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Continuous Biosynthesis of Farnesyl Laurate in Packed Bed Reactor: Optimization using Response Surface Methodology

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Abstract: This study is aimed to develop an optimal continuous procedure for lipase-catalyzed esterification of farnesol with lauric acid in a packed bed reactor in order to investigate the possibility of large scale production. Response Surface Methodology (RSM) based on Central Composite Rotatable Design (CCRD) was used to optimize the two important reaction variables which are packed bed height (cm) and substrate flow rate (ml/min) for the esterification of farnesol with lauric acid in a continuous packed bed reactor. The optimum conditions for the esterification of farnesol with lauric acid were found as the following: 18.18 cm packed bed height and 0.9 mL min^{-1} substrate flow rate. The optimum molar conversion of lauric acid to farnesyl laurate was 98.07±0.82%.

Key words: Esterification, farnesyl laurate, continuous, packed bed reactor, response surface methodology, central composite rotatable design

INTRODUCTION

Farnesyl laurate is a long-chain fatty acid ester as well as sesquiterpene ester that present great interest in food, cosmetic and pharmaceutical industries as flavor and fragrance compounds (Claon and Akoh, 1994; Stamatis *et al*., 1998; Chatterjee and Bhattacharrya, 1998). The demand of long-chain fatty acids esters has been growing continuously over the past 10 years. Although lipase-catalyzed long-chain fatty acid esterification has significant benefits, the industrial application of the technology has been slow (Laudani *et al*., 2007). Many examples of biocatalytic synthesis have been documented, though few were optimized in bioreactors in spite of their importance for large scale preparation (Khaled *et al*., 1991; Xu *et al*., 2001; Petzelbauer *et al*., 2002; Xi and Xi, 2005). Packed bed reactors have been extensively investigated by several researchers and the process has to be technically and economically feasible for use in industrial scale application (Watanabe *et al*., 2001; Nie *et al*., 2006; Royon *et al*., 2007).

The packed bed reactor is one of the most commonly employed for solid-fluid contacting in heterogeneous catalysis (1) because it allows reuse of the enzyme without need of a prior separation, (2) it permits to handle substrates of low solubility by using large volumes containing low concentrations of substrate, (3) it leads to more consistent product quality and improved enzyme stability due to the ease of automation and control, (4) it is suitable for long-term and industrial scale production, differently from a stirred-tank reactor where enzymes particles would be susceptible to breaking because of the mechanical shear stress and (5) it is more cost effective than the batch operation (Roca *et al*., 1996; Mu and Hoy, 1998; Xu *et al*., 1998; Shimada *et al*., 1999; Laudani *et al*., 2007).

In this study, we developed a packed bed reactor system for production of farnesyl laurate. The present work was focused on the reaction parameters that affected lipase-catalyzed esterification of farnesol with lauric acid using iso-octane as a solvent in a continuously packed bed reactor. Present objectives were to better understand the relationships between the reaction variables (packed bed height and substrate flow rate) and the response, molar conversion of lauric acid (%) and to achieve the optimal continuous esterification condition in a packed bed reactor system by using statistical experimental design and RSM analysis. Halim *et al*. (2009) have also conducted the experiment using the same reaction variables for transesterification of waste palm cooking oil with methanol in packed bed reactor using RSM. Response Surface Methodology (RSM) is a collection of mathematical and statistical techniques widely used to determine the effects of several variables and to optimize different chemical and biotechnological processes (Montgomery, 2001; Aziz *et al*., 2007).

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MATERIALS AND METHODS

Materials: Immobilized lipase (Lipozyme RM IM, 150 IUN g^{-1}, 0.2-0.6 mm particles size) from *Rhizomucor miehei* on macroporous anion exchange resin, was obtained from Novozymes (Bagsvaerd, Denmark). Farnesol (≥95% purity), lauric acid (≥96% purity) and iso-octane (99.8% purity) were purchased from Sigma Aldrich (Germany), Fluka (Switzerland) and Fischer Scientific (UK), respectively. The chemicals purchased were of highest purity.

Packed bed reactor setup: The packed bed reactor column with dimensions of 1.2 cm (i.d) × 24 cm length which consisted of a jacketed glass tube was used in the esterification of farnesol with lauric acid. The substrate mixture was fed upwards through the column using a peristaltic pump. The column was packed with immobilized enzyme with small amount of cotton wool placed at the reactor ends. Temperature for the reaction was maintained by the recirculating water bath (RCWB).

Esterification reaction in a continuous reactor: The substrate mixture typically comprised of lauric acid and farnesol with 1.4:1 mol ratio (lauric acid to farnesol molar ratio). Iso-octane was used as the solvent. Substrate flow rate and packed bed height (immobilized enzyme quantity) were varied to study the effect of changing reactor column condition esterification of farnesol with lauric acid. The reaction was carried out at 45°C. Samples were collected after 3 h of reaction time. All the constant parameters were obtained during experimental batch studies.

Gas chromatography analysis: The concentrations of the reactants were determined using a gas chromatography (GC-2014, Shimadzu, Japan) equipped with a Flame Ionization Detector (FID). The column was a capillary column of Inert Cap Wax with dimension of 0.25 mm ID × 30 m × 0.25 μm film thickness (GL Sciences, Japan). Nitrogen was used as carrier gas. The temperature of injector and the detector were 250°C. A temperature program was used, initial temperature 180°C, 10°C min^{-1} up to 240°C and hold for 6 min. Consumption of the lauric acid in the reaction was calculated from the gas chromatography analysis of the sample in order to determine the ester produced. All of measurements were carried out in three replicates to reduce the error in the analytical procedure.

Experimental design: A-five-level-two-factor, Centre Composite Rotatable Design (CCRD) was employed in this optimization study. Packed bed height (A) and substrate flow rate (B) were the independent variables selected to be optimized for the esterification of farnesol with lauric acid. Molar conversion of lauric acid (Y) was taken as the response of the design experiments. The coded and uncoded (actual) levels of the independent variables are given in Table 1. Thirteen experiments were augmented with five replications were carried out at the center points to evaluate the pure error. Once the experiments are performed, second order model (quadratic) was selected since it suggested by the design of experiment analysis with significant value less than 0.05. The response variable (molar conversion of lauric acid) was fitted a second-order model in order to correlate the response variable to the independent variable. The general form of the second order polynomial equation is as follows:

Table 1: Independent variables for coded and uncoded value in CCRD

		Levels				
Variables	Symbol	-2.0 (-α)	-1.0	0.0	+1	+2.0 (+α)
Packed bed height (cm)	A	4.0	9.0	14.0	19.0	24.0
Substrate flow rate (mL min^{-1})	B	0.6	0.9	1.2	1.5	1.8

$$Y = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ij} X_i X_j + \varepsilon \quad (1)$$

where, Y is the response (dependent variable), b_0 the constant coefficient, b_i, b_{ii} and b_{ij} the coefficient for the linear, quadratic and interaction effect, x_i and x_j the factors (independent variables) and ε is the error.

RESULTS AND DISCUSSION

In our previous study, the optimum condition for farnesyl laurate production was studied in a batch system. The optimum conditions from the batch study (Lipozyme RM IM as catalyst, 1.4:1 lauric acid to farnesol molar ratio, 0.93 g of lipase loading, temperature of 45°C and iso-octane as solvent) were used as a basis to conduct the continuous packed bed reactor studies.

Statistical analysis: In the present study, the relationship between response (molar conversion of lauric acid) and two independent variables (packed bed height and substrate flow rate) were studied. The results at each point are based on the experimental design are shown in Table 2. The experimental sequence was randomized in order to minimize the effects of the uncontrolled factors. Regression analysis is the general approach to fit the empirical model with the collected response variable data (Montgomery, 2001). By using multiple regression analysis, the response obtained in Table 2 was correlated with the two independent variables using the polynomial equation as in Eq. 1. The coefficients of the full regression

Table 2: Experimental design results for esterification of farnesol with laurica acid

	Factors		Response
Run	Packed bed height (cm) (A)	Substrate flow rate (mL min^{-1}) (B)	Molar conversion of lauric acid (%) (Y)
1	14.00 (6.83 g)[a]	1.20	89.52
2	24.00 (11.41 g)	1.20	92.00
3	14.00 (6.83 g)	1.20	88.95
4	14.00 (6.83 g)	1.20	89.61
5	19.00 (9.17 g)	1.50	87.01
6	9.00 (4.38 g)	1.50	84.75
7	14.00 (6.83 g)	0.60	97.93
8	14.00 (6.83 g)	1.20	88.49
9	19.00 (9.17 g)	0.90	98.15
10	14.00 (6.83 g)	1.80	90.87
11	4.00 (2.13 g)	1.20	67.44
12	14.00 (6.83 g)	1.20	89.01
13	9.00 (4.38 g)	0.90	80.23

[a]No. in parentheses represent weight of particles along with the packed bed height

Table 3: ANOVA for the regression model and respective model terms

Source	Sum of squares	Degree of freedom	Mean of square	F-value	Prob>F
Model	722.65	5	144.53	206.00	<0.0001[a]
A	400.21	1	400.21	570.42	<0.0001[a]
B	35.85	1	35.85	51.09	0.0002[a]
A^2	127.46	1	127.46	181.67	<0.0001[a]
B^2	39.41	1	39.41	56.17	0.0001[a]
AB	61.31	1	61.31	87.38	<0.0001[a]
Residual	4.91	7	0.70		
Lack of fit	4.07	3	1.36	6.48	0.0514
Pure Error	0.84	4	0.21		

[b]Prob>F-value less than 0.05 is significant

model equation and their statistical significance were determined and evaluated using Design-Expert 6.0.7 software from State-Ease Inc. The final model in terms of actual value is:

$$Y = 38.35132 + 6.92856A - 4.19352B - 0.09434A^2 + 14.57184B^2 - 2.61000AB \quad (2)$$

The results obtained were then analyzed by ANOVA to assess the goodness of fit. The significant quadratic models and the corresponding significant model term for all responses are tabulated in Table 3. Based on a 95% confidence level, the model was tested to be significant indicating that the regression model is reliable in predicting the molar conversion of lauric acid (Lee *et al*., 2005). Apart from that, each term in the model was also tested to be significant at a 95% confidence level. From these statistical tests, it was found that the model is adequate for predicting the molar conversion of lauric acid within the range of the variable studied. Values of Prob>F less than 0.05 indicate model terms are significant. For model validation, all the model terms should be significant whereas the lack of fit should be non significant. Thus, both criteria for quadratic model showed by Table 3 were valid for the present study.

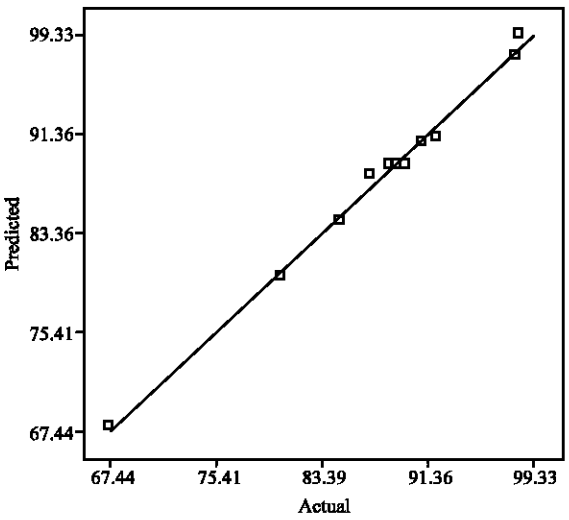

Fig. 1: Predicted versus actual

To test the fit of the model, the regression equation and determination coefficient (R^2) were evaluated. In this case, the value of the determination coefficient (R^2 = 0.9932) indicates that the sample variation of 99.32% for molar conversion of lauric acid is attributed to the independent variables and only 0.68% of the total variations are not explained by the model. Figure 1 shows the predicted versus actual. The value of the adjusted determination coefficient (Adj. R^2 = 0.9884) is also very high to advocate for a high significance of the model. A higher value of the correlation coefficient (R = 0.9205) justifies an excellent correlation between the independent variables (Yuan *et al*., 2008).

On the other hand, a relatively lower value of the coefficient variation (CV = 0.95%) indicates a better precision and reliability of the experiments carried out (Yuan *et al*., 2008). The CV as the ratio of the standard error of estimate to the mean value of the observed response (as a percentage) is a measure of reproducibility of the model and as general rule a model can be considered reasonably reproducible if its CV is not greater than 10% (Beg *et al*., 2003). By applying diagnostic plots including normal probability plot of residual, plot of residuals versus predicted, the assumptions of normality, independence and randomness of the residual were satisfied. The fitted model for molar conversion of lauric acid was accepted.

Figure 2 shows the changes of molar conversion of lauric acid with varying packed bed height and substrate flow rate. It is observed that, the molar conversion of lauric acid increase when there is an increase in packed bed height. The maximum molar conversion of lauric acid of 98.15% was achieved at 19 cm packed bed height with substrate flow rate 0.9 mL min^{-1}. A significant decrease in

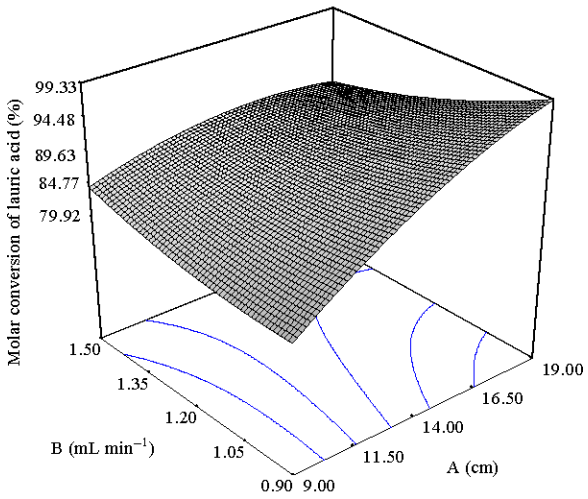

Fig. 2: Three-dimension response surface contour plot indicating the effect of interaction between packed bed height and substrate flow rate for esterification of farnesol with lauric acid

Table 4: The preset goal with the constraints for all the independent factors and response in numerical optimization

		Experimental region	
Variables	Ultimate goal	Lower limit	Upper limit
Packed bed height (A)	In range	9.00	19.00
Substrate flow rate (B)	In range	0.90	1.50
Molar conversion of lauric acid (Y)	Maximized	67.44	98.15

Table 5: Optimum condition found by Design-Expert® for esterification of farnesol with lauric acid

	Factors					Response	
Run	Packed bed height (cm)	Substrate flow rate (mL min^{-1})	$Y_{observed}$	$Y_{predicted}$	Error[c]	Standard deviation[d], σ	95% Confidence level[e]
1	18.18	0.90	98.95	98.34	+0.61	0.66	0.82
2	18.18	0.90	97.40	98.34	-0.94		
3	18.18	0.90	97.62	98.34	-0.72		
4	18.18	0.90	98.57	98.34	+0.23		
5	18.18	0.90	97.82	98.34	-0.52		

c = Error = ($Y_{observed}$ - $Y_{predicted}$); d = σ = √[(Σ($Y_{observed}^2$) – ((Σ$Y_{observed}$)^2/n))/(n-1)]; e = Confidence level = ± (2.776 σó)/√n where n represents the sample size

molar conversion of lauric acid at any point along the line of 19 cm of packed bed height was observed when the substrate flow rate increased more than 0.9 mL min^{-1}. This behavior also has been observed by other researchers (Laudani *et al*., 2007; Ciftci *et al*., 2008).

Based on the experimental data obtained, mass transfer coefficient and reaction rate constant were also calculated in this study using an average value of 0.4 mm of particle diameter. The calculated value of expected mass transfer coefficient and the effective reaction rate constant were 0.23 m h^{-1} and 0.33 l g^{-1} h^{-1}, respectively. Generally, at low flow rates, low conversions were obtained because of mass transfer resistance at liquid film layer. An increased in substrate flow rate caused reduction in these mass transfer limitations so higher reaction rates were obtained resulting in increasing the conversion (Tepe and Dursun, 2008; Halim *et al*., 2009). However, too high substrate flow rate resulted in a decrease in conversion. This is due to the insufficient residence time for the substrate to interact with the enzyme. As a consequence, the substrate failing to bind at the enzyme active site so that yielding a low conversion.

It was also observed that increasing the packed bed height beyond 19 cm does not have much effect on increasing the molar conversion of lauric acid. This could be due to a saturated effect of the enzyme amount. Gandhi *et al*. (1995) explained by considering that the active sites of the enzyme molecules present in excess would not be exposed to the substrate and remain inside the bulk of enzyme particles without contributing significantly to the reaction.

Optimization analysis: The optimum conditions for two variables, packed bed height and substrate flow rate were obtained using numerical optimization feature of

Design-Expert 6.0.7 software. All the factors and the response with respectively high and low limit experimental region have to satisfy the creations defined for the optimum working condition as stated in Table 4. The goal was set to optimize the molar conversion of lauric acid. The optimized conditions found by DOE were 18.18 cm packed bed height, 0.9 mL min^{-1} substrate flow rate and 98.34% of conversion. The value of molar conversion of lauric acid obtained from experiment was compared with the one predicted by DOE as shown in Table 5. There was an error of ±0.66% for molar conversion of lauric acid value within 95% confidence level (i.e., 98.07±0.82%).

CONCLUSION

The response surface methodology based on central composite rotatable design was employed for optimization and analysis of esterification of farnesol with lauric acid in packed bed reactor system. The optimum conditions of packed bed height and substrate flow rate were 18.18 cm and 0.9 mL min^{-1} and 98.07±0.82% molar conversion of lauric acid was determined under this condition. Work is in progress in this direction to study the influence of external mass transfer on the production of farnesyl laurate.

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