http://dx.doi.org/10.5935/0103-5053.20140165

J. Braz. Chem. Soc., Vol. 25, No. 9, 1704-1711, 2014.
Printed in Brazil - ©2014 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

Production of Biodiesel Catalyzed by *Candida rugosa* Lipase at Interface of w/o Microemulsion System

Zhongqin Tan,[a] Miaoli Fang,*[,a,b] Huan Du,[a] Lelian Song,[a] Dahai Ren,[c] Xiujuan Tang,[d] Xiaoxiang Han*[,a] and Xinle Liang[a]

[a]*Department of Applied Chemistry, Zhejiang Gongshang University, 310018 Hangzhou, P. R. China*

[b]*Zhejiang Medical College, 310053 Hangzhou, P. R. China*

[c]*Department of Chemical & Biomolecular Engineering, Clarkson University, 13699-5705 Potsdam, New York, USA*

[d]*College of Environmental Science and Engineering, Zhejiang Gongshang University, 310018 Hangzhou, P. R. China*

A síntese de biodiesel foi realizada entre o ácido palmítico e o metanol em microemulsão reversa (w/o) preparada a partir da mistura de ácido dodecilbenzenossulfônico (DBSA)/isooctano/água. O planejamento Box-Behnken foi adotado para avaliar o efeito de importantes fatores que afetam o rendimento de palmitato de metila e a metodologia de superfície de resposta (RSM) foi empregada para descrever os parâmetros do processo de esterificação. Os resultados mostraram que as condições ideais para preparação do palmitato de metila foram: 3,33 w_0 ([H_2O]/[surfactante]), tempo de reação (4,2 h), razão molar 5:1 de metanol/ácido e concentração de lipase de 130 mg g^{-1} ([lipase]/[ácido]) obtendo-se nestas condições 98% de rendimento de biodiesel. As constantes cinéticas do modelo foram determinadas a partir de experimentos à temperatura de 40 °C com concentrações iniciais de 0,025-0,25 mol L^{-1} de ácido palmítico e 0,025-0,3 mol L^{-1} de metanol no sistema de microemulsão. Os estudos cinéticos mostraram que a reação obedece ao mecanismo Ping-Pong bi-bi com inibição por metanol.

The synthesis between palmitic acid and methanol was carried out in a w/o reverse microemulsion prepared from the mixture of dodecylbenzenesulfonic acid (DBSA)/isooctane/water. Box-Behnken design was adopted to evaluate the effect of significant factors on the methyl palmitate yield and response surface methodology (RSM), which was employed to optimize the process parameters in the esterification. The conditions that showed optimal results for methyl palmitate preparation were: 3.33 w_0([H_2O]/[surfactant]), 4.2 h reaction time, 5:1 methanol/acid molar ratio, and 130 mg g^{-1} lipase ([lipase]/[acid]) concentration. The following verification experiment obtained a result of 97% in almost total agreement with the expected value (98%). The kinetic constants of the model were determined by experiments at 40 °C with initial concentrations of 0.025-0.25 mol L^{-1} palmitic acid and 0.025-0.3 mol L^{-1} methanol in the microemulsion system. The kinetic studies showed that the reaction obeyed the Ping-Pong bi-bi mechanism with inhibition by methanol.

Keywords microemulsion, lipase, esterification, response surface methodology (RSM), inhibition

Introduction

Alternative fuels for diesel engines are becoming very important due to diminishing petroleum reserves, environmental deterioration by exhaust gases from petroleum-fueled engines, and increases in the crude oil prices.[1,2] Fatty acid alkyl esters, also called biodiesel, are made from renewable resources such as vegetable oils and animal fats. These esters can significantly lower nitrogen oxide exhaust, emissions of particulate matter, and noxious gases such as NO_x, CO and SO_x.[3-5] Thus biodiesel is environmentally friendly and shows great potential as an alternative liquid fuel.[3]

*e-mail: hxx74@126.com, fangml1633@126.com

Biodiesel can be produced by thermal cracking, esterification of fatty acids, or transesterification of oils and fats with short chain alcohols.[6,7] Transesterification or esterification carried out by using different catalytic systems or in supercritical conditions are the most common methods for biodiesel production.[8,9] The catalysts used for trans/esterification may be grouped in four categories: alkalines, acids, inorganic heterogeneous catalysts and enzymes.[10] Out of these four, the alki or acid catalyzed processes are the most efficient with the shortest times and the highest yields.[3] However, the high energy requirement, reactant consumption, complex treatment of glycerol, and potential pollution to the environment can not be ignored.[11]

Utilization of lipase as a catalyst for biodiesel fuel production has a great potential of producing high yields in a short period of time without consuming as much energy or causing contamination like with the alkali and acid processes. In recent decades, an increased number of researchers have reported on its application.[12-16] To maintain and improve enzymatic activity is key to biosynthesis and biotransformation. Lipases have the unique feature in that their enzyme activity occurs between the aqueous/organic phase. For this reason, their activity generally depends on the available interfacial area.[17] Microemulsion (w/o) as a colloid dispersed system which contains an aqueous/organic phase is a suitable medium for enzyme-catalyzed reactions. The enzyme can be molecularly dispersed and entrapped in the polar core of the microemulsion avoiding direct contact with the organic solvent.[18] Hence, the microstructures of lipases in the reaction media will be protected.[19] The microemulsion containing mixtures of surfactant, water, and organic solvent is capable to solubilize nonpolar or polar substrates. Therefore, the reaction can occur with a large internal interface between the aqueous and organic phase.[20]

In this study, lipase from *Candida rugosa* (CRL) was applied to catalyze the esterification of palmitic acid and methanol for biodidesel production. The reagents used were ideal for this experiment since palmitic acid is a common fatty acid that is widely distributed in nature while methanol is more cost efficient than other alcohols. The reaction was carried out in dodecylbenzenesulfonic acid (DBSA) w/o microemulsion and included the deduction of the reaction mechanism by kinetic modeling. Signal factors in the reaction such as time, methanol/acid molar ratio, w_0, and lipase concentration were investigated to explore their effects on the esterification yield. Box-Behnken design and response surface methodology (RSM)[21] were applied to optimize the esterification conditions to describe the effects and relationships of the main reaction variables to obtain optimum methyl palmitate yield.

Experimental

Materials

Lipase from *Candida rugosa* was purchased from Sigma Aldrich. Palmitic acid and methanol (≥ 99.5%) were obtained from Qiangsheng (Jiangsu Province, PR China). All other chemicals used in the study were of analytical grade and used without further purification.

Experiment design and statistical analysis

The Software program Stat-Ease Design Expert (Version 6.0.5, Stat-Ease. Inc., USA) was used in the statistical experimental design. Significant factors were picked out and RSM analysis was carried out employing a Box-Behnken design, which included 29 experiments of four variables at three levels (−1, 0, 1). This was done to evaluate the effect of those factors on the methyl palmitate yield of the subsequent reaction and determine the optimal conditions.[22,23]

The experimental data was fitted to the quadratic polynomial model. The interaction between the variables was elucidated and the second-order polynomial equation for predicting the optimal point was given as shown in equation 1:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum \sum_{i<j=1}^{4} \beta_{ij} X_i X_j \qquad (1)$$

where Y (%) is the response value of esterification yield, X_i represents independent factors, and β_0, β_i, β_{ii}, β_{ij} are intercept, linear, quadratic and interaction constant coefficients, respectively. The accuracy and general ability of the quadratic polynomial equation was evaluated by the coefficient of determination (R^2), and its regression coefficient significance was checked by a F test. The connection between the response and experimental levels of each factor was expressed visually as response surface curves and contour plots, by which the optimal point for each independent variable was deduced.

Preparation of microemulsion and enzymatic catalysis

The microemulsion system was formulated by mixing isooctane, water and DBSA in appropriate proportions at room temperature in a 50 mL round-bottom flask for a period of time until it became optically transparent and homogeneous. Due to the solubility difficulty of palmitic acid, some of the palmitic acid that was added to the flask was pre-incubated in a water-bath at 40 °C with magnetic stirring. After the palmitic acid was dissolved, methanol

and CRL were also added to the flask. Subsequently, the mixture was stirred for 4 h and the sample was gathered by adding *n*-caprylic alcohol to break the emulsion.

GC analysis

The yield of methyl palmitate was analyzed by gas chromatography (Agilent 6890N GC), equipped with a FID detector and HP-5 capillary column (30.0 m × 320 nm × 0.25 μm). A sample of 20 μL was drawn out and mixed with 280 μL of isooctane and 300 μL of a standard solution for GC analysis. The oven temperature was programed as follows: initially set at 50 °C for 2 min, increased to 130 °C at 5 °C min^{-1} for 4 min, and then increased again to 300 °C at 15 °C min^{-1} and maintained at this temperature for 6 min. Nitrogen was used as the carrier gas at 500 kPa. The temperature of the injector and detector was set at 280 °C and 300 °C, respectively. The product was identified by comparing it with an authentic sample methyl palmitate (Aladdin). Methyl laureate (Aldrich) was used as internal standard for quantitative analysis.

Results and Discussion

Effect of water content w_0

Various w_0 (defined as the molar ratio of water to surfactant) over a range from 2.3 to 3.5 were performed to prepare the microemulsion for catalyzing methyl palmitate production. The results are presented in Figure 1. The methyl palmitate yield increased gradually from 41% to 95% with the w_0 increasing from 2.3 to 3.3. When the w_0 further increased to 3.5, the methyl palmitate decreased to 53%. These results can be explained because when the w_0 was below 3.3 the water content could not meet the lipase conformation requirements to maintain its catalytic activity in continuous reactions.[24] With the increase of w_0, the amount of water available for oil to form oil-water droplets increased, thereby increasing the available interfacial area and activating lipases which were beneficial for the methyl palmitate production.[25] However, when the w_0 was above 3.3 the coalescence of the emulsion water droplets would occur decreasing the interfacial area of the organic and aqueous layers. This had an inhibitory effect on the enzyme activity and disturbed the esterification by hydrolysis.[26,27]

Effect of the molar ratio

In order to research the optimal molar ratio of the alcohol to palmitic acid, the ratios from 1:1 to 8:1 were tested. On one hand, increase of methanol helped the reaction equilibrium move to the product side and improved the conversion of palmitic acid. On the other hand, when the alcohol was in excess, the conversion decreased due to do inhibitory reasons. This is observed (Figure 2), with the increase of molar ratios of methanol to acid. At first, an increase of esterification yield is noticed before the yield subsequently decreased for this reaction. A maximum yield of 94% was acquired at a molar ratio of 4:1. The decrease of yield of methyl palmitate when the methanol concentration was high could be attributed to the inhibitory effect of methanol on the biocatalyst leading to a decline of active centers in the entire volume of the solution.

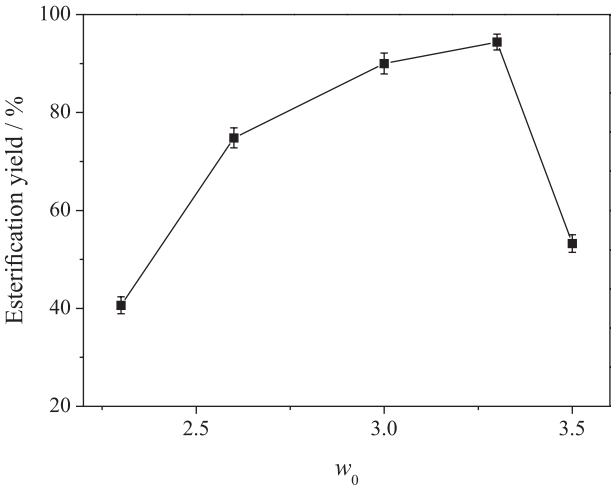

Figure 1. Effect of w_0 on esterification catalyzed by CRL in DBSA microemulsion system (reaction conditions: molar ratio (alcohol/acid) 4, time 4 h, lipase 120 mg g^{-1}).

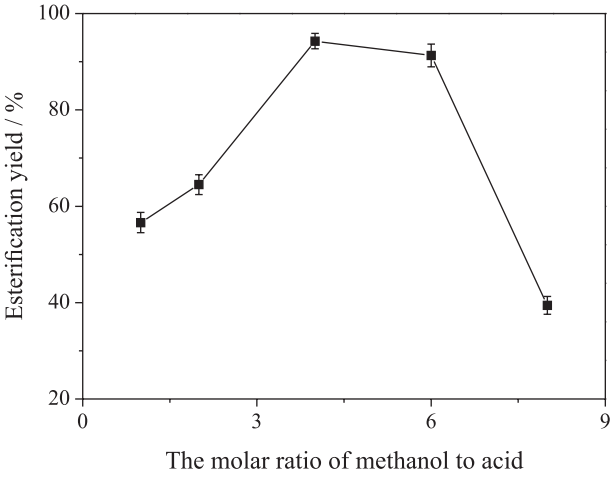

Figure 2. Effect of molar ratio on esterification catalyzed by CRL in DBSA microemulsion system (reaction conditions: w_0 3.3, time 4 h, lipase 120 mg g^{-1}).

Effect of time

The influence of reaction time in the range of 1-5 h was examined along with comparing DBSA microemulsion

with and without lipase as a catalyst. As seen in Figure 3, obviously DBSA microemulsion with lipase exhibited the maximal reaction rate in a short period of time. The yield of methyl palmitate in DBSA microemulsion with lipase was much higher than DBSA system without lipase by nearly 70% at 4 h. After that, the percentage of conversion did not show any significant difference. The equilibrium time was reached within 4 h.

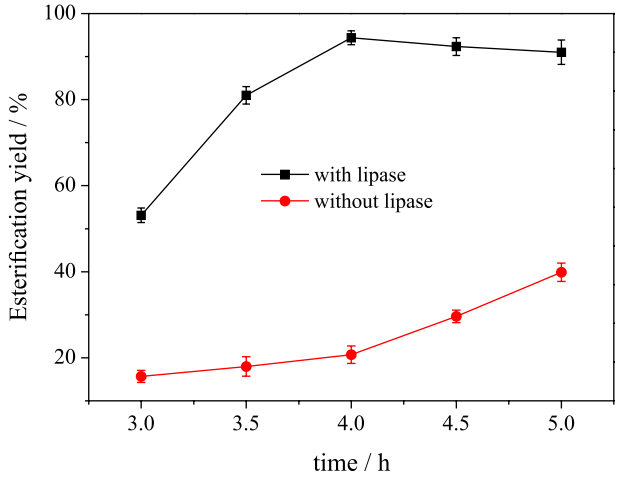

Figure 3. Effect of reaction time on esterification catalyzed by CRL and without CRL in DBSA microemulsion system (reaction conditions: molar ratio (alcohol/acid) 4, w_0 3.3, lipase 120 mg g^{-1}).

Effect of concentration of lipase

The concentration of enzyme played a vital role in the esterification reaction. The reaction rate increased with the increasing concentration of enzyme. However, superfluous lipase might also have a negative effect and its industrial production cost is high. Thus, the optimum concentration of lipase was studied and results listed in Figure 4. From Figure 4, it was found that the yield of methyl palmitate increased with the increasing lipase concentration. The maximal yield of methyl palmitate (94%) was obtained at lipase concentration of 120 mg g^{-1}. When the lipase concentration was over 120 mg g^{-1}, the yield of methyl palmitate decreased slightly. This is due to the excess lipase aggregated with each other causing the active sites of the lipase to be less exposed to the substrates which hindered the full contact of individual lipase macromolecules with reactants.[28-30]

Study on different reaction system

The yields of methyl palmitate in different reaction systems are presented in Figure 5. It was determined that DBSA/isooctane/water microemulsion system achieved the best results. There was a tiny decrease between

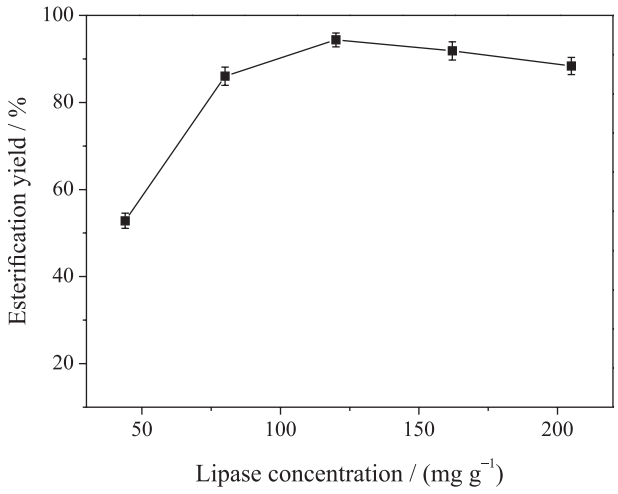

Figure 4. The effect of concentration of lipase on esterification in DBSA microemulsion system (reaction conditions: molar ratio (alcohol/acid) 4, w_0 3.3, time 4 h).

isooctane and *n*-heptane and a major decrease between cyclohexane and *n*-hexane. A possible explanation for these results was the difference in the molecular structure of the organic media used. *n*-heptane and *n*-hexane are straight, short-chain alkanes which can embed in the interfacial septum formed by the DBSA molecules. The hydrocarbons can then form an additional layer in the interfacial membrane.[31] Penetration of the mostly saturated hydrocarbons into the surfactant layer of the microemulsion impedes the contact and/or interaction between lipase and its substrates resulting in a smaller product yield.[32] In addition, the log *P*-value of the solvent also has a little effect on the yield, as the log *P*-value of the solvent increases the extent of esterification yield.[33] Therefore, there was a subtle drop in esterification yield when *n*-heptane (log*P*-value 4.0) was replaced by *n*-hexane (log*P*-value 3.5). Cyclohexane

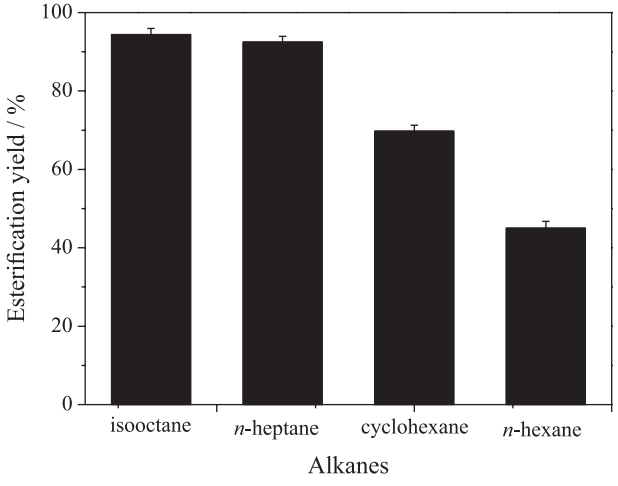

Figure 5. Effect of different kinds of microemulsion system on esterification (reaction conditions:molar ratio (alcohol/acid) 4, w_0 3.3, time 4 h, lipase 120 mg g^{-1}).

has an unique ring structure that does not have a proper structure to penetrate the DBSA interfacial membrane. Thus, lipase activities in the *n*-heptane, cyclohexane, and *n*-hexane medias were considerably lower compared with the lipase activity in isooctane. The biocatalysis in microemulsion had an organic-solvent dependency.[31] Thus, isooctane was the preferable organic phase in the DBSA microemulsion system in further research.

Optimization of the reaction conditions

A three-level four-factor Box-Behnken experimental design and RSM were employed to study this reaction. According to the results of the single factor tests, w_0, reaction time, reactant ratio, and lipase concentration were proven to have significant effects on yield of methyl palmitate. Thus, RSM was used based on these four independent factors. In total, 29 experiments were required with each experiment performed in triplicate. The experimental design and results are presented in Tables S1 and S2. As can be seen, the yield of methyl palmitate ranged from 69 to 98% and the design points of run 14 and run 29 gave the minimum and maximum yields, respectively.

The standard analysis of ANOVA indicated that the model was significant. The model *F*-value of 24.58 and a low probability P (< 0.0001) implied that the model was significantly suitable. The "lack of fit *F*-value" of 3.26 demonstrated that the lack of fit was not significantly relative to the pure error. There was a 13% chance that a "lack of fit *F*-value" could occur due to noise. The coefficient of determination (R^2) of the model was 96%, which indicated a good accuracy and a general fitness of the polynomial model, and the response trends could be analyzed by with this model. Regression coefficients of the predicted quadratic polynomial model are shown in Table 1.

The experimental data was well qualified for the model equation which could be expressed as follows:

$$Y = +96.35 - 1.34X_1 + 3.56X_2 + 4.39X_3 + 052X_4 - 2.15X_1^2 - 6.97X_2^2 - 10.15X_3^2 - 9.70X_4^2 + 0.43X_1X_2 + 1.92X_1X_3 + 3.33X_1X_4 + 4.36X_2X_3 + 2.38X_2X_4 + 6.81X_3X_4 \quad (2)$$

where X_1, X_2, X_3 and X_4 were the coded values of the test variables w_0, reaction time, alcohol/acid mole ratio, and lipase concentration respectively and Y was the response of yield of methyl palmitate. Equation 2 indicated that linear terms X_2, X_3, X_4 and quadratic terms $X_1 \cdot X_2$, $X_1 \cdot X_3$, $X_1 \cdot X_4$, $X_2 \cdot X_3$, $X_2 \cdot X_4$, $X_3 \cdot X_4$ (positive coefficients) had positive effects on the increase of Y. However, X_1, as well as X_1^2; X_2^2, X_3^2 and X_4^2 had negative effects. Thus, both the linear effect between the independent variables and the interaction between the four independent factors were significant.

Table 1. Regression coefficient of predicted quadratic polynomial model

Term	Coefficient estimated	Standard error	Probe > *F*
Intercept	96.35	1.06	< 0.0001[a]
X_1	−1.34	0.69	0.0705
X_2	3.56	0.69	0.0001[a]
X_3	4.39	0.69	< 0.0001[a]
X_4	0.52	0.69	0.4605
X_1^2	−2.15	0.93	0.0372[b]
X_2^2	−6.97	0.93	< 0.0001[a]
X_3^2	−10.15	0.93	< 0.0001[a]
X_4^2	−9.7	0.93	< 0.0001[a]
X_1X_2	0.46	1.19	0.7061
X_1X_3	1.92	1.19	0.1290
X_1X_4	3.33	1.19	0.0140[b]
X_2X_3	4.36	1.19	0.0025[a]
X_2X_4	2.38	1.19	0.0650
X_3X_4	6.81	1.19	< 0.0001[a]

[a]Significant at 1% level; [b]significant at 5% level.

The interaction between corresponding factors was reflected by the shape of the contour lines. Response surface contour plots obtained from the predicted model are shown in Figure 6, which displays the effects of four independent factors and various composites on the esterification yield.

The variation of yield of methyl palmitate with w_0, reaction time, alcohol/acid molar ratio and lipase concentration are given in Figure 6a, Figure 6b and Figure 6c, respectively. When the w_0 was kept at 3.3, an increase in methyl palmitate yield was observed with the increase of reaction time and alcohol/acid molar ratio initially. However, the trend reversed when the reaction time, alcohol/acid molar ratio, and lipase concentration surpassed a certain value. For the reaction time, yield increased until reaching 4 hours when equilibrium was obtained. In addition, both the alcohol/acid molar ratio and lipase concentration improved the reaction rate of esterification initially. However, when the alcohol/acid molar ratio and lipase concentration exceeded 4:1 or a 120 mg g^{-1} value respectively, the trend reversed because too much methanol caused lipase denaturation and superfluous lipase aggregated. As can be seen in Figure 6c, the interaction effect of w_0 and lipase concentration on production of methyl palmitate was significant.

The interaction of reaction time and lipase concentration on methyl ester synthesis was presented in Figure 6e. Compared with other contour lines, they were rounded

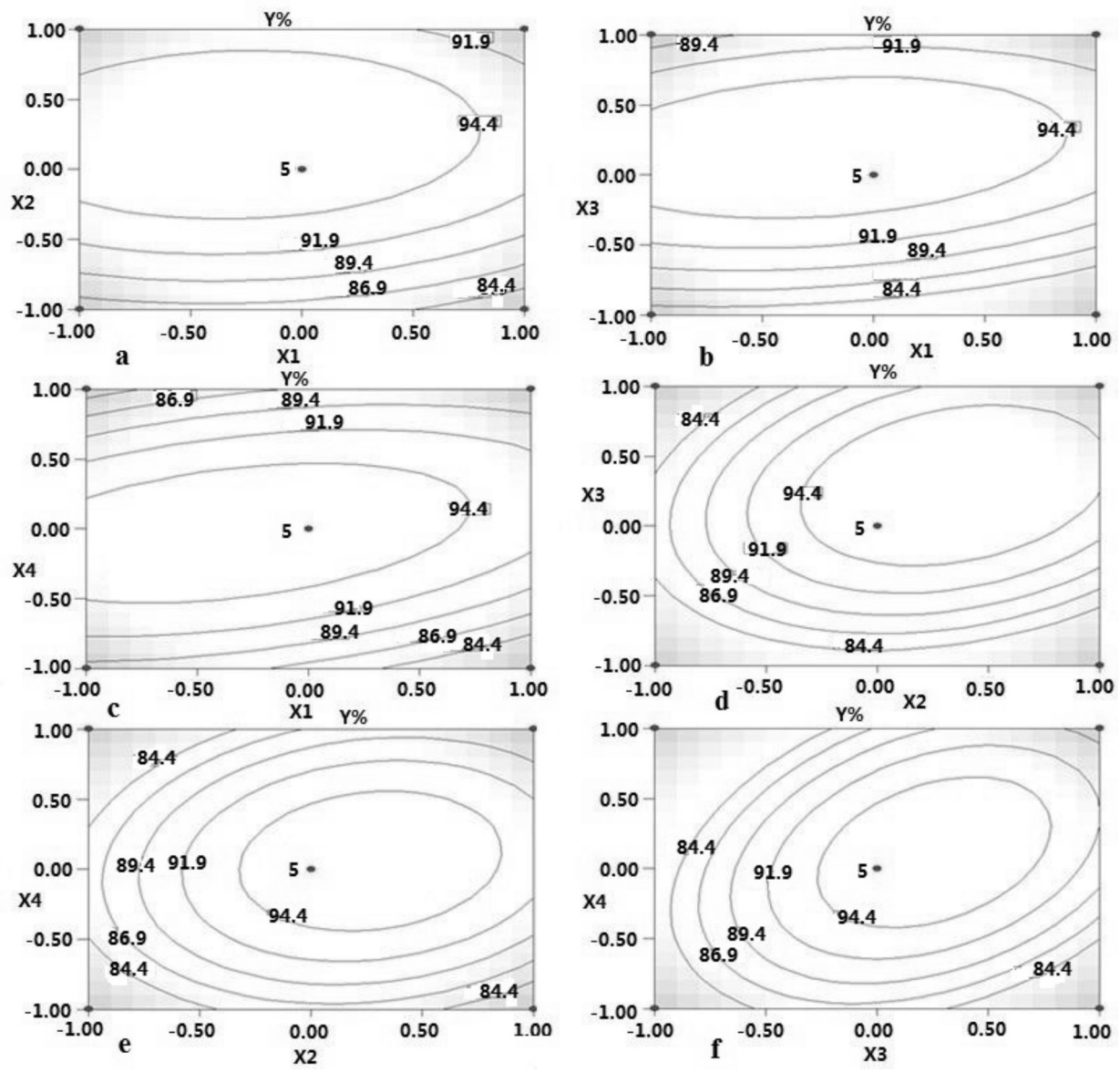

Figure 6. Response surface contour plots of interaction between the four independent factors on yield of biodiesel. (a) Effect of w_0 and reaction time; (b) effect of w_0 and methanol/acid molar ratio; (c) effect of w_0 and lipase concentration; (d) effect of reaction time and methanol/acid molar ratio; (e) effect of reaction time and lipase concentration; (f) effect of methanol/acid molar ratio and lipase concentration.

which meant the interaction effect of these two factors was insignificant. Figure 6d presented a contour plot of the effect of reaction time and alcohol/acid molar ratio of the reaction. Figure 6f presented the effect of alcohol/acid molar ratio and lipase concentration on the reaction. From the analysis of the response surface, reaction time, alcohol/acid molar ratio, and lipase concentration had a significant effect on the response surface. In comparison to reaction time and lipase concentration, alcohol/acid molar ratio was more significant. At first, the conversion of palmitic acid increased with increasing alcohol/acid molar ratio, which reflected a general effect of ascending reaction time on the reaction. Subsequently, the yield of methyl palmitate emerged a peak with a maximum value and declined. The cause could be explained because the activation center of lipase potentally is damaged by methanol in the reaction, but becasue the methanol was dissolved in the microemulsion system due to it being in low enough concentration (methanol/acid molar ratio = 4:1) no lipase damage occured and the reaction rate was accelerated.

Validation of the model

According to the reaction result, the regression model showed a perfect fitness for the esterification yield. The optimal reaction parameters evaluated from the regression model (equation 2) were as follows: 3.327 w_0, 4.21 h reaction

time, 4.78:1 methanol/acid molar ratio and 129.2 mg g^{-1} lipase concentration. Three parallel experiments were conducted under the model optimal conditions of 3.33 w_0 ([H_2O]/[surfactant]), 4.2 h reaction time, 5:1 methanol/acid molar ratio, 130 mg g^{-1} lipase concentration and the average yield of methyl palmitate was 97%, which was consistent with the predicted value (98%). For this reason, the regression model was considered to be effective and accurate to predict the yield of methyl palmitate.

Kinetic study

During the study of molar ratio, there was a decrease of the conversion with the excess methanol. So the effect of concentration of methanol and palmitic acid on the rate of reaction was researched systematically. For determination of initial rates of esterification, the concentration of the methanol varying from 0.025 to 0.3 mol L^{-1}, a palmitic acid concentration between 0.025-0.25 mol, and 130 mg g^{-1} *Candida rugosa* lipase were adopted in the microemulsion system. The initial rates were determined from the quantified data.

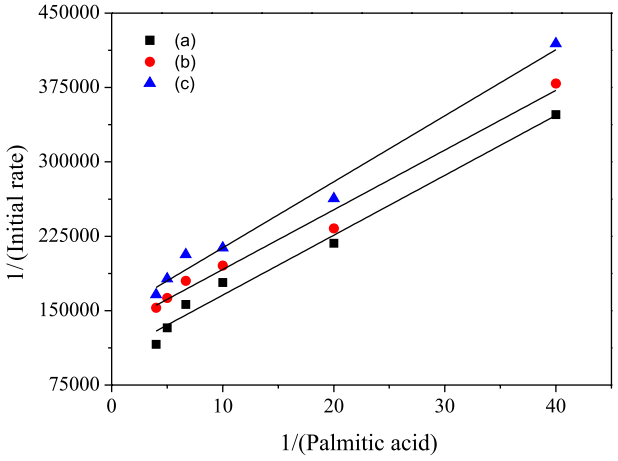

Figure 7. Lineweaver-Burk plot: 1/[Initial rate] *vs.* 1/[P] at different concentration of methanol, (a) 0.2 mol L^{-1}; (b) 0.25 mol L^{-1}; (c) 0.3 mol L^{-1}.

The double reciprocal (Lineweaver-Burk) plot of the initial velocity *vs*. methanol concentrations at several palmitic acid concentrations is shown in Figure 7. The rate of reaction increased with the increasing concentration of methanol at constant concentration of palmitic acid. When it reached the maximum at critical concentration any further increase in methanol concentration caused the reaction rate to decrease and thus the substrate inhibition was notable. The Lineweaver Burk plot $1/r_0$ versus $1/[P_0]$ for varied initial concentrations of methanol gives parallel lines (Figure 7), where r_0 is the initial rate of reaction and $[M_0]$ is the initial concentration of methanol. When one of the reactants forms a complex with the lipase that can participate in the reaction, it is called Ping-Pong bi-bi with dead end inhibition.[34] Figure 7 showed that the results were in accord with the Ping-Pong bi-bi mechanism which was postulated with dead end alcohol inhibition. This mechanism is depicted below in Cleland's notation:[35]

$$E \rightarrow P \underset{k1'}{\overset{k1}{\rightleftarrows}} EP \underset{k2'}{\overset{k2}{\rightleftarrows}} EA + W \rightarrow EA + M \underset{k3'}{\overset{k3}{\rightleftarrows}} EAM \underset{k4'}{\overset{k4}{\rightleftarrows}} EQ \rightarrow Q + E$$
$$\downarrow$$
$$M \underset{}{\overset{Ki}{\rightleftarrows}} EM$$

where P and M are the substrates palmitic acid and methanol, W and Q are the products water and methyl palmitate, E and EA are the free lipase and modified lipase, EP and EM are the lipase-palmitic acid complex and lipase-methanol dead-end complex, and EAM and EQ are the modified lipase-methanol complex and the lipase-methyl palmitate complex. The rate equation of the esterification reaction is as follows:

$$v_0 = \frac{v_{max}[P][M]}{K_{mM}[P] + K_{mp}[M]\left(1 + \frac{[M]}{K_i}\right) + [P][M]} \quad (3)$$

where v_0 is the initial rate of reaction, v_{max} is the maximum rate, [P] and [M] are the initial substrate concentrations palmitic acid and methanol, K_{mP} is the Michaelis constant for palmitic acid, K_{mM} is the Michaelis constant for methanol, and K_i is the inhibition constant due to methanol.

To verify the application of Ping-Pong bi-bi mechanism, the data was calculated (FigureS1 and FigureS2 Supplementary Information) and the kinetic parameters determined for above mechanism were obtained as: $v_{max} = 1.55 \times 10^{-5}$ mol (L min mg)$^{-1}$, $K_{mP} = 0.10221$ mol L^{-1}, $K_{mM} = 0.1612$ mol L^{-1}, $K_i = 0.1083$ mol L^{-1}. The experiment parameters gave a straight line passing through the origin with a good correlation coefficient.

Conclusions

In this study, RSM was employed to optimize methyl palmitate preparation conditions of methanol and palmitic acid catalyzed by the lipase from *Candida rugosa* in a DBSA microemulsion system. The results showed that there was a high consistency between the predicted and experimental values. By vibration test, a methyl palmitate yield of 97% was obtained under the model optimal conditions: w_0 3.33, reaction time 4.2 h, methanol/acid molar ratio 5:1, lipase concentration 130 mg g^{-1}, which was in almost total agreement with the expected value (98%). Data for the lipase catalysis in w/o microemulsions

supports that this esterification reaction's mechanism was the Ping-Pong bi-bi mechanism.

Acknowledgements

The supports of this work from National Natural Science Foundation of China (No. 21177110) and National Natural Science Foundation of Zhejiang province, China (No. LY13B070005) are gratefully acknowledged.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

References

1. Fukuda, H.; Kondo, A.; Noda, H.; *J. Biosci. Bioeng*. **2001**, *92*, 405.
2. Sidney, A. N.; Adalgisa, R. D. A.; *J. Braz. Chem. Soc.* **2013**, *24*, 1891.
3. Tan, T.; Liu, J.; Nie, K.; Deng, L.; Wang, F.; *Biotechnol. Adv.* **2010**, *28*, 628.
4. Rosa, C. D.; Morandim, M. B.; Ninow, J. L.; Oliveira, D.; Treichel, H.; Oliveira, J. V.; *J. Supercrit. Fluids* **2008**, *47*, 49.
5. Yang, L.; Wei, D.; Dehua, L.; *J. Mol. Catal. B: Enzym*. **2013**, *91*, 67.
6. Ma, F.; Hanna, M. A.; *Bioresour. Technol*. **1999**, *70*, 1.
7. Lee, J. H.; Kim, S. B.; Kang, S. W.; Song, Y. S.; Park, C.; Han, S. O.; Kim, S. W.; *Bioresour. Technol*. **2011**, *102*, 2105.
8. Rosset, I. G.; Tavares, M. C. H.; Assaf, E. M.; Porto, A. L. M.; *Catal. Lett*. **2013**, *143*, 863.
9. Demirbas, A.; *Energ. Convers. Manage.* **2008**, *49*, 125.
10. Gog, A.; Roman, M.; Tosa, M.; Paizs, C.; Irimie, F. D.; *Renew. Energ*. **2012**, *39*, 10.
11. Lara Pizarro, A. V.; Park, E. Y.; *Process. Biochem.* **2003**, *38*, 1077.
12. Rosset, I. G.; Tavares, M. C. H.; Assaf, E. M.; Porto, A. L. M.; *Appl. Catal. A* **2011**, *392*, 136.
13. Lua, X. F.; Vorab, H.; Khosla, C.; *Metab. Eng*. **2008**, *10*, 333.
14. Kaieda, M.; Samukawa, T.; Matsumoto, T.; Ban, K.; Kondo, A.; Shimada, Y.; Noda, H.; Nomoto, F.; Ohtsuka, K.; Izumoto, E.; Fukuda, H.; *J. Biosci. Bioeng*. **1999**, *88*, 627.
15. Rosset, I. G.; Assaf, E. M.; Porto, A. L. M.; *Curr. Catal.* **2013**, *2*, 53.
16. Anschau, A.; Aragão, V. C.; Porciuncula, B. D. A.; Kalil, S. J.; Burkert, C. A. V.; Burkert, J. F. M.; *J. Braz. Chem. Soc.* **2011**, *22*, 2148.
17. Stamatis, H.; Xenakis, A.; Provelegiou, M.; Kolisis, F. N.; *Biotechnol. Bioeng.* **1993**, *42*, 103.
18. Eli, R.; Prakash, K.; *Biotechnol. Lett*. **1990**, *12*, 241.
19. Stamatis, H.; Xenakis, A.; Kolisis, F. N.; *Biotechnol. Adv.* **1999**, *17*, 293.
20. Orlich, B.; Schomäcker, R.; *Enzyme Microb. Technol*. **2001**, *28*, 42.
21. Khuri, A. I.; Cornell, J. A.; *Response Surfaces: Design and Analyses,* 10th ed.; Marcel Dekker Inc: New York, 1987.
22. Kiran, K. R.; Karanth, N. G.; Divakar, S.; *Appl. Microbiol. Biotechnol*. **1999**, *52*, 579.
23. Manohar, B.; Divakar, S.; *Process. Biochem*. **2004**, *39*, 847.
24. Jiang, Y.; Liu, X.; Chen, Y.; Zhou, L.; He, Y.; Ma, L.; Gao, J.; *Bioresour. Technol.* **2014**, *153*, 278.
25. Su, E. Z.; Zhang, M. J.; Zhang, J. G.; Gao, J. F.; Wei, D. Z.; *Biochem. Eng. J.* **2007**, *36*, 167.
26. Jung, H.; Lee, Y.; Kim, D.; Han, S. O.; Kim, S. W.; Lee, J.; Kim, Y. H.; Park, C.; *Enzyme Microb. Technol*. **2012**, *51*, 143.
27. Binks, B. P.; Whitby, C. P.; *Langmuir* **2004**, *20*, 1130.
28. Li, Q.; Yan, Y.; *Appl. Energy* **2010**, *87*, 3148.
29. Kumari, A.; Mahapatra, P.; Garlapati, V. K.; Banerjee, R.; *Biotechnol. Biofuels* **2009**, *2*, 1.
30. Verdugo, C.; Luque, R.; Luna, D.; Hidalgo, J. M.; Posadillo, A.; Sancho, E. D.; Rodriguez, S.; Ferreira-Dias, S.; Bautista, F.; Romero, A. A.; *Bioresour. Technol.* **2010,** *101*, 6657.
31. Park, K. M.; Kim, Y. N.; Choi, S. J.; Chang, P. S.; *Food Chem*. **2013**, *138*, 733.
32. Hirai, M.; Kawai-Hirai, R.; Sanada, M.; Iwase, H.; Mitsuya, S.; *J. Phys. Chem. B* **1999**, *103*, 9658.
33. Manohar, B.; Divakar, S.; *Process Biochem.* **2005**, *40*, 3372.
34. Zhang, H. Y.; Wang, X.; Ching, C. B.; *Chirality* **2007**, *19*, 245.
35. Cleland, W. W.; *Biochim. Biophys. Acta* **1963**, *67*, 104.

Submitted on: May 13, 2014
Published online: July 15, 2014