Research Article

Continuous Production of Lipase-Catalyzed Biodiesel in a Packed-Bed Reactor: Optimization and Enzyme Reuse Study

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An optimal continuous production of biodiesel by methanolysis of soybean oil in a packed-bed reactor was developed using immobilized lipase (Novozym 435) as a catalyst in a *tert*-butanol solvent system. Response surface methodology (RSM) and Box-Behnken design were employed to evaluate the effects of reaction temperature, flow rate, and substrate molar ratio on the molar conversion of biodiesel. The results showed that flow rate and temperature have significant effects on the percentage of molar conversion. On the basis of ridge max analysis, the optimum conditions were as follows: flow rate 0.1 mL/min, temperature 52.1°C, and substrate molar ratio 1 : 4. The predicted and experimental values of molar conversion were 83.31 ± 2.07% and 82.81 ± .98%, respectively. Furthermore, the continuous process over 30 days showed no appreciable decrease in the molar conversion. The paper demonstrates the applicability of using immobilized lipase and a packed-bed reactor for continuous biodiesel synthesis.

1. Introduction

Biodiesel, also known as fatty acid methyl esters (FAME), has attracted great attention in recent years because of the depletion of fossil fuels, increased crude oil price, and its environmental benefits. It is mainly synthesized by transes- terifying triglycerides from soybean, rapeseed, and palm oils with methanol [1]. The advantages of biodiesel over diesel fuel are its renewability, biodegradability [2], lower emission of toxic compounds [3], and higher combustion efficiency [4]. However, the industrial scale production of biodiesel is limited due to the undesirable byproducts, the recovery of glycerol, the removal of inorganic salts and water, the treatment of wastewater, and the requirement of high energy. Many research works related to enzymatic catalysts have been carried out to overcome these drawbacks [5–7].

Lipases classified as hydrolysis E.C. 3.1.1.3 have both hydrolytic and synthetic activity. They can catalyze trans- esterification of triglycerides as well as esterification of free fatty acids to produce biodiesel. Compared to chemical catalysis, the lipase-catalyzed synthesis of biodiesel is more compatible with variations in the quality of raw materials. It requires only simple purification steps and conducts under moderate reaction conditions. The main obstacle for com- mercialization of the lipase-catalyzed process is the cost of the enzyme [7, 8]. The cost of Novozym 435 lipase is approx. 1000 USD per kg [7]. The alkaline catalyst (NaOH) is approx. 0.62 USD per kg [9]. To compensate for the high cost of the enzyme, an effective way is to extend the operational life of the lipase, which would significantly increase productivity of a given amount of enzyme and lower the biodiesel production price. This can be achieved by using immobilized enzymes. In the batch system, Novozym 435, an immobilized *Candida antarctica* lipase B on acrylic resin, could be repeat- edly used 5–50 cycles under their optimal reaction conditions [10–12]. Li et al. [13] reported that the enzyme activity of Novozym 435 showed no obvious loss after being used for 200 cycles. Furthermore, Royon et al. used 13.5% cotton

oil and 54% methanol with Novozym 435 in *tret*-butanol solvent system to produce biodiesel at 50°C reaction temperature and 24 hours reaction time with 97% yield. Chang et al. reported that canola oil and methanol (molar ratio 1 : 3.5) was employed to biosynthesize biodiesel with 42% Novozym 435 at reaction temperature of 38°C and reaction time of 12.4 hours in hexane system. Therefore, we chose Novozym 435 as the biocatalyst in a pack-bed reactor system in this study.

For industrial scale production, the use of immobilized enzyme with packed-bed reactor is more cost effective than the batch operation [14]. The immobilized lipase is packed into a column and the reaction mixture is continuously pumped through the column. The enzyme can be reused without a prior separation. Besides, the advantages of using a packed-bed reactor also include continuous removal of glycerol and excess alcohol, effective reuse of enzymes, and the protection of enzyme particles from mechanical shear stress [14–16]. However, long-term operation of a packed-bed reactor is still a challenge. In a solvent-free system, the packed-bed reactor can be operated for 3–7 days without decreasing ester yield [17, 18]. The stepwise addition of methanol prevented the deactivation of the lipase, which allowed a constant enzyme activity during 100 days of operation [19]. Furthermore, Chen and Wu [20] regenerated lipase by periodically washing with *tert*-butanol, and the lipase maintained its activity for 70 days. The stability of immobilized lipase also can be improved using *tert*-butanol as the solvent in the continuous packed-bed reactor. The enzyme activity was maintained through 5 and 20 days by Halim et al. [21] and Royon et al. [15], respectively.

In this study, we developed a continuous packed-bed reactor, which was packed with Novozym 435 lipase, to produce biodiesel from soybean oil with methanol. *tert*-butanol was used as the solvent, because it is an ideal medium that enhances miscibility of methanol with vegetable oils as well as being regenerating agent of lipase [10, 13, 15, 20]. Statistical experiment design and RSM analysis were employed to investigate the affinities between the reaction variables (reaction temperature, flow rate, and substrate molar ratio) and response (molar conversion percentage), and to obtain the optimal conditions for continuous packed-bed reactor operation. This paper provides a feasible model for long-term operation of packed-bed reactor and sheds light on industrial scale production of biodiesel with enzymatic catalysts.

2. Materials and Methods

2.1. Materials. Immobilized lipase (triacylglycerol hydrolase, EC 3.1.1.3; Novozym 435) from *Candida antarctica*, supported on acrylic resin beads, was purchased from Novo Nordisk Bioindustrials, Inc. (Bagsvaerd, Denmark). According to the commercial production manual, its catalytic activity was 10,000 PLU/g (propyl laurate units per gram), and it contained 1%-2% (w/w) moisture. Soybean oil was purchased from Taisun Enterprise Co., Ltd. (Chang-Hua, Taiwan). Methanol and *tert*-butanol were purchased from Katayama Chemical Co., Ltd. Tributyrin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Molecular sieves

(4 Å) were purchased from Davison Chemical (Baltimore, MD, USA), and *n*-hexane was obtained from Merck Chemical Co. (Darmstadt, Germany). All other chemicals were analytical reagent grade.

2.2. Experimental Design. A 3-level-3-factor Box-Behnken design with three replicates at the center was employed in this study, requiring 15 experiments [22, 23]. The variables and their levels selected for the study of biodiesel synthesis were reaction temperature (40°C–60°C), flow rate (0.1–0.5 mL/min), and substrate molar ratio (1 : 3–1 : 5). All experiments in this paper were performed in a 32.5% *tert*-butanol system with a packed-bed reactor. Table 1 presents the independent factor (X_i) levels and the experimental design in terms of coded and uncoded values. To avoid bias, 15 runs were performed in a fully random order.

2.3. Enzymatic Transesterification in a Continuous Reactor. All materials were dehydrated by molecular sieves (4 Å) for 24 hours. Soybean oil (0.05 mole), *tert*-butanol (32.5%, based on the weight of soybean oil), and different molar ratios of methanol were well mixed in a feeding flask. The transesterification reaction was carried out in a packed-bed reactor consisting of a stainless steel tube 25 cm in length and with a 0.46 cm inner diameter. The mixture was pumped through the continuous reactor (a packed-bed column with 1.7 g Novozym 435 lipase) at the designed conditions. The system was placed into the temperature-controlled chamber to prevent temperature gradients.

2.4. Determination of Biodiesel Yield. The biodiesel formation was determined by injecting a 1 μL aliquot in splitless mode into a gas chromatograph (Hewlett Packard 7890, Avondale, PA, USA) equipped with a flame-ionization detector (FID) and an MXT-65TG-fused silica capillary column (30 m $\times$ 0.25 mm i.d.; film thickness 1 μm; Restek, Bellefonte, PA, USA). Injector and FID temperatures were set at 300°C and 320°C, respectively. The oven temperature was maintained at 195°C for 10 minutes. Nitrogen was used as the carrier gas. The percentage of molar conversion was defined as (mmol of biodiesel/3 mmol of initial soybean oil) $\times$ 100% and was estimated using peak area integrated by on-line software Hewlett Packard 6890 Series II Chem Station (Hewlett Packard 6890, Avondale, PA, USA).

2.5. Statistical Analysis. The experimental data (Table 1) were analyzed by the response surface regression (RSREG) procedure of SAS software to fit the following second-order polynomial equation

$$Y = \beta_{k0} + \sum_{i=1}^{3} \beta_{ki}x_i + \sum_{i=1}^{3} \beta_{kii}x_i^2 + \sum_{i=1}^{2}\sum_{j=i+1}^{3} \beta_{kij}x_ix_j, \qquad (1)$$

where Y is the response (percent molar conversion), β_{k0}, β_{ki}, β_{kii}, and β_{kij} are constant coefficients, and x_i is the uncoded independent variable. The ridge max option was used to compute the estimated ridge of maximum response for increasing radii from the center of the original design.
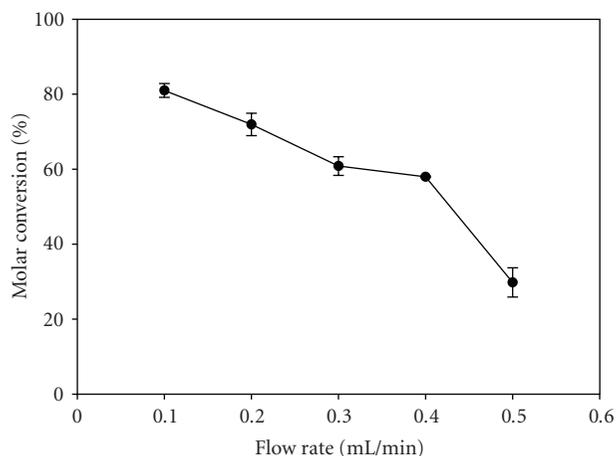
FIGURE 1: The effects of flow rate on the conversion of soybean oil to biodiesel. Reaction conditions: temperature 50°C, substrate molar ratio 1 : 4, and 1.7 g enzyme.

3. Results and Discussion

3.1. Effect of Reaction Parameters. Reaction temperature and molar substrates ratio are crucial parameters affecting the yield of enzymatic synthesis of biodiesel [6]. Many researches [10, 24, 25] showed that lipase such as Novozym 435 deactivated at a methanol-to-oil ratio more than 3. The deactivation was resulted from the contact between lipase and insoluble methanol in the reaction mixture. However, the molar conversion increased with the increase of methanol to soybean oil ratio and did not decrease even at the ratio above 4 : 1 (data not shown). The addition of *tert*-butanol to the mixture enhanced miscibility of methanol with vegetable oils and therefore protected lipase from deactivation [10, 26]. *Tert*-butanol also enhanced mass transfer by reducing viscosity of oils [10]. Thus, the lipase maintained high activity even at high methanol concentration.

Another important parameter affecting the conversion efficiency in a packed-bed reactor is substrate flow rate. Figure 1 showed the conversion decreased with increasing flow rate. The highest conversion (81%) was obtained at a flow rate of 0.1 mL/min. The increase in substrate flow rate caused reduction in the residence time of substrate in the packed-bed reactor, which resulted in poor contact between lipases and substrates.

The choice of temperature, flow rate, and substrate molar ratio range (Table 1) needs to be examined precisely in Box-Behnken design. The optimal conditions of synthesis can be found inside the experimental region through the analyses of statistics and contour plots.

3.2. Model Fitting. The major objective of this study is the development and evaluation of a statistical approach to better understand the affinity between the variables of a continuous lipase-catalyzed transesterification reaction in continuously packed-bed reactor. On the basis of this concept, the large-scale and continuous process can be optimized to obtain greater efficiency and to decrease human resource requirements. Compared with one-factor-at-a-time design, the RSM employed in this study was more efficient in reducing the number of experimental runs and time required to determine the optimal parameters for optimal biodiesel production.

The RSREG procedure was employed to fit the second-order polynomial (1) to the experimental data (Table 2) with a coefficient of determination, $R^2 = 0.985$. Among the various treatments, the highest molar conversion ($81.23 \pm 1.49\%$) was treatment no. 2 (0.1 mL/min, 60°C, and substrate molar ratio 1 : 4), and the lowest molar conversion ($27.53 \pm 1.76\%$) was treatment no. 3 (0.5 mL/min, 40°C, substrate molar ratio 1 : 4). Moreover, treatments no. 2, no. 11, and no. 12 showed that the optimal flow rate was 0.1 mL/min, resulting in approximately 80% molar conversion. From the SAS output of RSREG, the second-order polynomial (2) obtained was given below:

$$\begin{aligned} Y = {} & 7.188 + 1.613x_1 - 163.456x_2 + 13.633x_3 \\ & - 0.023x_1^2 + 1.236x_1x_2 + 0.292x_2^2 \\ & + 0.222x_1x_3 + 1.125x_2x_3 - 2.708x_3^2. \end{aligned} \quad (2)$$

The analysis of variance (ANOVA) data were presented in Table 2. The results indicated that the second-order polynomial model was an adequate representation of the actual relationship between the response percentage and the significant variables with very small *P*-value (.0006). Furthermore, the overall effect of the three synthesis variables on the molar conversion of biodiesel was further analyzed by a joint test (Table 3). These results revealed that the flow rate and temperature were important parameters, which have a statistically significant overall effect ($P < .01$) on the response molar conversion of biodiesel.

3.3. Mutual Effect of Parameters. The relationships between reaction factors and response could be better understood by examining the planned series of contour plots generated from the predicted model. Figures 2(a)–2(c) represented the same range of temperature (40°C–60°C) and substrate molar ratio (1 : 3–1 : 5). Overall, the three contour plots presented a similar behavior in that predicted molar conversion increased. At a flow rate of 0.1 mL/min, the maximum molar conversion obtained was up to 80% (Figure 2(a)) whereas the yield was significantly reduced at 0.5 mL/min (Figure 2(c)). Thus, the most suitable flow rate would be around 0.1 mL/min.

3.4. Obtaining Optimal Synthesis Conditions. The optimal point of synthesis was determined by ridge max analysis, which approximates the estimated ridge of maximum response for increasing radii from the center of original design. Table 4 indicated that molar conversion increased with a reduced flow rate, which is in agreement with our previous study [23]. The ridge max analysis indicates that maximum molar conversion was $83.31 \pm 2.07\%$ at 0.1 mL/min, 52.1°C, with a 1 : 4 substrate molarratios.

TABLE 1: 3-level-3-factor Box-Behnken design of experiments.

Treatment[a] no.	Temperature (°C) X_1	Factors Flow rate (mL/min) X_2	Molar ratio (alcohol/oil) X_3	Yield (%) $Y_1 \pm$ SD	Predicted Yield[c] (%) Y_2
1	1[b] (60)	1 (0.5)	0 (4)	55.00 ± 2.53	58.04
2	1 (60)	−1 (0.1)	0 (4)	81.23 ± 1.49	82.26
3	−1 (40)	1 (0.5)	0 (4)	27.53 ± 1.76	26.50
4	−1 (40)	−1 (0.1)	0 (4)	70.53 ± 1.69	67.49
5	1 (60)	0 (0.3)	1 (5)	72.40 ± 1.69	71.65
6	1 (60)	0 (0.3)	−1 (3)	63.65 ± 1.49	60.34
7	−1 (40)	0 (0.3)	1 (5)	39.72 ± 0.93	43.03
8	−1 (40)	0 (0.3)	−1 (3)	41.90 ± 0.09	42.65
9	0 (50)	1 (0.5)	1 (5)	56.94 ± 1.09	54.66
10	0 (50)	1 (0.5)	−1 (3)	41.14 ± 0.41	41.42
11	0 (50)	−1 (0.1)	1 (5)	80.14 ± 2.32	79.87
12	0 (50)	−1 (0.1)	−1 (3)	79.13 ± 3.88	81.42
13	0 (50)	0 (0.3)	0 (4)	63.98 ± 1.26	63.98
14	0 (50)	0 (0.3)	0 (4)	63.22 ± 0.35	63.98
15	0 (50)	0 (0.3)	0 (4)	64.73 ± 0.71	63.98

[a]The treatments were run in random order.
[b]The values (−1), (0), and (1) are coded levels.
[c]Calculated from the refined model.

(a) Flow rate 0.1 mL/min   (b) Flow rate 0.3 mL/min   (c) Flow rate 0.5 mL/min
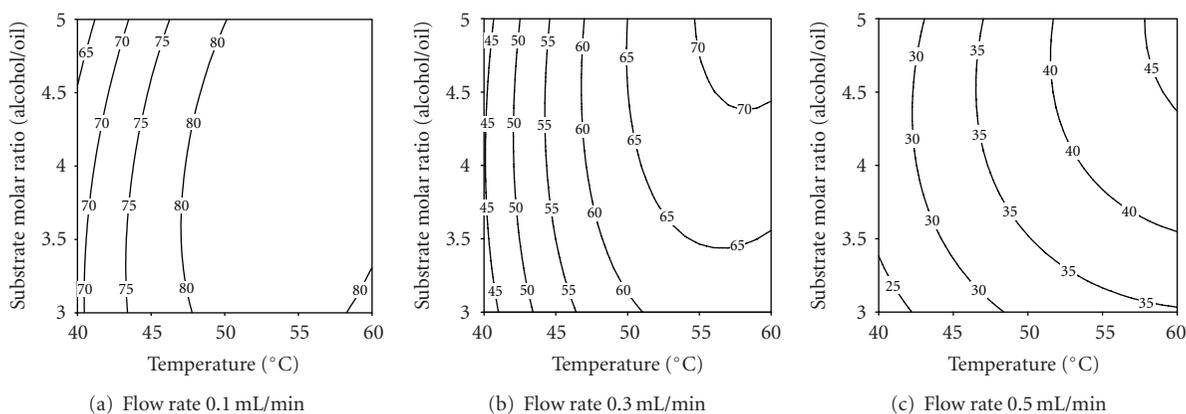
FIGURE 2: Contour plots of molar conversion of continuous synthesized fatty acid methyl esters. Reaction conditions: constant flow rate with 1.7 g enzyme. Numbers inside the plots indicate the molar conversion at given reaction conditions.

TABLE 2: Analysis of variance for synthesis variables pertaining to the response of percent molar conversion.

Source	df	Sum of squares	Prob > F
Model	9	0.21783	0.0006
Linear	3	0.18552	<0.0001
Quadratic	3	0.01473	0.0314
Crossproduct	3	0.01757	0.0221
Pure error	2	0.00003	
Total error	5	0.00355	
R^2	0.985		

TABLE 3: Analysis of variance for the joint test.

Factor	df	Sum of squares	Prob > F[a]
Temperature (X_1)	4	1388.879994	0.0010
Flow rate (X_2)	4	2269.962598	0.0003
Molar ratio (X_3)	4	166.191998	0.0898[b]

[a]Prob > F: level of significance.
[b]Not significant at $P > .01$.

3.5. Model Verification. The biocatalysis of such esters by lipase-catalysis reactions under milder conditions has become a current industrial interest. Many reactions were conducted by immobilized lipase in a continuous packed-bed bioreactor for minimizing labors and overhead costs in the industry [27]. An optimized enzymatic catalysis of biodiesel production with higher yield at reduced cost in the optimal condition would be more appealing to the consumers and benefit to the manufacturers. The validity of the

TABLE 4: Estimated ridge of maximum response for variable percent molar conversion.

| Coded radius	Estimated response (% corporation)	Standard error	Uncoded factor values		
			X_1 (°C)	X_2 (mL/min)	X_3 (methanol/oil)
0.0	63.98	1.92	50.00	0.30	4.00
0.1	65.97	1.92	50.53	0.28	4.01
0.2	67.91	1.90	50.98	0.27	4.03
0.3	69.82	1.87	51.34	0.25	4.03
0.4	71.71	1.84	51.63	0.23	4.04
0.5	73.59	1.81	51.84	0.21	4.04
0.6	75.49	1.79	51.98	0.19	4.04
0.7	77.39	1.80	52.07	0.17	4.03
0.8	79.33	1.84	52.12	0.15	4.02
0.9	81.30	1.92	52.12	0.13	4.01
1.0	83.31	2.07	52.09	0.10	4.00
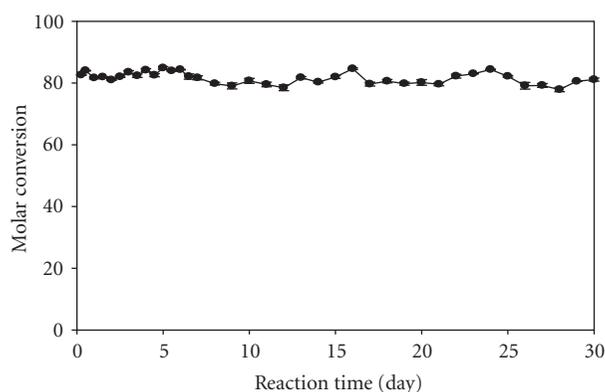
FIGURE 3: The effects of operating time on the conversion of soybean oil to biodiesel. Reaction conditions: temperature 52.1°C, flow rate 0.1 mL/min, substrate molar ratio 1 : 4, and 1.7 g enzyme.

predicted model was examined by conducting experiments at the suggested optimum synthesis conditions. The value predicted by ridge max analysis was 83.31 ± 2.07%, and the actual value was 82.81 ± 0.98%.

3.6. Enzyme Reuse. The operational stability of the continuous packed-bed reactor was shown in Figure 3. The immobilized lipase showed excellent stability under its optimal conditions. The ridge max analysis suggested the optimal reaction conditions were flow rate of 0.1 mL/min, temperature of 52.1°C, and a methanol-to-oil ratio of 1 : 4. A high molar conversion (80%) was obtained and the immobilized lipase was operated over 30 days without losing catalytic activity. Watanabe et al. [19] achieved a high conversion (90%) over 100 days in the stepwise methanolysis with three columns of continuous packed-bed reactor system; however, they required a large amount of immobilized lipase (10 g). Chen and Wu [20] studied the methanolysis reaction with a continuous stirred tank reactor and obtained a 70% conversion over 70 days. The drawback is the requirement for periodical regeneration of the lipase by *tert*-butanol washing. Halim et al. [21] reported a conversion of 79%-80% over 5 days by methanolysis of waste palm oils in a *tert*-butanol mediated packed-bed system. These results, applied to large-scale biodiesel production, will increase costs associated with reactor design and poor operational stability. The packed-bed reactor proposed in this paper not only extended the usability of the lipase but also improved the conversion efficiency in the continuous one-step operation system.

4. Conclusions

We developed an optimal packed-bed reactor for continuous production of biodiesel from soybean oil in a *tert*-butanol solvent system. RSM and 3-factor-3-level Box-Behnken design were employed for optimization of transesterification of soybean oil. The ridge max analysis indicates that maximum molar conversion was 83.31% at 0.1 mL/min, 52.1°C, with a 1 : 4 substrate molar ratio. The continuous packed-bed reactor can operate more than 30 days without an appreciable loss in substrate conversion. This system demonstrates the potential for industrial scale production of biodiesel by using a packed-bed reactor with a *tert*-butanol solvent system.

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