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Optimization of the lipase-catalyzed irreversible transesterification of *Pistacia chinensis* Bunge seed oil for biodiesel production

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Production of biodiesel, a mixture of alkyl esters of fatty acids, by a lipase-catalyzed irreversible transesterification of *Pistacia chinensis* Bunge seed oil using dialkyl carbonate as an acyl acceptor is studied. Response surface methodology (RSM) based on central composite design (CCD) was used to optimize five important reaction variables. A quadratic model was established to modify those variables for high biodiesel yield. From the analysis of variance (ANOVA), the most important variables in the experimental design response were the reaction time, temperature, the molar ratio of oil to diethyl carbonate, and the amount of water added. The biodiesel yield predicted by the model was 98.2% under the optimal conditions and the subsequent verification experiments gave the average biodiesel yield of 97.6% thus confirming the validity of the predicted model.

Key words: optimization, lipases, irreversible transesterification, diethyl carbonate, plant oils, biodiesel, *Pistacia chinensis* Bunge.

The high demand of the industrialized world in energy and the pollution caused by fossil fuel utilization have made it necessary to develop alternative and renewable energy sources. One of the particular considered alternatives is biodiesel. Biodiesel shows many salient properties: its cetane number, flash point, and volumetric heating value are comparable to those of fossil fuels.[1] There are several advantages associated with the use of biodiesel as alternative, reversible energy source. Biodiesel is biodegradable, nontoxic, and eco-friendly, its lower CO_2 emission helps to reduce the impacts to global warming.[2]

Several processes are currently available for the production of biodiesel *via* transesterification, *e.g.*, chemical and enzymatic catalysis and non-catalytic supercritical alcohol treatment.[3—5] Chemical-catalyzed processes give high conversions of triacylglycerols to the corresponding esters but they suffer from several drawbacks. Chemical reactions are energy-intensive, require labor-consuming separation/purification steps to remove by-product (glycerol), and generate significant amount of wastewaters to be treated from catalyst and glycerol. Therefore, nowadays the enzymatic production of biodiesel receives growing attention and rapidly develops. The synthesis of biodiesel mediated by lipases occurs under mild conditions, moreover, the wastes are minimized, and glycerol is more easily purified. The enzymatic process tolerates the wide range of amount of water present in oil and increases the biodiesel yield by avoiding the soap formation. In addition, the immobilization of lipase on a support combines these advantages with the advantages of heterogeneous catalysis.[6,7] However, there are two bottlenecks in enzymatic approach for biodiesel production. One problem is short operational life of the lipase caused by the negative influence of an excessive short-chain alcohols.[8] It has been demonstrated that methanol is insoluble in vegetable oil, therefore, and the immobilized lipases are easily inactivated by contacting with insoluble methanol existing as drops in the oil. Another problem is the high cost of the feedstock. Presently, biodiesel is mainly produced from soybean, canola, palm, peanut, physical nut (*Jatropha curcas* L.), cotton, and sunflower oils. Some of those oils are commodities, whose prices are strongly depend on the market. This results in a direct competition for these feedstocks between the food industry and biodiesel production, which

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may be critical for a world with exponentially increasing population. Therefore, non-edible oil sources are preferable for the biodiesel production. Special attention should be given to the crops with low agronomic demand and a low cost of cultivation and harvesting, having a reasonable plant cycle, favorable geographic adaptability, and high oil content.[9]

Pistacia chinensis Bunge (Anacardiaceae) widely distributed in China is a deciduous tree with a scale bark. *P. chinensis* bears fruits from September to November producing the inverted, egg-shaped globular drupes. It has been observed that the fruits of *P. chinensis* are pink-colored in the initial stage. The ripe fruits can be either nearly blue (or green) in color when the seed is fully developed and plump or pink-colored when the fruit is empty and the seed inside is undeveloped.[10] *P. chinensis* is a kind of heliophile, can endure wind and is easy to sprout. The trees grow in slightly acidic, neutral or slightly alkaline soil (usually in limestone areas) indicating that it has strong adaptability to unfavorable nature environment and anti-adverse circumstances. According to investigation in 11 provinces, China has more than 66 700 ha of *P. chinensis* forest resources.* The oil content in seeds and kernels of *P. chinensis* is 42.5 and 56.5%, respectively. The oil-producing rate of *P. chinensis* seeds ranges from 20 to 30%. The oil of *P. chinensis* seeds is not edible because of its bitter taste. *P. chinensis* seeds have been seldom used as feedstock for biodiesel but offer good potential for this purpose. Therefore, it was of interest to study production of biodiesel from *P. chinensis* seed oil in detail.

Optimization of the reaction parameters involved in lipase-catalyzed biodiesel production is commonly made by varying only one factor (variable) at a time while keeping other factors fixed. This method has the flaw of not explaining the relationships between the explanatory variables and the response variables. Response surface methodology (RSM) is an effective statistical method to optimize complex processes. The main advantage of RSM is a reduced number of experiments to gain the information sufficient for the statistically acceptable results. Response surface methodology is also faster and less expensive method to collect research data than the traditional method.[11] Previously, RSM has been successfully applied for the optimization of biodiesel production from rapeseed oil, soybean oil, cottonseed oil, *etc.*[12,13] It should be noted that the central composite design (CCD) and response surface methodology have been successfully applied for the optimization of several biotechnological and chemical processes.[2]

In our previous study,[14] we reported on the biodiesel production by the lipase-catalyzed irreversible transesterification of cottonseed oil with dialkyl carbonates in organic solvent. This approach eliminates the risk of deactivation of the lipase by short-chain alcohol since it is generated *in situ* from short-chain dialkyl carbonates. This modification of the procedure results in an irreversible and significantly accelerated reaction. The present study was planned (i) to evaluate the precision of RSM analysis for optimization of lipase-catalyzed irreversible transesterification of *P. chinensis* seed oil in the solvent-free system and (ii) to describe the effects and relationships of the main reaction variables on the biodiesel yield with the aim to find the conditions providing the optimum biodiesel yield. Carrying out the transesterification in the solvent-free system allows cost reduction and improvement in the control of industry processes. The obtained data will then be used to optimize the biodiesel production conditions.

Experimental

Novozym 435 (lipase from *Candida Antarctica*), Lipozyme TL IM (lipase from *Thermomyces lanuginosus*), and Lipozyme RM IM (lipase from *Rhizomucor miehei*) were purchased from Novo Nordisk Co. (Denmark). Dimethyl carbonate (DMC), diethyl carbonate (DEC), and methyl acetate were obtained from Shanghai Chemical Co. (China). Methyl and ethyl esters of different fatty acid used as reference standards for gas chromatography were purchased from Sigma—Aldrich. All other commercially available chemicals and reagents were of analytical grade. All organic solvents were dried for several days over molecular sieves 4 Å before use.

The *P. chinensis* seeds were obtained from Shuyang East Lake Garden Center, Jiangsu Province, China. The *P. chinensis* seeds (500 g) were crushed using a commercial grinder and charged to a Soxhlet apparatus fitted with a 2 L round-bottom flask. The extraction was carried out for 6 h with refluxing *n*-hexane (water bath heating). The solvent was removed under vacuum (T_{bath} = 45 °C) using a rotary evaporator. Deionized water (taken in amount of 7% of the oil weight) was added to the crude oil heated to 60 °C and the mixture was kept at 70 °C for 6 h. The oil sediment was removed and the hydrated oil was evaporated under vacuum to remove volatiles. The resulted *P. chinensis* seed oil was used in this study. The acid number, saponification value, and fatty acid components of this oil were determined according to the Chinese national standards GB5534-85, GB5530-1998, and GB/T17376-1998, respectively. The acid number was 3.13 (mg KOH) g^{-1}, saponification value was 211.72 (mg KOH) g^{-1}. Triacylglycerides found in this oil contained the residues of the following acids: myristic (14:0) (0.4%), palmitic (16:0) (15.3%), palmitoleic (16:1) (1.4%), stearic (18:0) (1.1%), oleic (18:1) (51.4%), linoleic (18:2) (28.0%), and linolenic (18:3) (2.4%) acids. The average molecular weight of the oil calculated according to the fatty acid composition is 806.84. The moisture content determined by Karl Fisher titration is 0.0363 vol.%.

Lipase-catalyzed irreversible transesterification of *P. chinensis* seed oil was carried out varying five variables according to the CCD given in Tables 1 and 2. The 10 ml screw-caped glass vials

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* The data of Deutsche Gesellschaft für Technische Zusammenarbeit, 2006.

Table 1. Variables, their codes, and experimental values of the variable used in the central composite design

Variable	Code	Variable level				
		−2	−1	0	1	2
Reaction time/h	x_1	4	8	12	16	20
Reaction temperature/°C	x_2	25	35	45	55	65
Lipase amount (wt.%)*	x_3	5	7.5	10	12.5	15
DEC—oil ratio	x_4	1.5 : 1	3 : 1	4.5 : 1	6 : 1	7.5 : 1
Amount of added water (wt.%)*	x_5	0	0.75	1.5	2.25	3

* Based on the oil weight.

were charged with *P. chinensis* seed oil (2 g) and different amounts of DEC followed by addition of different amounts of water (0—3 wt.%) and lipase (5—15 wt.%). The obtained mixtures were stirred using an orbital shaker (180 rpm) varying the reaction temperature and reaction time. Samples (3 μL) taken from the reaction mixture were mixed with 20 m*M* methyl heptadecanoate in *n*-heptane (7.5 μL) (internal standard), and *n*-heptane (139.5 μL) and analyzed by gas chromatography.

Gas chromatography of the samples was performed with an Agilent 6890 instrument equipped with HP-5 capillary column

Table 2. Central composite design matrix and responses (biodiesel yield) found experimentally and predicted by the regression model

Run		Point type	Block	Variable					Biodiesel yield (%)	
Formal	Actual			x_1	x_2	x_3	x_4	x_5	Experiment	Prediction
1	*25*	−1	2	0	−2	0	0	0	23.4	24.6299
2	*31*	−1	2	0	0	0	0	−2	94.7	89.4966
3	*33*	0	2	0	0	0	0	0	81.2	78.9005
4	*27*	−1	2	0	0	−2	0	0	62.0	65.3633
5	*26*	−1	2	0	2	0	0	0	94.7	93.9299
6	*29*	−1	2	0	0	0	−2	0	46.4	57.4466
7	*28*	−1	2	0	0	2	0	0	87.3	84.3966
8	*23*	−1	2	−2	0	0	0	0	26.5	40.5633
9	*32*	−1	2	0	0	0	0	2	32.5	38.1633
10	*30*	−1	2	0	0	0	2	0	84.7	74.1133
11	*24*	−1	2	2	0	0	0	0	87.9	74.2966
12	*14*	1	1	1	−1	1	1	−1	83.3	91.2392
13	*8*	1	1	1	1	1	−1	−1	96.8	99.8309
14	*17*	0	1	0	0	0	0	0	82.2	81.8233
15	*6*	1	1	1	−1	1	−1	1	28.7	28.5142
16	*11*	1	1	−1	1	−1	1	1	69.6	66.8392
17	*21*	0	1	0	0	0	0	0	81.9	81.8233
18	*12*	1	1	1	1	-1	1	−1	84.4	91.2725
19	*18*	0	1	0	0	0	0	0	80.4	81.8233
20	*19*	0	1	0	0	0	0	0	82.0	81.8233
21	*7*	1	1	−1	1	1	−1	1	77.6	70.9975
22	*13*	1	1	−1	−1	1	1	1	27.3	25.6059
23	*16*	1	1	1	1	1	1	1	86.7	92.4225
24	*15*	1	1	−1	1	1	1	−1	82.9	84.4225
25	*5*	1	1	−1	−1	1	−1	−1	57.4	53.0142
26	*2*	1	1	1	−1	−1	−1	−1	65.4	66.3642
27	*1*	1	1	−1	−1	−1	−1	1	23.8	15.1309
28	*3*	1	1	−1	1	−1	−1	−1	77.8	72.3475
29	*20*	0	1	0	0	0	0	0	80.9	81.8233
30	*9*	1	1	−1	−1	−1	1	−1	52.7	52.1559
31	*22*	0	1	0	0	0	0	0	81.7	81.8233
32	*10*	1	1	1	−1	−1	1	1	33.7	37.3559
33	*4*	1	1	1	1	−1	−1	1	69.7	68.4475

(5% methylphenylsiloxane, 30 m×320 μm, film thickness 0.25 μm). The column temperature was maintained at 180 °C for 1 min, heated to 300 °C at heating rate of 10 °C min^{-1}, and then maintained for 2 min. The temperatures of the injector and detector were 260 °C and 280 °C, respectively. All samples (1 μL, see above for the sample preparation) were measured in triplicate with reproducibility being always within 3%. Biodiesel yield was defined as an amount of the produced fatty acid esters divided by initial amount of *P. chinensis* seed oil.

Experimental design. All experiments on the lipase-catalyzed irreversible transesterification of *P. chinensis* seed oil for biodiesel production was carried out applying a CCD. Design matrix and responses for CCD are given in Table 2. Central composite design helps in investigating linear, quadratic, cubic, and cross-product effects of the reaction conditions on the biodiesel yield. Five independent variables studied were the reaction time, reaction temperature, the lipase amount, the oil—DEC molar ratio (further, substrate molar ratio), and the amount of water added. Codes, ranges, and levels of the five variables are listed in Table 1. The levels of the variables were selected based on the results of our previous work.[14] All variables at zero level constitute the center points and the combinations of each of the variables at either their lowest (–2.0) or highest (+2.0) level with other variables at zero level constitute the axial points. The experiment sequence was randomized to minimize the effects of the uncontrolled factors.

Statistical Analysis. The experimental data obtained following the above procedure were analyzed by RSM second-order polynomial equation (1)

$$Y = \beta_{k0} + \sum_{i=1}^{5}\beta_{ki}x_i + \sum_{i=1}^{5}\beta_{kii}x_i^2 + \sum_{i=1}^{4}\sum_{j=i+1}^{5}\beta_{kij}x_ix_j \qquad (1)$$

where Y is the response (biodiesel yield, wt.%); x_i and x_j are the coded independent variables (see Table 1), β_{k0} is the intercept, β_{ki} are the linear effects, β_{kii} are the quadratic effects, β_{kij} are the interaction effects. For regression analysis and analysis of variance (ANOVA), we used MINITAB release 14 software. Response surface plots were visualized using the fitted quadratic polynomial equation obtained from regression analysis, holding two of the independent variables at a constant value corresponding to the stationary point and changing the other two variables. Confirmatory experiments were carried out to validate the obtained equation using combinations of independent variables that were not included in the original experimental design but were within the factor space.

Results and Discussion

Selection of lipase for irreversible transesterification. Although lipases exhibit high catalytic activity and selectivity, it is widely known that these properties are strongly dependent on the particular reaction. To select a suitable lipase for the irreversible transesterification of *P. chinensis* seed oil in the solvent-free system, three commercially available lipases (Novozym 435, Lipozyme TL IM, and Lipozyme RM IM) were tested. Novozym 435 showed highest activity towards transesterification of the *P. chinensis* seed oil providing the biodiesel yield of 96.9 and 95.2% for the DMC and DEC acyl acceptors, respectively (Table 3). Therefore, we used only Novozym 435 for the following optimization of biodiesel production.

Comparison of different acyl acceptors for biodiesel production form *P. chinensis* seed oil. Some short-chain alcohols (especially methanol and ethanol) have conventionally been used as acyl acceptors in the lipase-catalyzed transesterification of oils for biodiesel production.[15] However, excessive alcohols could to some extent inactivate the lipases.[16,17] Although stepwise addition of alcohols may decrease the inactivation of lipases, this must simultaneously increase the operational complexity. Xu *et. al.* described a new enzymatic route for production of fatty acid methyl esters from soybean oil using methyl acetate as an acyl acceptor,[18] but this method requires considerable amounts of lipases and excessive amounts of methyl acetate to achieve high conversions. It should be emphasized that the drawback of the oil transesterification with alcohol or methyl acetate is a reversibility of the reaction. Therefore, the transesterification involving DMC or DEC as the acyl acceptors is irreversible thus it was shown on the example of biodiesel production in the organic solvent.[14] In order to investigate the possibility to utilize DMC and DEC as acyl acceptors for our purpose in the solvent-free system, we compared different acyl acceptors under the same condition. In fact, application of DMC or DEC provides ~1.5 and ~10 fold higher yields of the target esters than in the

Table 3. Effects of the nature of lipase and acyl acceptor on transesterification of *P. chinensis* seed oil*

Acyl acceptor	Biodiesel yield (%)					
	Novozym 435		Lipozyme TL IM		Lipozyme RM IM	
	8 h	24 h	8 h	24 h	8 h	24 h
DMC	82.5	96.9	0.67	1.47	2.78	12.1
DEC	77.0	95.2	1.97	6.40	4.43	14.8

* Reaction conditions: ratio of oil : dialkyl carbonate = 1 : 3, lipase 10 wt.% (based on the oil weight), 45 °C, frequency of rotation of 200 rpm.

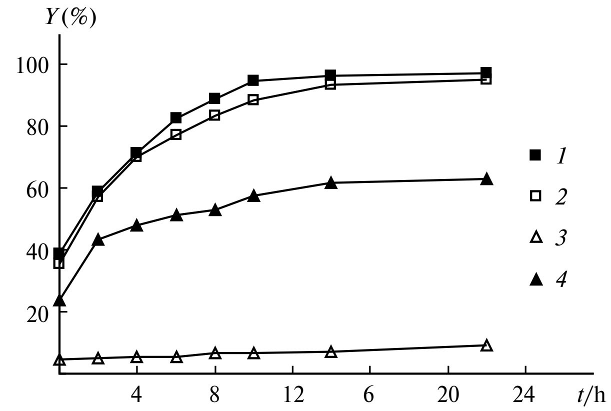

Fig. 1. Solvent-free lipase-catalyzed transesterification of *P. chinensis* seed oil with DMC (*1*), DEC (*2*), methanol (*3*), and methyl acetate (*4*) as acyl acceptor. Reaction conditions: ratio of seed oil to acyl acceptor of 1 : 3, lipase Novozym 435 (10 wt.%, based on the oil weight), 45 °C, 24 h, frequency of rotation of 200 rpm. *Y* is a biodiesel yield, *t* is a reaction time.

case of methyl acetate and methanol, respectively (Fig. 1). Note that the advantage of DMC and DEC over other acyl acceptors is more obvious in the solvent-free system than in organic solvent system.[14] The differences in the yields of biodiesel provided by DMC and DEC are insignificant (see Fig. 1); therefore, in the present study we used DEC since transesterification using DMC in the organic solvent system had been earlier investigated in detail.[14]

Optimization of the process variables by the application of response surface methodology. ***Designing the experiments: central composite design.*** To obtain regression model, five-level five-factor rotatable design was employed to evaluate the effects of the following variables: (1) the reaction time, (2) the reaction temperature, (3) the lipase amount, (4) the substrate molar ratio, and (5) the amount of water added. Experimental design matrix consist of 33 sets of coded conditions: 16 factorial points, 6 center points, 10 star points, and 1 added center point (see Table 2). High biodiesel yield were observed for all combinations of the variables, consequently, these variables significantly affected the irreversible transesterification of *P. chinensis* seed oil.

Interpretation of the regression analysis data. The coefficients of the quadratic polynomial model calculated from CCD and the corresponding *t*- and *p*-values are given in Table 4. The *t*-value was used to determine the significance of the regression coefficients and the *p*-value was defined as the smallest level of significance leading to rejection of the null hypothesis. In general, the larger the magnitude of the *t*-value and the smaller the *p*-value, the more significant was the corresponding coefficient term.[19]

The intercept is equal to 80.3619, which was significant because the *t*- and *p*-values are 21.848 and 0.000,

Table 4. Regression coefficient found experimentally using the central composite design*

Regression term	Regression coefficient	Standard error for the coefficient	*t*-Value	*p*-Value
Intercept	80.3619	3.678	21.848	0.000
x_1	8.4333	1.999	4.218	0.001
x_2	17.3250	1.999	8.666	0.000
x_3	4.7583	1.999	2.380	0.036
x_4	4.1667	1.999	2.084	0.061
x_5	−12.8333	1.999	−6.419	0.000
x_1x_1	−5.3676	1.782	−3.013	0.012
x_2x_2	−4.9051	1.782	−2.753	0.019
x_3x_3	−1.0051	1.782	−0.564	0.584
x_4x_4	−3.2801	1.782	−1.841	0.093
x_5x_5	−3.7676	1.782	−2.115	0.058
x_1x_2	−1.2625	2.449	−0.516	0.616
x_1x3	1.3125	2.449	0.536	0.603
x_1x_4	1.9750	2.449	0.807	0.437
x_1x_5	−2.4125	2.449	−0.985	0.346
x_2x_3	1.3375	2.449	0.546	0.596
x_2x_4	−1.2500	2.449	−0.511	0.620
x_2x_5	6.6875	2.449	2.731	0.020
x_3x_4	1.0000	2.449	0.408	0.691
x_3x_5	−1.0375	2.449	−0.424	0.680
x_4x_5	0.7250	2.449	0.296	0.773

* The standard deviation between experimental and predicted values $S = 3.794$, the coefficient of determination $R^2 = 98.1\%$, the adjusted coefficient of determination $R^2_{\text{adj}} = 95.9\%$.

respectively. The intercept (equal to the arithmetical mean of all experimentally found response values (80.3619%)) does not depend on the variables and the interaction of the variables. This fact indicates that the average biodiesel yield also does not depend on the experimental variables. The linear effects (β_i) of the regression, *i.e.*, the coefficients for the reaction time (x_1), the reaction temperature (x_2), the lipase amount (x_3), and the amount of water added (x_5), were found to be significant ($p = 0.001$, 0.000, 0.036, and 0.000, respectively) since their p-values are less than the adopted significance level of 0.05 (see Tables 1 and 4). Thus, the biodiesel yield is linearly dependent on these four variables. Whereas the effect for the substrate molar ratio (x_4) ($p = 0.061$) was not found to be significant. The quadratic effects (β_{ii}) of the regression were analyzed likewise and it was found that the quadratic terms of the reaction time and the reaction temperature were significant ($p = 0.012$ and 0.019, respectively). Since the reaction time and the reaction temperature are significant, the relationship between the biodiesel yield and these parameters have to be curvilinear. All interaction effects (β_{ij}), with exception for the coefficient for the x_2x_5 term (reaction temperature—amount of the added water, $p = 0.020$), were found to be insignificant. The sign on the coefficient (positive or negative) indicates the direction of the effect. It was found that the coefficients for the linear terms (amount of added water), all quadratic variables, and the interaction terms x_1x_2 (time—temperature), x_1x_5 (time—amount of water added), x_2x_4 (temperature—substrate molar ratio), x_3x_5 (lipase amount—amount of added water) are negatively associated with the biodiesel yield. That means that with an increase in these variables the biodiesel yields decreases. Whereas the coefficients for other linear terms (time, reaction temperature, lipase amount, and substrate molar ratio) and the interaction terms x_1x_3 (time—lipase amount), x_1x_4 (time—substrate molar ratio), x_2x_3 (temperature—lipase amount), x_2x_5 (temperature—amount of added water), x_3x_4 (lipase amount—substrate molar ratio), x_4x_5 (substrate molar ratio—amount of water added) are positive, consequently, an increase in these variables leads to an increase in the biodiesel yield. The final regression equation (2) comprising found coefficients is:

$$
\begin{aligned}
Y = {} & 80.3619 + 8.43333x_1 + 17.3250x_2 + 4.75833x_3 + \\
& + 4.16667x_4 - 12.8333x_5 - 5.36765x_1^2 - 4.90515x_2^2 - \\
& - 1.00515x_3^2 - 3.28015x_4^2 - 3.76765x_5^2 - \\
& - 1.26250x_1x_2 + 1.31250x_1x_3 + 1.97500x_1x_4 - \\
& - 2.41250x_1x_5 + 1.33750x_2x_3 - 1.25000x_2x_4 + \\
& + 6.68750x_2x_5 + 1.00000x_3x_4 - 1.03750x_3x_5 + \\
& + 0.725000x_4x_5. \qquad (2)
\end{aligned}
$$

The low value of standard deviation between the experimental and predicted results ($S = 3.794$) showed that Eq. (2) adequately represented actual relationship between the response and significant independent variables. Furthermore, the high values of coefficient of determination R^2 (98.1%) and adjusted coefficient of determination R^2_{adj} (95.9 %) indicated a high dependence and correlation between the observed and predicted values of response. This also indicated that the statistical model explained 98.1% of the total variations.

The statistical difference between the means of the regression was tested using analysis of variance (ANOVA). The main statistics in ANOVA is the F-test which is denoted as a ratio of the mean square for the regression term (MSR) and mean square error (MSE). According to Table 5, the F-statistics for the regression is high enough (88.0) indicating that the most of the response variance could be explained by the regression model. To estimate that the F-statistics was high enough to be statistically significant, the corresponding p-value was used. If $p < 0.05$, the model

Table 5. Analysis of variance for the variables affecting the biodiesel yield*

Source of variance	Degrees of freedom	SS_{seq}	SS_{adj}	MS_{adj}	F-Value	p-Value
Regression	20	16877.1	16877.1	843.85	88.0	0.000
linear	5	13823.4	13823.4	2764.68	28.82	0.000
square	5	2034.3	2034.3	406.86	4.24	0.021
Interaction	10	1019.4	1019.4	101.94	1.06	0.458
Residual	11	1055.2	1055.2	95.93	—	—
lack of fit	6	1052.7	1052.7	175.45	349.73	0.000
pure error	5	2.5	2.5	0.50	—	—
Total	32	17994.9	—	—	—	—

* SS_{seq} is a sequential sum of squares, SS_{adj} is an adjusted sum of squares, MS_{adj} is an adjusted mean squares.

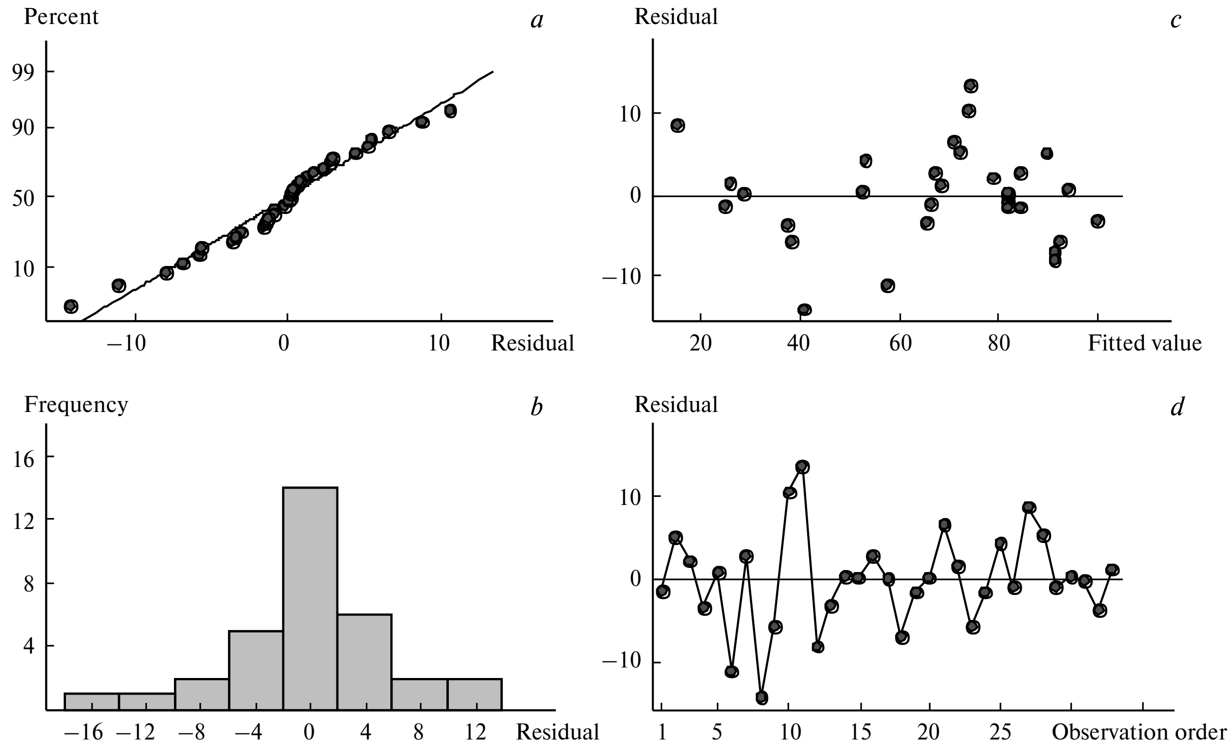

Fig. 2. *a*, Normal probability plot for the residuals; *b*, plot of the histograms of the residuals for all observations; *c*, plot of the residuals *vs.* fitted values; *d*, plot of the residuals *vs.* the order of the data collecting.

is statistically significant.[20] Data given in Table 5 also indicates that the linear ($p = 0.000$) and square ($p = 0.021$) effects are highly significant confirming the applicability of the predicted model. The ANOVA table (see Table 5) also showed no residual error; consequently, the data for the response variance can be very well explained by the regression model.

Interpretation of the residual graph. The normality of the data can be checked by plotting the normal probability plot (NPP) of the residuals. The NPP is a graphical technique for assessing whether or not a data set is approximately normally distributed.[21] The residual is the difference between the observed and predicted values of the regression. If the points on the plot form a nearly linear pattern, the data are normally distributed. Figure 2, *a* shows normal probability plot of the residuals. It can be seen that the experimental points are reasonably aligned suggesting normal distribution. Figure 2, *b* presents the same results but as a histogram of residuals showing the distribution of the residuals for all observations. The bell shaped symmetrical histogram (see Fig. 2, *b*) is characteristic of a zero-mean normal distribution. The residuals *versus* the fitted values (predicted responses) are plotted on Fig. 2, *c*. The residuals are randomly distributed about zero, *i.e.*, the errors have a constant variance. On Fig. 2, *d*, the plot of the residuals *versus* the order of the observations in CCD is given. This plot can be useful if the order of the observations influences the results. This can occur when the data were collected in a line sequence; therefore, this plot can be used to design a non-randomized experiment. In our case concerning the optimization of biodiesel production, the residuals are randomly distributed around zero and therefore the regression terms do not correlate with each other.

Analysis of the 3D response surface plots. The 3D response surfaces, a three dimensional graph representing the response as a function of two variables, were used to explore the individual and combined effects of the variables and the mutual interactions between the variables and the response (Fig. 3). Upon the response surface analysis, we explore the geometry of the surface, the maxima and minima in the response function, and the significance of the coefficients of the canonical equation. To obtain the optimum parameters, the minimum/maximum optimization problem should be solved (finding the absolute maximum or minimum value of a function over a given interval).[19]

Equation (2) suggests the presence of the following two-factor interactions: x_1x_3 (reaction time—lipase amount), x_1x_4 (reaction time—substrate molar ratio), x_2x_3 (temperature—lipase amount), x_2x_5 (temperature—amount of water added), x_3x_4 (lipase amount—substrate molar ratio), and x_4x_5 (substrate molar ratio—amount of water added). It should be emphasized that the x_2x_5 two-factor interaction is the most significant. Therefore, it was of great interest to characterize further the interactions in the studied range of the process variables. To investigate the combined effect of two variables on the biodiesel yield, three-dimensional plots were drawn using Minitab software.

A 3D surface response plot showing the effects of the reaction time (x_1) and the lipase amount used (x_3) on the biodiesel yield is given on Fig. 3, *a*. It is obvious that the effect of the lipase loading on the biodiesel yield is not significant: the increase in the amount of lipase (x_3) leads only to a small increment in the yield. However, increasing the x_3 value and decreasing the reaction time (x_1) can be used to attain the highest biodiesel yield. The effect of the reaction time (x_1) and the substrate molar ratio (x_4) on the biodiesel yield is shown on Fig. 3, *b*. With the increase in the substrate molar ratio (x_4), the yield of biodiesel increases until the maximum yield is obtained and then decreases. This pattern of the plot can be explained by the inhibitory effect of DEC similarly to the inhibitory effect of DMC found earlier[14] during the enzymatic transesterification of vegetable oils in the organic solvent system. Figure 3, *c* represents the effect of the x_2x_3 two-factor interaction (temperature—lipase amount) on the biodie-

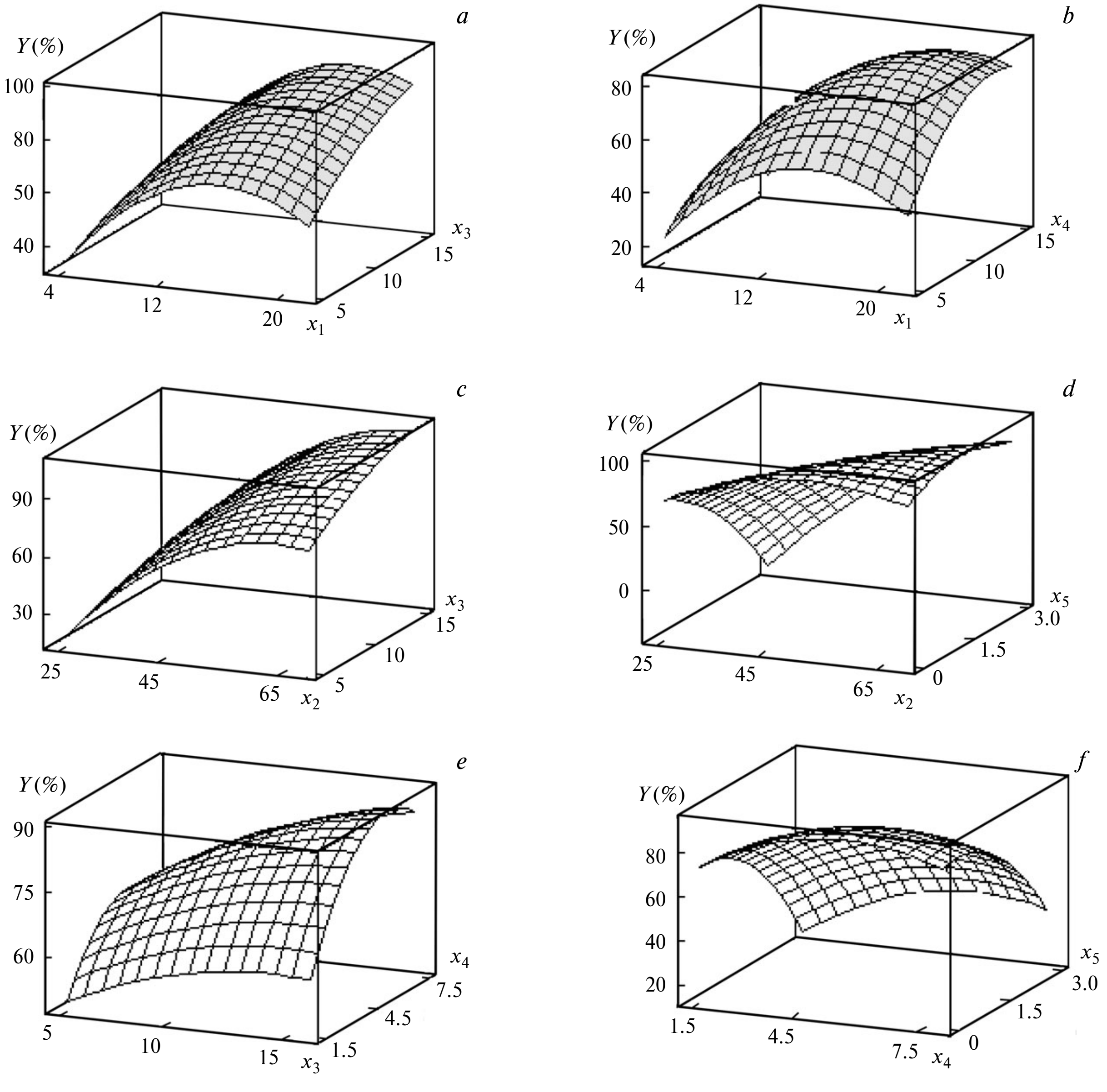

Fig. 3. 3D Response surface plots for the biodiesel yield (*Y*) as a function of x_1 and x_3 (*a*), x_1 and x_4 (*b*), x_2 and x_3 (*c*), x_2 and x_5 (*d*), x_3 and x_4 (*e*), and x_4 and x_5 (*f*); x_1 is the reaction time (h), x_2 is the reaction temperature (°C), x_3 is the lipase amount (wt.%), x_4 is the DEC—oil molar ratio, x_5 is the amount of the added water (wt.%).

sel yield. From Fig. 3, *c* it follows that the reaction temperature (x_2) is a very significant variable and the high biodiesel yield can be achieved increasing the reaction temperature even at small loadings of the lipase (*e.g.*, 5%). The analysis of the 3D response surface shown on Fig. 3, *d* indicates that the amount of water added (x_5) is also a significant variable. At 25 °C and large amounts of water added (3%), the biodiesel yield is very low (nearly equal to zero). However, at the temperature elevated to 65 °C, the amount of water added exerts less pronounced effect on the biodiesel yield. This fact can be due to both the formation of fine dispersion of water in the oil and evaporation of water at the elevated temperature, which is reduced the water content in the reaction mixture. The inhibitory effect of the DEC excess is less pronounced at the large loadings of the lipase since catalytic effect of the lipase partly offsets the inhibitory effect of DEC (Fig. 3, *e*). High substrate molar ratio can weaken to some extent the negative effect of water added because of its dilution effect (Fig. 3, *f*).

Process optimization. In the production of biodiesel, relatively high product yields were expected for economic feasibility. The yield of biodiesel can be enhanced by manipulating the transesterification conditions such as the reaction time, the temperature, the catalyst loading, the substrate molar ratio, and the amount of added water. The optimal values of the selected variables were obtained by solving the regression Eq. (2) using MINITAB software. The optimal conditions for the biodiesel production from *P. chinensis* seed oil estimated by the model Eq. (2) were as follows: the reaction time (x_1) of 12.9 h, the temperature (x_2) of 43.8 °C, the lipase amount (x_3) of 14.6 wt.%, the ratio of DEC to *P. chinensis* seed oil (x_4) of 3.9 : 1, and the amount of added water (x_5) of 0 wt.%. The theoretical yield of fatty acid ethyl ester under the these conditions predicted by Eq. (2) is $Y = 98.2\%$. In order to verify the obtained model, five independent replicates for biodiesel production from *P. chinensis* seed oil were performed under the optimal reaction conditions. An average biodiesel yield was found to be 97.6%; this value is within the values calculated by of model Eq. (2) with a relatively insignificant error of 0.6%. It can be concluded that the proposed regression model is statistically acceptable for the predicting the biodiesel yield produced *via* solvent-free lipase-catalyzed irreversible transesterification.

This study focused on the application of RSM for the optimization of the conditions for the biodiesel production from *P. chinensis* seed oil using lipase as a catalyst. This investigation provided useful information regarding the development of economic and efficient processes for the biodiesel production from *P. chinensis* seed oil by a solvent-free lipase-catalyzed irreversible transesterification.

In summary, this study showed that (i) *P. chinensis* Bunge seed oil can be considered as an alternative feedstock for the biodiesel production, and (ii) the lipase-catalyzed irreversible transesterification using DEC as an acyl acceptor is a promising technology for the biodiesel production. Based on the central composite design, quadratic polynomial model was obtained to predict the biodiesel yield. Analysis of the experimental data by RSM revealed that the optimum reaction conditions for the irreversible transesterification of *P. chinensis* seed oil to biodiesel were as follows: the reaction time of 12.9 h, the temperature of 43.8 °C, the lipase amount of 14.6%, the seed oil : DEC = 3.9 : 1, and the absence of moisture. Under these predicted optimum conditions, the theoretically calculated biodiesel yield reached to 98.2%; while, the experimentally obtained biodiesel yield was 97.6%. The developed mathematical model was validated and proven to be statistically adequate and accurate to predict the optimal yield of biodiesel.

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