Optimization of Biodiesel Production Using Immobilized *Candida Rugosa* Lipase on Magnetic Fe_3O_4-Silica Aerogel

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ABSTRACT: *Hydrophobic magnetic silica aerogel was used as a support to immobilize Candida rugosa lipase by adsorption method. Physical and chemical properties of the support and immobilized lipase were determined by Field Emission Scanning Electron Microscope (FESEM), Brunauer–Emmett–Teller (BET) analysis and Fourier Transform InfraRed (FT-IR) spectroscopy and the results showed that the lipase was successfully immobilized onto the support. Biodiesel production from sunflower oil using immobilized lipase was investigated. Response Surface Methodology (RSM) was employed to evaluate the effect of process variables namely methanol/oil molar ratio (4:1-6:1), enzyme concentration (4-10 % mass fraction of oil) and water concentration (3-10 % mass fraction of oil) on biodiesel yield and predict the optimal reaction conditions. A second-order regression model with high coefficient determination value (R^2= 0.99) was fitted to predict the response as a function of reaction parameters. The results indicated that optimum values for methanol/oil molar ratio, enzyme concentration, and water concentration were obtained at 4.5:1, 9.4% and 7.4 %, respectively, in which biodiesel yield was predicted at 72.3%. As the difference between the experimental and predicted values were shown as non-significant, the response surface model employed could be considered as adequate.*

KEYWORDS: *Biodiesel; Magnetic silica aerogel; Candida Rugosa Lipase; Transesterification; Response SurFace methodology (RSM); Optimization.*

INTRODUCTION

Biodiesel production has received considerable attention during the past decade as an alternative energy source to conventional fuel. It is nonpolluting fuel and combines environmental friendliness with biodegradability, low toxicity and renewability [1,2]. Several sources are available as feedstock for biodiesel

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production, such as animal fats and vegetable oils. Vegetable oils are more widely used than other sources [3]. Biodiesel is generally produced through a transesterification reaction. In this reaction, triglycerides react with alcohol to form a mixture of glycerol and fatty acid alkyl esters, called biodiesel [4]. This reaction can be catalyzed either chemically or enzymatically. Enzymatic transesterification using lipase as a biocatalyst eliminates the reactions catalyzed under acid or alkali conditions by yielding product of very high purity in mild temperatures and allows the synthesis of specific alkyl esters and easy recovery of glycerol [1, 5]. Lipases have been found in many species of animals, plants, bacteria, yeast, and fungi. Among them, microbial lipases have advantages including their small generation time, high yields and catalytic activities, simple cultivation conditions and ease of genetic manipulation which make them suitable for industrial applications. Funguses are the main microbial sources to produce lipase. Yeast C. *rugosa* lipases are very important biocatalytic tool in different fields of industry and science due to their high activity and non-genotoxic or cancerogenic effects on human health [6]. However, utilization of lipases for biodiesel production is often limited owing to its lack of stability under processing conditions and the difficulty in its recovery and recycling from the reaction mixture. These problems can be overcome by immobilization of lipase in solid support with appropriate method [7]. Different techniques of immobilization of lipases have been used in biodiesel syntheses such as adsorption [8-11], Entrapment [12], Encapsulation [13] and covalent binding [14]. Among them adsorption is one of the most widely used methods for the immobilization of lipases because of its simple and cheap procedure [1]. On the other hand, lipases strongly adsorb on the hydrophobic interfaces by their lids and protein chains. For this reason, hydrophobic interaction has become the most popular method for lipase immobilization [11].

Silica aerogels are extremely porous materials with a high specific surface area. Almost all aerogels are derived from gels made through sol-gel chemistry. To prevent the collapse of the gel structure, drying is made to take place under special conditions. If the gels are dried at ambient pressure and temperature without any modification, they lose their porosity because of the effects of the capillarity forces. In ambient pressure drying method, the hydroxyl groups of the wet gel surface, which can lead to shrinkage during the subsequent drying process, are chemically substituted by hydrophobic functional groups. Thus the resulting aerogel in this method has hydrophobic surfaces providing excellent properties to use as lipase immobilization supports [15, 16]. Furthermore, magnetic composite of these materials allows selective and easy enzyme separation from the reaction medium with no need for expensive separation methods [17].

Many parameters could influence the immobilized lipase-catalyzed transesterification reaction and biodiesel production yield. Process optimization is important and helpful for the industrialization and development of biodiesel production. An optimized enzymatic process for biodiesel manufacture could improve the conversion yield and reduce the cost of production. RSM method can provide a research strategy for studying parameter interaction [3, 18].

In this work, immobilized *Candida rugosa* lipase on magnetic silica aerogel was used as biocatalyst in transesterification of sunflower oil with methanol. Central Composite Design (CCD) and RSM analysis were employed to understand the relationships between the reaction variables (methanol/oil molar ratio, enzyme concentration and added water content) and the response (conversion yield) and to obtain the optimum conditions for biodiesel synthesis.

EXPERIMENTAL SECTIONS

Materials

Lipase from *Candida rugosa* (type VII) was purchased from Sigma Aldrich (Saint Louis, MO, USA). Sunflower oil with high purity was purchased from a local market. Iron oxide particles were prepared from US Research Nanomaterials Company (Fe_3O_4, high purity, 99.5+%, 15-20 nm). All the other chemicals used were of analytical grade.

Methods

Biocatalyst preparation

Hydrophobic magnetic silica aerogel was used for immobilization of *Candida rugosa* lipase. This support was synthesized by ambient pressure drying method. For synthesis support, initially, 0.5 g iron oxide particles were dispersed in 20 ml deionized water by an ultrasonic mixer (Bandelin, Sonopuls 3100). The power of the mixer was

70 watt, and solutions were mixed for 1 hr. Then sodium silicate was added to the mixture in 1 to 4 ratios. In the next step, the removal of unwanted Na^+ ions from the sodium silicate solution was carried out by mixing the diluted sodium silicate solution with the ion exchange resin in equal volume proportion. After formation of silicic acid with pH around 2, ammonium hydroxide solution (1.0 M) was added to raise its pH to 4 for gelation. The obtained silica sol was transferred to the Teflon vessels immediately and a hydrogel formed. The gel was kept in the oven at 50°C for 180 min to strengthen the silica network. The water present in the pores of the gel was exchanged with isopropyl alcohol and normal hexane (1:1 v/v) by placing the gel in oven for 18 h at 50 °C. After solvent exchanging and aging, the wet gels were immersed in HMDZ/n-hexane (1:4 v/v) solutions for 12 h at 50 °C in order to modify the surface. Finally, the modified gels were dried at room temperature for 24 h and then at 50, 80 and 120°C for 2 h, respectively [19].

In immobilization step, for pre-wetting hydrophobic supports, 5 ml of ethanol solution (37, %v/v) was added to the supports and then left in a closed conical flask for 2 hours. Next, the alcohol solution was discharged and phosphate buffer (20mL, 25 mM, pH 7.0) solution containing lipase (1.35 mg/mL) was mixed with pre-wetted supports for 100 min to complete the adsorption process. The ratio of enzyme to support was considered 0.45 (w/w) based on optimization experiments. The immobilization was conducted at 25°C, stirring rate of 500 rpm. After that, the suspension was filtrated and the supports with immobilized lipase were separated, washed with phosphate buffer to remove the unabsorbed enzyme and dried in the air [6, 15, 16].

Characterization of support and immobilized lipase

Organic and inorganic bonds present in the support and immobilized lipase were studied by Fourier Transform InfraRed spectroscopy (FTIR, PU 9800, from Philips, Netherlands). The pore structure and particle morphology were characterized by field emission scanning electron microscopy (FESEM, Mira 3-XMU, Tescan USA Inc.) The specific surface area was determined by the Brunauer-Emmett-Teller (BET) method (BEL Sorp-II mini, BEL Japan Inc., Osaka, Japan) from the amount of N_2 gas adsorbed at various partial pressures.

Experimental design, optimization, and statistical analysis

A five level central composite design with three factors was employed to determine the effect of variables, such as methanol/oil molar ratio (A), enzyme concentration (B), and water concentration (C) on FAME (Fatty Acid Methyl Ester) yield. RSM was used to evaluate the effects of independent variables on the response variable. The independent variables and their levels are shown in Table 1. A second order polynomial equation (1) was used to express the biodiesel yield as a function of the studied variables.

$$Y = a_0 + a_1x_1 + a_2x_2 + a_3x_3 + a_{11}x_1^2 + a_{22}x_2^2 + a_{33}x_3^2 + a_{12}x_1x_2 + a_{13}x_1x_3 + a_{23}x_2x_3 \quad (1)$$

Where, Y and x_i represent the response and independent variables, respectively, a_0 is a constant, a_i , a_{ii} and a_{ij} are the linear, quadratic and interaction coefficients, respectively. The analysis was done using decoded factors.

Adequacy of the models was examined taking into account the coefficient of determination (R^2) and adjusting the coefficient of determination (R^2-adj) besides. To fit the second order polynomial equation, analysis of variance (ANOVA) was used [20, 21]. A small P-value ($p< 0.05$) represents the significance of each term in the model. For graphical explanation of the independent variable interactions, three dimensional surface plots of the model were used. This is useful to visualize the relationship between the response and the experimental levels of each factor. Numerical optimization was performed by the response optimizer for determining the exact optimum concentrations of independent variables leading to maximum biodiesel yield. The experimental design, data analysis, and optimization procedure were performed using the Design Expert version 7 statistical software (Stat-Ease Inc., NY, USA).

Enzymatic transesterification reaction

Transesterification reactions were carried out at 40 °C in a 50 mL capped flask with varying amounts of methanol/oil molar ratio, water, and immobilized enzyme, according to the experimental design (Table 2) on a shaking incubator under batch operation. Methanol addition was done as a three-step process. The first portion of methanol and the whole amount of oil were added at the start of the reaction; the second portion of

Table 1: The main independent variables and their levels used in central composite design.

Factor	Variables	Levels				
		High axial (+α)	High factorial (+1)	Center (0)	Low factorial (-1)	Low axial (-α)
x_1	Methanol/oil molar ratio	6.68	6	5	4	3.32
x_2	Enzyme concentration	12.05	10	7	4	1.95
x_3	Water concentration	12.39	10	6.5	3	0.61

Table 2: Three factor central composite design and the experimental and predicted values of the response.

Run	Methanol/oil molar ratio	Enzyme concentration	Water concentration	Yield (%)	
				Experimental	Predicted
1	4	4	3	57.2	57.443
2	6	4	3	57.1	57.278
3	4	10	3	63.4	63.556
4	6	10	3	64.3	64.841
5	4	4	10	58.3	58.239
6	6	4	10	58.2	58.524
7	4	10	10	67.7	68.003
8	6	10	10	69.5	69.738
9	3.32	7	6.5	68.4	68.250
10	6.68	7	6.5	70.1	69.570
11	5	1.95	6.5	56.4	56.225
12	5	12.05	6.5	71.3	70.795
13	5	7	0.61	53.7	53.267
14	5	7	12.39	58.3	58.054
15	5	7	6.5	70.6	70.786
16	5	7	6.5	71.1	70.786
17	5	7	6.5	71	70.786
18	5	7	6.5	70.4	70.786
19	5	7	6.5	71	70.786
20	5	7	6.5	70.5	70.786

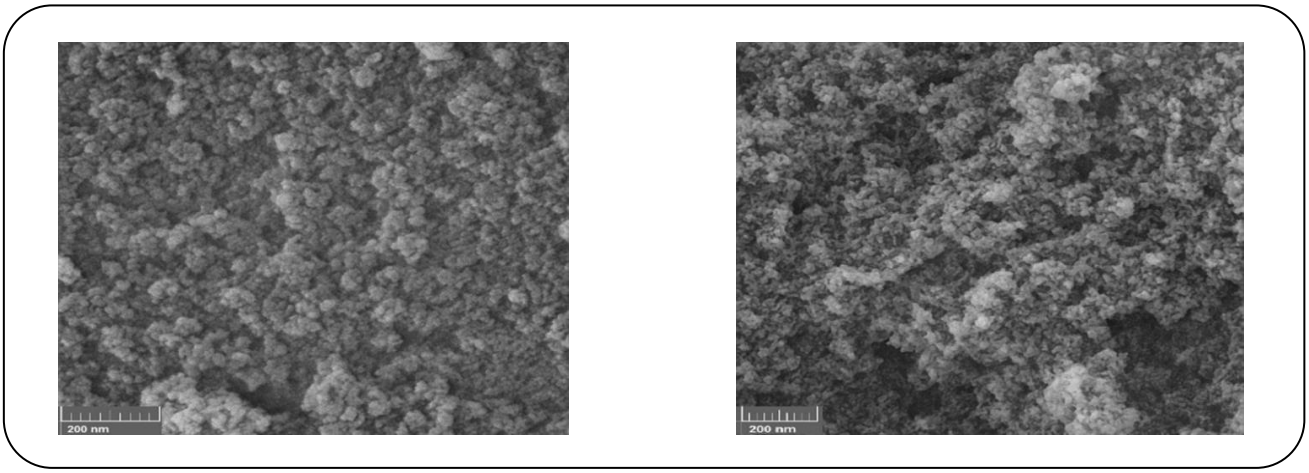

Fig. 1: FESEM micrographs of (a) magnetic silica aerogel; (b) magnetic silica aerogel-lipase.

methanol was added after 10 h, while the third portion was added after 25 h. The reaction has been carried out for 50 h. After finishing the reaction, 5mL distilled water was added, followed by centrifugation [22, 23]. The upper phase, containing methyl esters, was analyzed using an Agilent 6890N, GC apparatus (Agilent Technologies, Wilmington, DE, USA) equipped with a FID detector and split/splitless injector. The injector temperature was 250°C, split ratio = 50:1 and FID detector temperature was 250°C. The carrier gas used was hydrogen at a flow of 40 cm s^{-1}. The chromatographic conditions were: initial column temperature of 100°C, heating rate of 3 °C min^{-1} until reaching a final temperature of 250 °C. The percentage yield was defined as (mg biodiesel ÷ mg initial sunflower oil) × 100% [18].

RESULTS AND DISCUSSIONS

Physiochemical properties of support and immobilized lipase

Fig. 1 presents FESEM features of magnetic silica aerogel before and after the immobilization of *Candida rugosa* lipase. This picture showed that the particles and the size of the pores were less than 50 nm. It seems that after lipase immobilization the pore size and porosity of support decreased because of the creation of some bonds after pre-wetting and lipase adsorption. On the other hand, after immobilization a layer of enzyme may cover the surface of the support and decrease the porosity.

The BET analysis confirmed these results. Specific surface area and mean pore size of support decreased after enzyme immobilization (Table 3).

FT-IR analysis was used to confirm the adsorption of *Candida rugosa* lipase on magnetic silica aerogel. Fig. 2 shows the FT-IR spectra of the support before and after immobilization. The lipase enzyme has two characteristic bands at 1653 and 1600 cm^{-1} (primary and secondary amide groups) [24]. These peaks are clearly visible after lipase immobilization. This indicates the adsorption of lipase onto the support. In magnetic silica aerogel before and after immobilization, there was a characteristic peak at 585cm^{-1} indicating the existence of a Fe−O bond that confirmed the presence of Fe_3O_4 particles.

Fitting the response surface models

The biodiesel production yield obtained from experimental runs is shown in Table 2. Equation (1) was used to fit the data. The estimated regression coefficients for the final reduced model as well as the corresponding significance of regression were given in Table 4. It should be considered that lower p value and higher F ratio corresponds to higher significance of a term on studied response variations. The reduced model was obtained after removing the non-significant terms. The coefficient of determination (R^2) of model for the response in this study, was obtained 0.9975. The obtained high R^2 value confirmed the suitability of the suggested model. Furthermore, the attained non-significant lack of fit and high F value for the suggested model ensured the model was significant (Table 4).

Analysis of response surfaces

As shown in Table 2, biodiesel production yield varied from 53.7 to 71.3 %. As clearly observed in Table 4,

Table 3: Specific surface area and mean pore size of the support with and without lipase immobilization.

Materials	Surface area (m^2/g)	Mean pore size (nm)
Magnetic silica aerogel	499	8.35
Magnetic silica aerogel- lipase	478	7.65

Table 4: The significance probability (p value, F ratio) of regression coefficients and Lack-of-fit, regression coefficients, R^2 and R^2 adjusted for the final reduced model.

Parameters	Variables	Yield (Y)		Regression coefficient Y	
		F ratio	P value		
Main				a_0	16.272
	x_1	10.75	0.0083	a_1	5.97
	x_2	1309.55	< 0.0001	a_2	4.276
	x_3	141.35	< 0.0001	a_3	5.313
Quadratic	x_1^2	32.39	0.0002	a_{11}	-0.663
	x_2^2	487.32	< 0.0001	a_{22}	-0.286
	x_3^2	2106.14	< 0.0001	a_{33}	-0.437
Interaction	x_{12}	5.37	0.0429	a_{12}	0.121
	x_{13}	0.52	0.4884	a_{13}	-
	x_{23}	34.04	0.0002	a_{23}	0.087
Regression		435.32	< 0.0001		
Lack-of-fit		3.32	0.1071		
R^2		0.9975			
R^2 adjusted		0.9952			

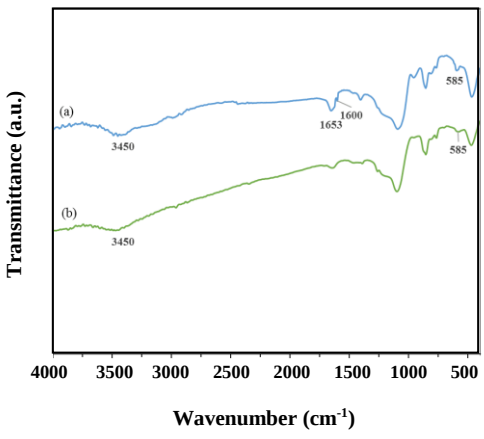

Fig. 2: FTIR spectra of the supports before and after immobilization: (a) magnetic silica aerogel- lipase, (b) magnetic silica aerogel

the linear and quadratic terms of all independent variables had significant ($p<0.05$) effects on biodiesel production yield. The results indicated that enzyme concentration, water content and methanol/oil molar ratio had more significant effects on biodiesel production yield, respectively, due to their high F ratio values. The resulted regression cofficients showed that all the main terms had a positive effect on yield percent. It means that at low enzyme concentration, water content and methanol/oil molar ratio, the biodiesel production yield increased by an increase in all the mentioned independent variables and vice versa. The opposite results were obtained for effects of all the independent variables at their high level on the yield (Table 4).

Figs. 3 and 4 show the interactive effect of parameters on biodiesel production yield. As clearly observed

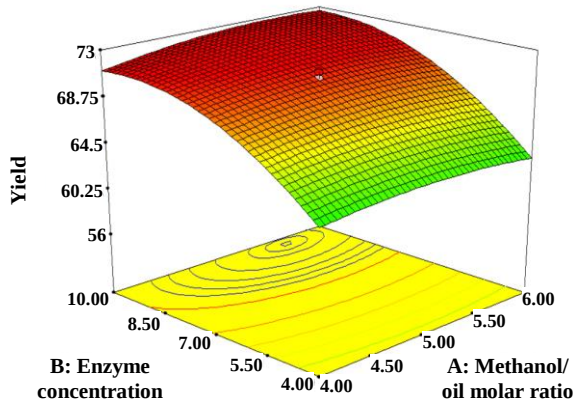

Fig. 3: Response surface plot of biodiesel production versus methanol/oil molar ratio and enzyme concentration (water concentration was fixed at 6.5%).

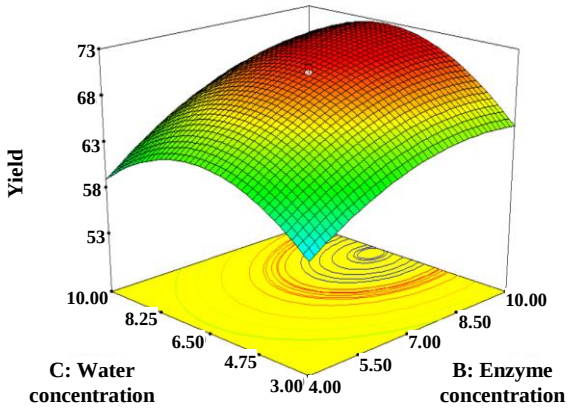

Fig. 4: Response surface plot of biodiesel production versus enzyme concentration and water concentration (methanol/oil molar ratio was fixed at 5).

in Table 4, the interaction of methanol/oil molar ratio and water concentration had a non-significant effect on biodiesel yield. While other interactions had significant effects on yield percent.

The interactive effect of methanol/oil molar ratio and enzyme concentration is shown in Fig. 3. This figure showed that in the low level of enzyme concentration, methanol/oil molar ratio didn't change the biodiesel production yield significantly. But in high level of enzyme concentration, increasing in methanol/oil molar ratio in low level, increased biodiesel production yield and in high level, decreased it. Thus the highest initial reaction rates were also obtained when using the highest level of biocatalyst content in combination with medium level for substrate molar ratio. The increase in the enzyme content favored the reaction rate, resulting in a larger number of active sites in the reaction medium, thus promoting greater conversion rates of fatty acids methyl esters.

Based on the stoichiometry of the transesterification reaction, at least three moles of alcohol are required to produce three moles of ester for a reaction with one mole of oil. Higher molar ratios result in greater ester conversion in a shorter time due to the reversibility of the reaction. On the other hand, an excessive amount of alcohol makes the recovery of glycerol difficult because of the effect of methanol on glycerol solubility. The existence of glycerol in the solution drives the equilibrium back to the left hand side and consequently lowers the biodiesel yield. Furthermore, the high amount of methanol negatively affects the lipase catalyst activity, inhibiting or even denaturing the enzyme. Thus, the yield reduced after the alcohol/oil ratio exceeded the optimum amount [23, 25]. *Poppe et al.* found a molar ratio of 5.6:1, to be the best for methanol/oil molar ratio similar to results obtained in this study [18].

The interactive effect of enzyme concentration and water content in methyl ester production yield is shown in Fig. 4. As indicated in this figure, at constant enzyme concentration, by the enhancement in water content, the yield increased at first to reach its maximum amount and then decreased. The results indicated that maximum biodiesel production yield (72.83%) was obtained at high enzyme concentration and middle water concentration.

Water content is another important factor in biocatalytic transesterification reaction by helping to avoid lipase deactivation. Water acts as a lubricant, increasing the flexibility of the internal structure of the enzyme. Water also functions as an allosteric regulator of lipase, inducing a conformational change that exposes the active site of the enzyme to the substrate, therefore altering the rate of reaction. However, there is a maximum amount of water that promotes the increase of catalytic activity; when this limit is exceeded, the reaction yield decreases. The reduction of the transesterification yield can be attributed to hydrolysis [3].

Optimization and verification of the immobilization conditions

The amount of methanol/oil molar ratio, enzyme and water concentration would be considered optimum if

methyl eater production yield attain the largest possible values. Numerical optimization was used to find the exact optimum levels of the studied variables. The optimum immobilization conditions were: 4.5:1 methanol/oil molar ratio, 9.4% enzyme concentration and 7.4 % water content. The highest production yield in these conditions predicted at 72.3%.

The experimental work at this condition was performed due to the maximum experimental yield. The resulting yield in optimal conditions (71.9%) was in good agreement with the predicted result, implying that the model derived from RSM can be used to adequately describe the relationship between the factors and response in biodiesel production from sunflower oil using immobilized lipase on hydrophobic magnetic silica aerogel.

CONCLUSIONS

In this research, the immobilization of *Candida rugosa* lipase was done on silica aerogel-iron oxide nanocomposites by adsorption method. Physical and chemical properties of support and the immobilized lipase showed that the lipase was successfully immobilized onto the support. Biodiesel production from sunflower oil using immobilized lipase was investigated. Response Surface Methodology (RSM) and Central Composite design (CCD) were employed to optimize the parameters that effect on biodiesel production yield. The optimum immobilization condition was: 4.5:1 methanol/oil molar ratio, 9.4% enzyme concentration and 7.4 % water content. The production yield in this condition was obtained 71.9%. Magnetic silica aerogel showed a promising future for immobilization lipase and biodiesel production as it allowed easy immobilization and separation from reaction media through a simple and inexpensive process.

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