254	0.003
$X_3$	37.68	4.383	8.597	0.000
$X_4$	2.415	0.353	6.848	0.000
$X_5$	4.736	0.654	7.24	0.000
$X_1^2$	-0.43	0.061	-7.051	0.000
$X_2^2$	-25.819	18.401	-1.403	0.198
$X_3^2$	-3.349	0.432	-7.755	0.000
$X_4^2$	-0.023	0.004	-6.292	0.000
$X_5^2$	-0.099	0.025	-4.017	0.004
$X_1X_2$	-1.018	0.376	-2.708	0.027
$X_1X_3$	-0.064	0.056	-1.14	0.287
$X_1X_4$	0.008	0.006	1.4	0.199
$X_1X_5$	0.027	0.014	1.923	0.091
$X_2X_3$	0.013	0.935	0.014	0.989
$X_2X_4$	-0.558	0.096	-5.84	0.000
$X_2X_5$	-1.31	0.234	-5.598	0.001
$X_3X_4$	-0.036	0.014	-2.531	0.035
$X_3X_5$	-0.015	0.035	-0.432	0.677
$X_4X_5$	-0.023	0.004	-6.416	0.000
$R = 0.998 \quad R^2 = 0.996 \quad R^2(\text{adj}) = 0.987$				

The statistical significance of this model was evaluated by the F-test (**Table 3**), which indicated that this regression is statistically significant at a 99.998 % probability level. The coefficient of determination ( $R^2$ ) was 0.996, indicating that the model can explain 99.7 % of the variability. The  $T$  and  $P$  values along with the constant and coefficients are also given in **Table 3**. The significance of the regression coefficients of the parameters is determined by the  $T$  value, and the  $P$  value is defined as the smallest level of significance leading to the rejection of the null hypothesis (10). In conclusion, the larger the magnitude of  $T$  and smaller the value of  $P$ , the more significant is the regression coefficient (6). The effects of the linear terms, i.e. lipase amount, *tert*-butanol volume ratio, methanol molar ratio, temperature, time, were found to be highly significant ( $P < 0.000$ ), suggesting that there was a linear relation of these five parameters with the FAME yield. Likewise, the effects of the quadratic term of lipase amount, *tert*-butanol volume ratio, methanol molar ratio, temperature, and time were also evaluated and indicated that the quadratic terms of lipase amount, methanol molar ratio, temperature and time were significant ( $P < 0.000$ ), suggesting that there was a non-linear relationship between fatty acid esters yield and these parameters. Interaction terms  $X_1X_4$ ,  $X_2X_3$ ,  $X_3X_4$ ,  $X_3X_5$  and  $X_4X_5$  had a significant effect on the methyl ester yield.

The experimental results of this analysis were used to develop a linear equation showing the relationships between degree of conversion, molar ratio of methanol to oil, reaction temperature, reaction time, *tert*-butanol-to-oil volume ratio and lipase amount. The regression equation below was prepared by considering the significant terms and was shown as below:

$$Y = -182.721 + 10.871X_1 + 84.512X_2 + 37.68X_3 + 2.415X_4 + 4.736X_5 - 0.43X_1^2 - 25.819X_2^2 - 3.349X_3^2 - 0.023X_4^2 - 0.099X_5^2 - 1.018X_1X_2 - 0.064X_1X_3 + 0.008X_1X_4 + 0.027X_1X_5 + 0.013X_2X_3 - 0.558X_2X_4 - 1.31X_2X_5 - 0.036X_3X_4 - 0.015X_3X_5 - 0.023X_4X_5 \quad (\text{Eq. 1})$$

### Analysis of 3D surface plots

Eq. 1 suggested the presence of interactions between lipase amount and *tert*-butanol volume ratio, lipase amount and temperature, *tert*-butanol volume ratio and time, methanol molar ratio and temperature as well as between methanol molar ratio and time. Thus, it was of interest to further characterize the interactions in the range of process parameters studied. To investigate the individual and interactive effects of these two factors on FAME yield, 3D surface plots were drawn (MATLAB R2009b) and the inferences obtained are discussed below.

The influence of the methanol molar ratio and temperature on FAME showed significant variation both above and below the optimum values (Fig. 1a). An increase in FAME yield was observed with the increasing of the methanol molar ratio and temperature at first. However, the trend was reversed when the methanol molar ratio reached the certain value. The methanol molar ratio was one of the most important variables affecting FAME conversion, and the stoichiometric ratio for methanol to oil is 3:1. Although a molar ratio higher than the theoretical value would be needed to drive the reaction to completion in practice (11), higher methanol concentrations were found to cause irreversible denaturation of the lipase because methanol is insoluble in the oil at high concentrations, which made proteins unstable and deprived the enzyme of “indispensable water” (7).

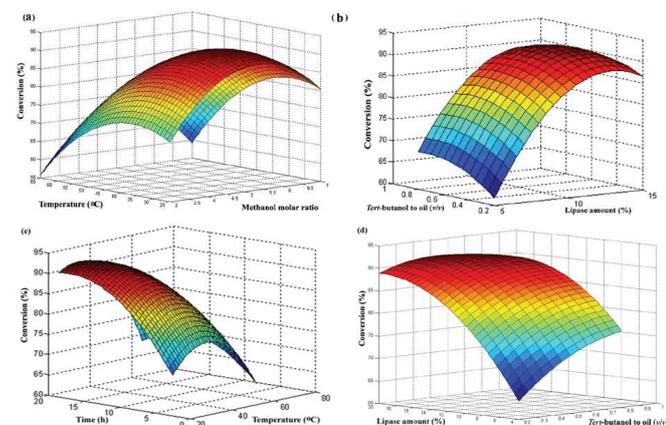


Fig. 1. 3D surface plots of the combined effect of methanol molar ratio and temperature (a), *tert*-butanol to oil (v/v) and the amount of lipase (b), time and temperature (c), lipase and *tert*-butanol to oil (v/v) (d).

The reaction temperature showed a strong effect on the activity and stability of the lipase. Relative higher temperature can activate the substrate molecules, reduce the viscosity of the reaction and lead to a higher ME yield. However, too high temperature might lead to lipase denaturation and loss of solvent through evaporation (11).

The effects of different lipase amount and *tert*-butanol volume ratio on the FAME yield are shown in Fig. 1b. The increase of *tert*-butanol volume ratio led to a small increment in the yield. This might be due to the ability of *tert*-butanol to improve the mixture of methanol and oil and ameliorate the lipase activity decrease caused by excessive presence of methanol. A further increase in the *tert*-butanol volume ratio caused a significant reduction [in the FAME yield], which might be due to dilution of the reactants with more *tert*-butanol present in the reaction mixture. It was also found that a lower dosage led to a lower FAME yield. When the lipase amount on the interface reached the maximum adsorptive capacity, the FAME yield could not be improved with increasing the lipase amount. Fig. 1c shows the effect of the interaction between *tert*-butanol and temperature. Longer time could promote the ME yield. A relatively longer time could reduce the effect of the dilution caused by adding too much *tert*-butanol. Fig. 1d depicts the effects of *tert*-butanol and lipase on the FAME yield. It was shown that the FAME yield was sensitive to the *tert*-butanol concentration and the temperature.

### Investigation of optimum operating conditions

The FAME yield was significantly affected by manipulation of the transesterification conditions, including the reaction time, temperature, the amount of lipase, methanol molar ratio and *tert*-butanol volume ratio. In order to acquire a relatively high production of FAME, the optimal values of the selected parameters were obtained by solving the regression Eq. 1 by MATLAB R2009b software. It was indicated that the optimum parameters for *J. curcas* L. seed oil were as follows: lipase amount ( $X_1$ ) of 12.435 % (w/w), *tert*-butanol-to-oil ratio of 0.577 (v/v), molar ratio of methanol to oil of 5.282, reaction temperature ( $X_4$ ) of 34.868 °C, and reaction time ( $X_5$ ) of 17.355 h. theoretical fatty acid ethyl ester content predicted under the above conditions was 88.5 %.

### Fourier transform infrared spectroscopy

The Fourier transform infrared spectrum of *J. curcas* L. seed oil is shown in Fig. 2a. The absorption bands at 2925.5  $\text{cm}^{-1}$  and 2855.5  $\text{cm}^{-1}$  were attributed to  $-\text{CH}_2-$  group and the band at 1745.2  $\text{cm}^{-1}$  to the carbonyl group. The band at 1163.3  $\text{cm}^{-1}$  was ascribed to C–O–C from the ester functional group and at 711.4  $\text{cm}^{-1}$  to the  $-(\text{CH}_2)_n-$  sequence of aliphatic chains of fatty acids. Fig. 2b displays the Fourier transform infrared spectrum of biodiesel from the studied *J. curcas* L. seed oil. The spectrum presented a band at 3007.3  $\text{cm}^{-1}$  ascribed to the H–C= group and a strong band at 1743.6  $\text{cm}^{-1}$  ascribed to the ester C=O axial deformation and two medium bands at 1171.1  $\text{cm}^{-1}$  and 1196.7  $\text{cm}^{-1}$  related to the C–O bond.

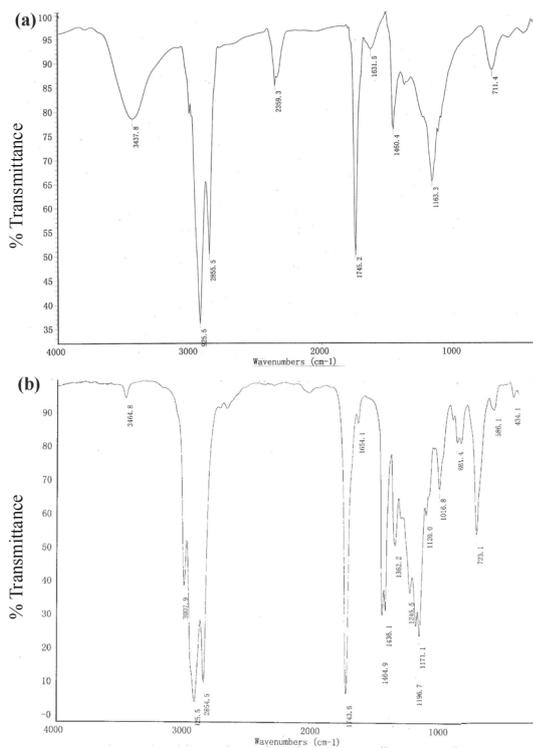


Fig. 2. Infrared spectroscopy of *Jatropha curcas* L. seed oil (a) and biodiesel (b).

### Qualitative analysis of FAME

The chromatogram of biodiesel from *J. curcas* L. seed oil (Fig. 3a) showed that there were seven fatty acid methyl esters, which were analyzed with MS. The composition of the biodiesel analyzed by GC-MS suggested that there were three main fatty acid methyl esters including hexadecanoic acid methyl ester ( $C_{17}H_{34}O_2$ ), 9,12-octadecadienoic acid methyl ester ( $C_{19}H_{34}O_2$ ) and 9-octadecadienoic acid methyl ester ( $C_{19}H_{36}O_2$ ) (Fig. 3b,c,d). These components made up more than 90 % of the total biodiesel. Some minor methyl esters were also detected and shown in Table 4. Polyunsaturated fatty acids with one or more double bonds, which are susceptible to oxidation during storage, thus reduce the acceptability for production of biodiesel (1). The GC-MS study demonstrated that the biodiesel from *J. curcas* L. seed oil contains mainly saturated and unsaturated fatty acids (~64 % of the total fatty acyl methyl esters), which advocated its high oxidative stability. Therefore, *J. curcas* L. seed oil could be considered as a potential alternative for biodiesel production.

### Fuel properties of biodiesel

Table 5 lists the fuel properties of biodiesel generated by the aforementioned optimized process. It was shown that *J. curcas* L. seed oil biodiesel meets the latest published American and European standards for biodiesel.

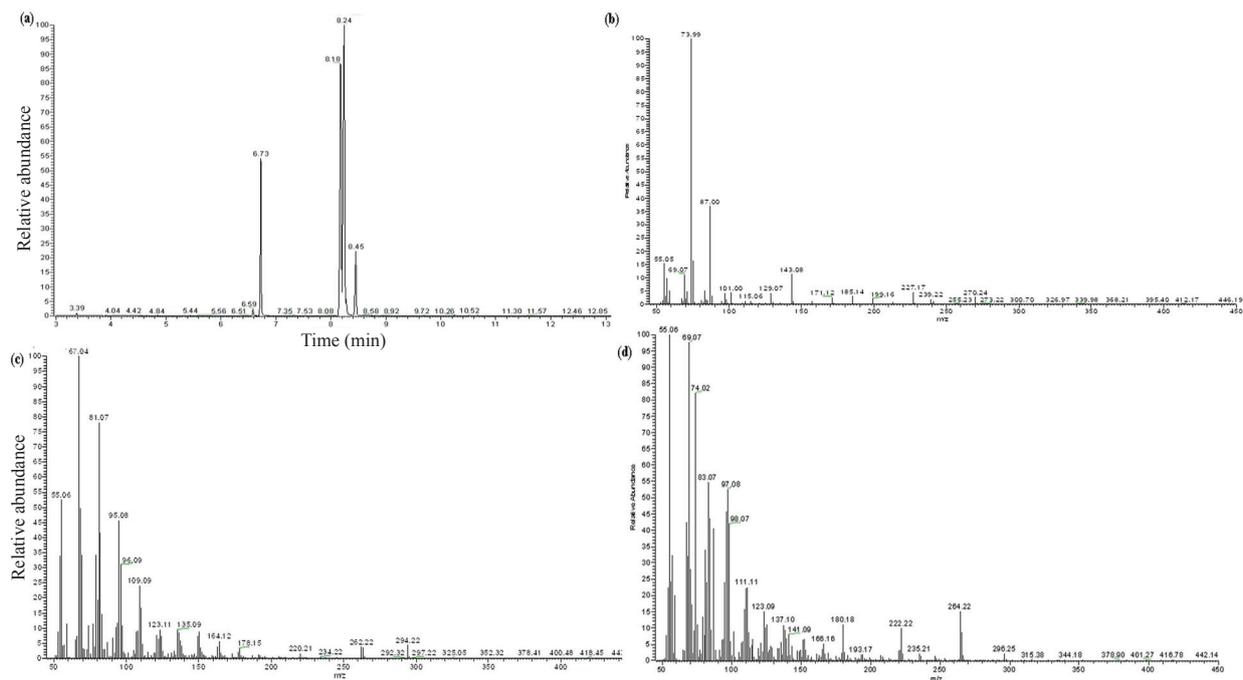


Fig. 3. GC-MS of biodiesel from *Jatropha curcas* L. seed oil. Gas chromatography spectrum of FAME (a); mass spectra of hexadecanoic acid methyl ester (b), 9,12-octadecadienoic acid methyl ester (c), and 9-octadecadienoic acid methyl ester (d).

TABLE 4

The retention times and content of each fatty acid methyl ester in biodiesel from *Jatropha curcas* L. seed oil

Fatty acid methyl ester content (%)	Retention time (min)	Content (%)
7-Hexadecanoic acid methyl ester (C <sub>17</sub> H <sub>32</sub> O <sub>2</sub> )	6.59	0.72
Hexadecanoic acid methyl ester (C <sub>17</sub> H <sub>34</sub> O <sub>2</sub> )	6.73	14.82
9,12-Octadecadienoic acid methyl ester (C <sub>19</sub> H <sub>34</sub> O <sub>2</sub> )	8.18	35.79
9-Octadecadienoic acid methyl ester (C <sub>19</sub> H <sub>36</sub> O <sub>2</sub> )	8.24	40.96
Octadecadienoic acid methyl ester (C <sub>19</sub> H <sub>38</sub> O <sub>2</sub> )	8.45	7.5
11-Eicosenoic acid methyl ester (C <sub>21</sub> H <sub>40</sub> O <sub>2</sub> )	10.26	0.07
Eicosenoic acid methyl ester (C <sub>21</sub> H <sub>42</sub> O <sub>2</sub> )	10.53	0.14

TABLE 5

Fuel properties of biodiesel from *Jatropha curcas* L. seed oil

Property	Value	DIN EN 14212	ASTM D6751-02
Viscosity at 40 °C (mm <sup>2</sup> /g)	4.87 ± 0.12	3.5–5.0	1.9–6.0
Density at 15 °C (g/cm <sup>3</sup> )	0.88 ± 0.2	0.86–0.90	0.87–0.89
Water content (mg/kg)	280 ± 7	<500	<300
Flash point (°C)	145 ± 9	>120	>130
Acid value (mg KOH/g)	0.041 ± 0.002	< 0.5	<0.8
Ash content (%)	0.0012	<0.02	<0.02
Carbon residue (%)	0.018 ± 0.002	<0.20	--
Free glycerol (wt%)	0.01 ± 0.002	<0.02	<0.02
Total glycerol (wt%)	0.15 ± 0.02	<0.25	<0.24

## Conclusions

In this study, *tert*-butanol was used as the reaction medium for the preparation of biodiesel production through immobilized lipase-catalyzed transesterification of rice bran oil and methanol. The optimized parameters were concluded from central composite design (CCD) and response surface methodology RSM analyses. Under the optimal conditions of RSM, biodiesel yield of 88.1 % could be obtained, and further verification experiments confirmed the validity of the predicted model. The fuel properties of biodiesel indicated that *J. curcas* L. seed oil can be considered as a potential alternative feedstock and the immobilized lipase-catalyzed irreversible transesterification reaction is a very promising technology for production of fatty acid esters.

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