Energies **2013**, *6*, 2052-2064; doi:10.3390/en6042052

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energies

ISSN 1996-1073

www.mdpi.com/journal/energies

Article

Optimizing the Production of Biodiesel Using Lipase Entrapped in Biomimetic Silica

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Received: 21 March 2013 / Accepted: 29 March 2013 / Published: 10 April 2013

Abstract: We entrapped lipase from *Pseudomonas cepacia* in polyallylamine-mediated biomimetic silica, and then applied entrapped lipase to the synthesis of biodiesel with soybean oil or waste cooking oil as a feedstock. The effects of reaction temperature, substrate molar ratio (methanol/oil) and *n*-hexane content (w/w of oil) were evaluated using response surface methodology (RSM) combined with Box-Behnken design. The optimal reaction conditions for soybean oil were 43.6 °C, substrate molar ratio of 4.3%, and 75% *n*-hexane. The predicted and experimental values of biodiesel conversion were 79% and 76%, respectively. The optimal reaction conditions for waste cooking oil were 43.3 °C, substrate molar ratio of 5%, and 38% *n*-hexane. The predicted and experimental values of conversion were 68% and 67%, respectively. The conversion efficiency remained the same even after 1-month storage of entrapped lipase at 4 °C or room temperature.

Keywords: biodiesel; lipase; biomimetic silica; response surface methodology

1. Introduction

The search for alternative fuels has drawn immense amount of attention during the past decade because of the inevitable depletion of fossil fuels. Biodiesel, fatty acid monoesters derived from renewable feedstocks, is one of the most promising alternative fuels; it is biodegradable, non-toxic, almost sulfur-less, and can be used alone or blended with conventional petrodiesel in unmodified

diesel engines [1]. Commercially, biodiesel is most often produced by transesterification of vegetable oils with short chain alcohols using alkaline catalysts. The alkali-catalyzed process has the advantages of short reaction time, high yield, and low cost for the catalysts. However, such process often required high quality food-grade vegetable oils with low level of free fatty acids (FFA) to prevent saponification, which leads to difficult glycerol separation and low ester conversion rate [2]. The high cost of virgin vegetable oils renders biodiesel unable to compete commercially with petrodiesel; biodiesel has over double the price of petrodiesel and the feedstock accounts for about 80% of the total operating cost [3].

Using lipases as catalysts can overcome many aforementioned drawbacks of the alkali-catalyzed process [4]. Lipases (E.C.3.1.1.3), are widely used in industry and capable of catalyzing a variety of reactions such as hydrolysis, alcoholysis, esterification, and transesterification [5]. The enzymatic process can utilize low quality feedstocks with high levels of FFA because FFAs can be directly converted to biodiesel via lipase-catalyzed esterification; it also requires less energy input and the glycerol byproduct is easier to separate. Even with many desirable properties, the enzymatic process has very limited commercial success mainly due to the high cost of lipases; one of the more cost effective approaches often adopted to reduce the cost is to recycle the enzyme through immobilization [6].

Various immobilization techniques have been applied to lipases. Adsorption is the attachment of enzymes onto the surface of support by weak forces [6]; materials such as phyllosilicates [7], Accurel MP1004 (porous polypropylene) [8], mesoporous silica [9], and silica zeolites [10] have been reported. The adsorption technique is facile; however, desorption of enzyme molecules is a common problem. Immobilization via electrostatic interaction onto ITQ-6 (delaminated derivative of lamellar precursor of ferrierite zeolite) has also been reported [11]. Strong covalent bonds have the advantage of minimal enzyme leakage; supports such as ITQ-6 [11], rice straw [12], magnetic nanoparticles [13], silica nanoparticles [14], and chitin [15] have been used. Cross-linked enzyme aggregates (CLEAs) can be formed by means of bifunctional or multifunctional reagents such as glutaraldehyde [16]. Nevertheless, decreased enzyme activity is often observed if the functional groups for forming covalent linkages are critical for catalysis. Lipases can also be entrapped in matrix of polymers like phyllosilicate sol-gel [17], cellulose acetate-TiO_2 gel fiber [18], and silica gels [19–21]. Porous membranes such as silica aerogel [22], surfactant micelles self-assembled with silica [23], and liposome covered with porous silica shell [24] have been applied to encapsulate lipases. The immobilization procedures of entrapment and encapsulation are often simpler than those of covalent linkages, but the polymeric matrices and membranes often have significant mass transfer resistance.

We have previously entrapped lipase from *Pseudomonas cepacia* in biomimetic silica [25]. Biomimetic silica, which mainly mimics the cell wall formation of diatoms, has been applied to entrap a variety of biomolecules and inorganic materials [26]. It can be formed *in vitro* by mixing silicic acid with silaffin peptides derived from diatom *Cylindrotheca fusiformis* such as R5 (H_2N-SSKKSGSYSGSKGSKRRIL-COOH) or their synthetic counterparts in the presence of phosphate [27]. Polymers such as poly-L-lysine and polyallylamine structurally resembling the biological templates have also been reported to catalyze silicification [28]. Such immobilization technique offers advantages such as mild reaction condition, rapid kinetics, excellent stability, and ease of operation [26].

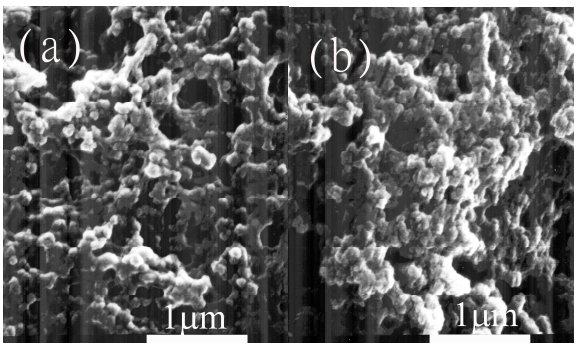
In this work, we optimized the synthesis of biodiesel by using lipase entrapped within biomimetic silica as a catalyst. Both soybean oil and waste cooking oil were used as feedstocks. In order to correlate the molar conversion with reaction variables (temperature, substrate molar ratio, and *n*-hexane content), Box-Behnken design and response surface methodology (RSM) analysis were used [29]. The addition of *n*-hexane can decrease the viscosity of the feedstocks and thus facilitate the mass transport; it has been reported as a suitable solvent for lipase-catalyzed transesterification [30]. We also evaluated the reusability and the storage stability of entrapped enzyme.

2. Results and Discussion

2.1. Characteristics of Entrapped Lipase

The average entrapment efficiency (amount of entrapped lipase/amount of added lipase) was 81% ± 3% (N = 4). The specific activities of free and entrapped lipases were 25 ± 2 and 3.7 ± 0.4 U/mg powder (N = 4), respectively; the corresponding activity recovery (specific activity of entrapped lipase/specific activity of free lipase) was 15%, significantly lower than the value we reported previously [25]. Scanning electron micrographs revealed the matrix was networks of fused silica particles (Figure 1); and the morphology remained the same after lyophilization. The yield of silica from a 100 μL reaction mixture were 0.87 ± 0.01 mg (N = 3) as determined with a modified molybdenum assay [31]; the corresponding enzyme loading was then calculated as 4.9 g lipase powder/g silica. The high enzyme loading could result in low activity recovery because the enzyme molecules buried in the interior of the silica matrix were devoid of available substrate. However, the commercial lipase preparation we used contained only 60 mg of protein per gram dry powder as assayed by the method of Lowry [32].

Figure 1. Scanning electron micrographs of biomimetic silica containing lipase from *Pseudomonas cepacia*. (**a**) Before; and (**b**) after lyophilization.



2.2. Model Fitting and Analysis of Variance

The design of experiments and the corresponding data are given in Table 1. Fitting the data with various models followed by analysis of variance (ANOVA), for soybean oil, the following quadratic polynomial most suitably described the correlation between molar conversion and the tested variables:

$$Y = 74.18 + 5.56A + 4.50B + 2.12C - 9.91A^2 - 5.13B^2 + 2.09C^2 - 2.04AB - 2.13AC - 1.17BC \quad (1)$$

where A, B, and C were temperature, substrate molar ratio (methanol/oil), and *n*-hexane content (%, w/w of oil), respectively. The *F*-value of 18.13 for the model was higher than $F_{0.01,9,7}$ of 6.72, indicating the model was significant at confidence level of 99%. The *F*-value for lack of fit was 3.02, much lower than $F_{0.01,3,4}$ of 16.69, indicating lack of fit was insignificant. Overall, the model had a small *P*-value of 0.0005 and a suitable coefficient of determination ($R^2 = 0.9589$), clearly showed that the model was highly significant and sufficient to describe the correlation between the molar conversion of biodiesel and the tested variables. Temperature, substrate molar ratio, *n*-hexane content, square of temperature, and square of substrate molar ratio were significant for the process with *P*-values smaller than 0.05 (Table 2).

For waste cooking oil, the following quadratic polynomial was the most suitable instead:

$$Y = 65.64 + 2.30A + 3.93B + 0.20C - 10.36A^2 - 1.50B^2 - 1.48C^2 + 0.17AB + 0.66AC - 1.71BC \quad (2)$$

where A, B, and C were as previously defined. The *F*-value of 13.28 for the model using waste cooking oil as a feedstock indicated the model was significant at confidence level of 99%. The *F*-value for lack of fit was 0.58, much lower than $F_{0.01,3,4}$, indicating lack of fit was insignificant. The model was highly significant and sufficient to describe the correlation between the molar conversion of biodiesel and the tested variables because of a small *P*-value of 0.0013 and a suitable coefficient of determination ($R^2 = 0.9447$). In this case, temperature, substrate molar ratio, and square of temperature were significant for the process (Table 3).

Table 1. 3-Level-3-factor Box-Behnken design of experiments and the corresponding conversions.

Treatment No. [a]	Variable [b]			Conversion (%)	
	Temperature (°C)	Molar ratio (methanol/oil)	*n*-Hexane (%, w/w of oil)	Waste cooking oil	Soybean oil
1	50 (1)	5 (1)	50 (0)	69.2	59.1
2	50 (1)	3 (−1)	50 (0)	60.7	52.6
3	35 (−1)	5 (1)	50 (0)	61.7	54.6
4	35 (−1)	3 (−1)	50 (0)	45.0	48.8
5	50 (1)	4 (0)	75 (1)	72.5	56.3
6	50 (1)	4 (0)	25 (−1)	70.9	56.3
7	35 (−1)	4 (0)	75 (1)	66.1	50.0
8	35 (−1)	4 (0)	25 (−1)	56.0	52.6
9	42.5 (0)	5 (1)	75 (1)	74.0	66.8
10	42.5 (0)	5 (1)	25 (−1)	73.7	68.1
11	42.5 (0)	3 (−1)	75 (1)	70.9	60.6
12	42.5 (0)	3 (−1)	25 (−1)	65.9	55.1
13	42.5 (0)	4 (0)	50 (0)	72.2	62.1
14	42.5 (0)	4 (0)	50 (0)	76.3	68.1
15	42.5 (0)	4 (0)	50 (0)	72.5	68.0
16	42.5 (0)	4 (0)	50 (0)	75.8	64.0
17	42.5 (0)	4 (0)	50 (0)	74.1	65.9

[a] The treatments were performed in random order; [b] The values of 1, −1, and 0 in parentheses were coded levels.

Table 2. ANOVA for joint test with soybean oil as a feedstock.

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	Prob > *F* [a,b]
Model	1039.99	9	115.55	18.13	0.0005
Temperature (A)	246.89	1	246.89	38.74	0.0004
Substrate molar ratio (B)	161.90	1	161.90	25.41	0.0015
n-Hexane (C)	36.04	1	36.04	5.66	0.0490
A^2	413.42	1	413.42	64.87	<0.0001
B^2	110.64	1	110.64	17.36	0.0042
C^2	18.46	1	18.46	2.90	0.1326
AB	16.71	1	16.71	2.62	0.1494
AC	18.16	1	18.16	2.85	0.1353
BC	5.49	1	5.49	0.86	0.3841
Residual	44.61	7	6.37	–	–
Lack of fit	30.96	3	10.32	3.02	0.1565
Pure error	13.65	4	3.41	–	–
Cor total	1084.60	16	–	–	–

[a] Significant at "Prob > F" lower than 0.05; [b] Insignificant at "Prob > F" higher than 0.1.

Table 3. ANOVA for joint test with waste cooking oil as a feedstock.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	Prob > F [a,b]
Model	668.11	9	74.23	13.28	0.0013
Temperature (A)	42.24	1	42.24	7.56	0.0286
Substrate molar ratio (B)	123.32	1	123.32	22.06	0.0022
n-Hexane (C)	0.32	1	0.32	0.06	0.8184
A^2	451.64	1	451.64	80.79	<0.0001
B^2	9.50	1	9.50	1.70	0.2335
C^2	9.25	1	9.25	1.65	0.2393
AB	0.11	1	0.11	0.02	0.8907
AC	1.72	1	1.72	0.31	0.5960
BC	11.66	1	11.66	2.09	0.1920
Residual	39.13	7	5.59	–	–
Lack of fit	11.80	3	3.93	0.58	0.6610
Pure error	27.33	4	6.83	–	–
Cor total	707.24	16	–	–	–

[a] Significant at "Prob > F" lower than 0.05; [b] Insignificant at "Prob > F" higher than 0.1.

2.3. Effects of Variables and Their Optimization

The correlation between the molar conversion of biodiesel and the tested variables can be better understood by examining the contour plots. For soybean oil, as elevating the temperature from 35 to 42.5 °C, the molar conversion increased (Figure 2a *vs.* 2b). Nevertheless, when increasing the temperature further to 50 °C, the molar conversion started to decrease, suggesting part of the *P. cepacia* lipase was inactivated at this temperature. The molar conversion increased with the substrate molar ratio, except when the ratio was close to 5. Comparing to temperature and substrate molar ratio, the content of *n*-hexane had much less effect on molar conversion. Similar effects were

observed with waste cooking oil although lower conversions were obtained and increased with substrate molar ratio up to 5 (Figure 3).

Figure 2. Contour plots of molar conversion using soybean oil as a feedstock at different temperature. (**a**) 35 °C; (**b**) 42.5 °C; (**c**) 50 °C.

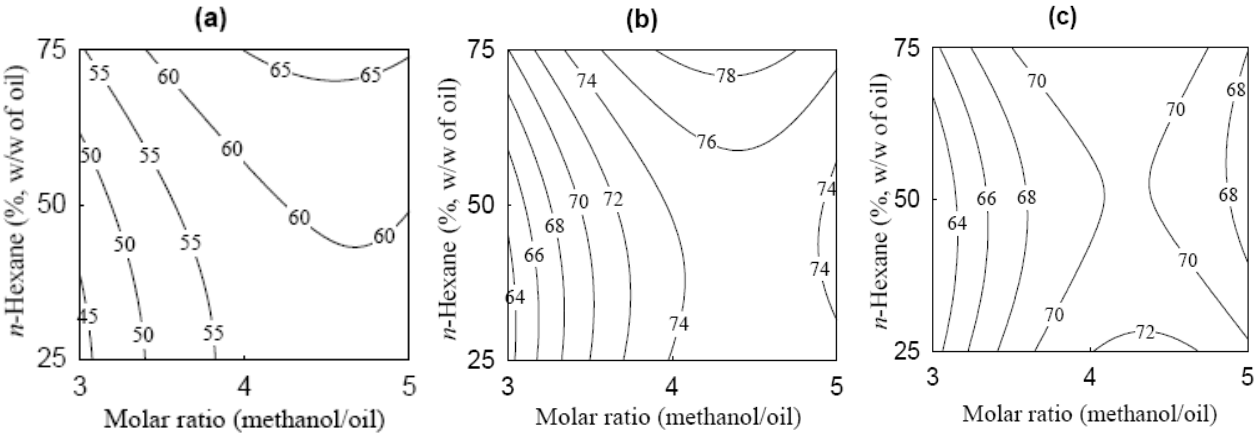
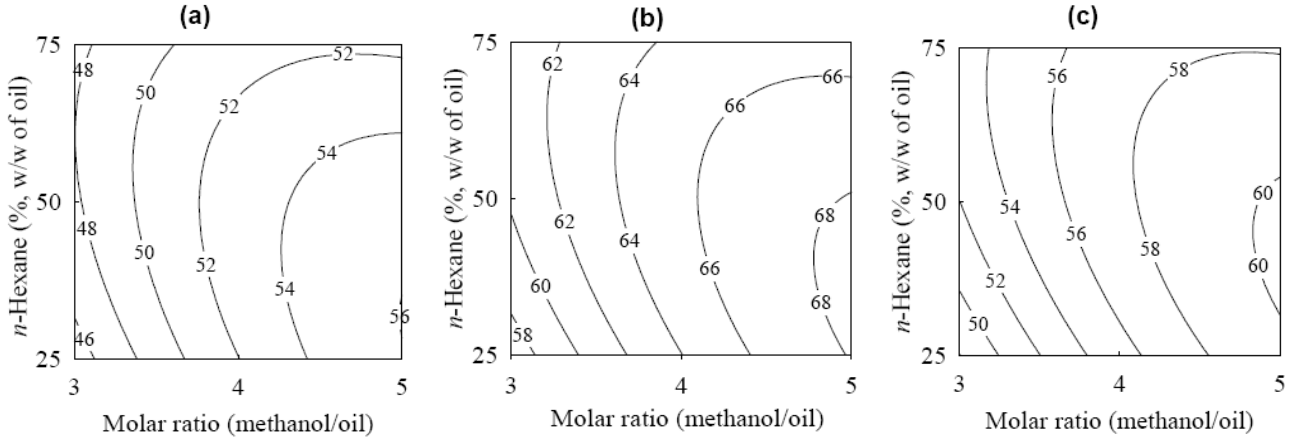


Figure 3. Contour plots of molar conversion using waste cooking oil as a feedstock at different temperature. (**a**) 35 °C; (**b**) 42.5 °C; (**c**) 50 °C.



The optimal reaction conditions for soybean oil were 43.6 °C, substrate molar ratio of 4.3%, and 75% *n*-hexane; the predicted and experimental values of biodiesel conversion were 79% and 76.0% ± 0.7% (N = 3), respectively. The optimal reaction conditions for waste cooking oil were 43.3 °C, substrate molar ratio of 5, and 38% *n*-hexane; the predicted and experimental values of conversion were 68 and 67.2% ± 1.5% (N = 3), respectively. Attempts to further increase the molar conversion by extending the incubation time or increasing the dosage of lipase had little effects, suggesting the reactions reached equilibrium. The lower conversion of the waste cooking oil could be related to its higher acid value of 1.8 than 0.3 mg KOH/g of soybean oil. Because FFA can be converted to polymers, aldehydes, and epoxides during frying, these products are not recognized as substrates by lipases [33]. The conversions of biodiesel, using either feedstock, were comparable to those using *P. cepacia* lipase entrapped in silica sol-gel or in silica aerogel reinforced with silica quartz fiber felt as catalyst [22,34].

After removing the *n*-hexane using a rotary evaporator, content of fatty acid methyl esters (FAME), kinematic viscosity at 40 °C, and density at 15 °C of the biodiesel synthesized from waste cooking oil were 72.3% ± 0.01% (w/w), 6.67 ± 0.04 mm^2/s, and 883 ± 3 kg/m^3 (N = 3), respectively. The content of FAME was lower than the lower limit of 96.5% as specified by the national standard of the Republic of China for FAME (CNS-15072) [35]; such result was expected because significant amount of feedstock was not converted. The viscosity affects the fuel spray, mixture formation, and combustion process [1]; high viscosity interferes with the injection process and leads to insufficient atomization. The measured viscosity was higher than the specified range of 3.5 to 5.0 mm^2/s. The high kinematic viscosity could be explained by the residual waste cooking oil which had a kinematic viscosity of 60 ± 3 mm^2/s (N = 3) at 40 °C. The density affects the injected fuel amount, injection timing, and injection spray pattern [1]. The density was within the specified range of 860 to 900 kg/m^3. These results clearly indicate the current conversion of the waste cooking oil needs to be increased in order to improve the properties of the biodiesel. Nevertheless, biodiesel produced from used frying oil which only partially fulfilled the European standard specification for biodiesel (EN-14214) could still be used as an alternative to fossil fuels [36].

2.4. Storage Stability and Reusability of Entrapped Lipase

The storage stability of entrapped lipase at 4 °C and room temperature was examined using soybean oil as a feedstock (Figure 4). Regardless of the storage temperature, the molar conversion remained unchanged within one month. The reusability of lipase with soybean oil as a feedstock is shown in Figure 5. After five reaction cycles, the molar conversion decreased to 16.5% when the recycled lipase was washed with *n*-hexane. A separate experiment confirmed the enzyme leakage from the matrix was minimal (data not shown); such results were in agreement with what was previously reported [37]. We also examined the activity of entrapped lipase as a function of incubation time under the optimal temperature (43.6 °C), the residual activities after 12, 24, and 36 h were 80%, 45%, and 39%, respectively. Besides particle-loss during washing, the inactivation of entrapped lipase at this temperature may also be partly responsible for the decreased conversion after recycling.

Figure 4. Storage stability of *Pseudomonas cepacia* lipase entrapped in biomimetic silica. Room temperature (●); 4 °C (○). Fifty milligrams of entrapped lipase were sampled every 3 d to carried out the transesterification of soybean oil at 43.6 °C, substrate molar ratio of 4.3 (stepwise additions at 0, 12 and 24 h, one third of the total amount each time), and 75% (w/w of oil) *n*-hexane; the reaction mixture also contained 960 μL of deionized water.

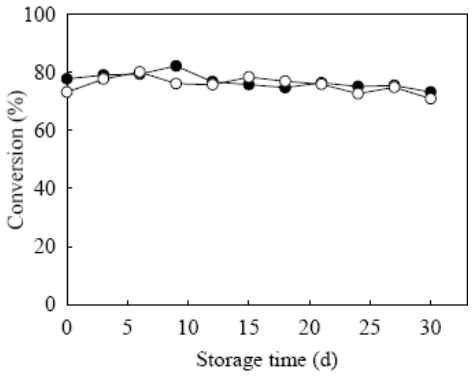
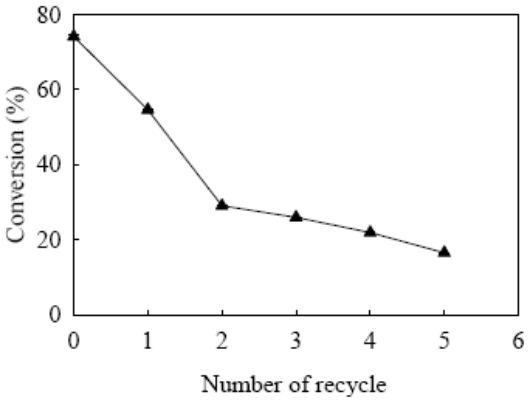


Figure 5. Reusability of *Pseudomonas cepacia* lipase entrapped in biomimetic silica. The transesterification of soybean oil was carried out at 43.6 °C, substrate molar ratio of 4.3 (stepwise additions at 0, 12 and 24 h, one third of the total amount each time), and 75% (w/w of oil) *n*-hexane; the dosage for entrapped lipase was 50 mg and the reaction mixture also contained 960 μL of deionized water.



3. Experimental Section

3.1. Entrapment of Lipase

The solution of 1 M tetramethoxysilane (TMOS) in 1 mM HCl freshly prepared at room temperature was used as the source of silicic acid. The entrapment reaction mixture typically contained 7.5 mL of 100 mM KH_2PO_4 with 0.1 N NaOH, 1.5 mL of hydrolyzed TMOS, 1.5 mL of deionized water, and 1.5 mL of Amano lipase PS (from *Pseudomonas cepacia*; Sigma-Aldrich, St. Louis, MO, USA) at the concentration of 500 mg/mL reconstituted in deionized water. The reaction was initiated by the addition of 3 mL of 5 mM polyallylamine (Sigma-Aldrich) with an average molecular weight of approximately 15 kDa in deionized water at room temperature. After 5 min of incubation, the precipitated biomimetic silica was collected by centrifugation at 100 *g* for 5 min. After washing twice with 15 mL of deionized water, the biomimetic silica was resuspended in 15 mL of deionized water. The amount of entrapped lipase was determined by subtracting the remaining lipase activity in the supernatant and the washing fraction from the initial lipase activity, and quantitating with an activity-concentration calibration curve. The morphology of the silica particles was studied with a Hitachi S-2400 scanning electron microscope at 20 kV; the samples were prepared by applying air-dried or lyophilized silica particles onto copper conductive tapes followed by sputtering with gold.

3.2. Assay for Lipase Activity

The assay was derived from that described by Pencreac'h *et al.* [32] with minor modifications. The assay mixture contained 90 μL of 10 mM *p*-nitrophenyl laurate in isopropanol and 810 μL of 50 mM Tris-HCl, pH 8.0, with 0.4% (w/v) Triton X-100 and 0.1% (w/v) arabic gum preheated to 37 °C. To initiate the reaction, 100 μL of lipase solution or suspension of entrapped lipase at appropriate concentration was added. The change in absorbance at 410 nm was monitored for 5 min at 37 °C using a thermostated V-550 spectrophotometer (JASCO, Tokyo, Japan). The activity was calculated from the

linear region of the absorbance-time curve with an apparent extinction coefficient of 13.66 mM^{-1} cm^{-1} for the hydrolysis product, *p*-nitrophenol. One activity unit was defined as the production of 1 μmol of *p*-nitrophenol per min at 37 °C.

3.3. Experimental Design

A 3-level-3-factor Box-Behnken design with five replicates at the center was applied in this work, requiring 17 experiments. The variables selected for the synthesis of biodiesel and the corresponding ranges were reaction temperature (35–50 °C), substrate molar ratio (methanol:oil = 3:1–5:1), and concentration of *n*-hexane (25%–75%, w/w of oil). Actual values of the variables, in terms of coded and uncoded values, were as shown in Table 1. Treatments were performed in a fully random order to avoid bias.

3.4. Transesterification of Oil to Biodiesel

In a typical reaction, entrapped lipase after lyophilization (50 mg) and soybean oil (4.8 g) were mixed in a 25 mL Erlenmeyer flask, followed by the addition of methanol (methanol:oil = 3:1–5:1, three separate additions at 0, 12, and 24 h, one third of the total amount each time). The mixture also contained *n*-hexane (25%–75%, w/w of oil) as a co-solvent and deionized water (960 μL); it has been reported that *P. cepacia* lipase requires a certain amount of water to be active [38]. The reaction mixture was incubated in the temperature range of 35–50 °C using a water bath with orbital shaking at 150 rpm for 36 h.

3.5. Analysis of FAME

The oil phase of the reaction mixture was first treated with sodium sulfate followed by centrifugation at 7500 *g* for 5 min. Fifty microliters of treated sample was mixed with 1 mL of 10 mg/mL methyl heptadecanoate in hexane as an internal standard. The molar conversion of biodiesel was determined by injecting 1 μL of the sample into a gas chromatograph (Shimadzu GC-14A, Kyoto, Japan) equipped with a flame-ionization detector (FID). A BPX70 capillary column (30 m × 0.25 mm i.d.; SGE Analytical Science, Ringwood, Australia) with hydrogen as carrier gas at a constant pressure of 6 kg/cm^2 was used. The injector and FID temperatures were set at 250 and 280 °C, respectively. The oven temperature was initially held at 150 °C for 30 s and then increased to 180 °C at 10 °C/min, finally to 198 °C at 1.5 °C/min. The amount of FAME in the sample was determined from the standard curves. The percentage of molar conversion was defined as follows:

$$Conversion\,(\%) = \frac{moles\ of\ FAME\ produced}{3 \times moles\ of\ oil} \times 100 \tag{3}$$

In order to characterize the biodiesel synthesized from waste cooking oil, the *n*-hexane was removed using a rotary evaporator (Büchi KRvrTD 65/45, Flawil, Switzerland) with the bath temperature at 60 °C. The content of FAME was measured according to the national standard of the Republic of China, CNS-15051 [35], which was similar to the procedure for determining the molar conversion describe previously. The content of FAME was calculated using the following equation:

$$FAME(\%) = \frac{\sum A - A_{EI}}{A_{EI}} \times \frac{C_{EI} \times V_{EI}}{m} \times 100 \quad (4)$$

where ΣA is the summation of peak area of all the FAME peaks (from C14:0 to C24:1); A_{EI} is the peak area of the internal standard, methyl heptadecanoate; C_{EI} is the concentration of methyl heptadecanoate; V_{EI} is the volume of methyl heptadecanoate; and m is the mass of the biodiesel sample. An Ostwald viscometer and a hydrometer were used to determine the kinematic viscosity and density according to CNS-12017 and CNS-3390 [35], respectively.

3.6. Statistical Analysis

The experimental data (Table 1) were fit to the following second-order polynomial equation using Design Expert software version 6.01 (Stat-Ease, Minneapolis, MN, USA.):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{ij} x_i x_j \quad (5)$$

where Y is response (molar conversion in percent); β_0, β_i, β_{ii}, and β_{ij} were constant coefficients; and x_i was the uncoded independent variable. ANOVA, regression analysis, and the plotting of response surface were performed using the same software.

4. Conclusions

In this work, we showed the potential of *P. cepacia* lipase entrapped in biomimetic silica as a catalyst for the synthesis of biodiesel. Using either soybean oil or waste cooking oil as a feedstock, the optimum conversions were similar to those using lipase entrapped in other silica-based supports as catalysts, but the properties of the synthesized biodiesel still need to be improved. The entrapped lipase also exhibited good storage stability. In comparison to common immobilization techniques such as covalent linkages and physical adsorption, the entrapment requires no prior chemical activation and has minimal enzyme leakege. In addtion, the process is facile and rapid. We could improve the reusability by entrapping magnetic nanoparticles together with lipase to facilitate the recovery. Alternatively, the reusability could be improved by using a packed-bed reactor, which removes the products continuously, minimize enzyme particle-loss during washing, and protect the enzyme from mechanical shear.

Acknowledgments

Financial support from National Science Council (NSC 100-2221-E-036-034) is gratefully acknowledged.

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