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# Solvent-Free Enzymatic Preparation of Feruloylated Monoacylglycerols Optimized by Response Surface Methodology

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The ability of immobilized lipase B from *Candida antarctica* (Novozym 435) to catalyze the direct esterification of glyceryl ferulate (FG) and oleic acid for feruloylated monoacylglycerols (FMAG) preparation in a solvent-free system was investigated. Enzyme screening and the effect of glycerol on the initial reaction rate of esterification were also investigated. Response surface methodology (RSM) was used to optimize the effects of the reaction temperature (55–65 °C), the enzyme load (8–14%; relative to the weight of total substrates), oleic acid/(FG + glycerol) (6:1–9:1; w/w), and the reaction time (1–2 h) on the conversion of FG and yield of FMAG. Validation of the RSM model was verified by the good agreement between the experimental and the predicted values of FG conversion and FMAG yield. The optimum preparation conditions were as follows: temperature, 60 °C; enzyme load, 8.2%; substrate ratio, 8.65:1 (oleic acid/(FG + glycerol), w/w); and reaction time, 1.8 h. Under these conditions, the conversion of FG and yield of FMAG are 96.7 ± 1.0% and 87.6 ± 1.2%, respectively.

**KEYWORDS: Feruloylated monoacylglycerols (FMAG); lipase-catalyzed esterification; solvent-free; response surface methodology**

## INTRODUCTION

Ferulic acid (4-hydroxy-3-methoxy cinnamic acid, FA) is a phenolic component of the cinnamic acid family occurring in most higher plants. FA has a maximum ultraviolet (UV) absorbance at 322 nm, which falls between the UVB (290−320 nm) and UVA (320−400 nm) regions (*1, 2*), and hence can be used as a potential all-natural UV-absorbing ingredient (*3*) that can be added, e.g., into cosmetics to help prevent human skin against UV radiation induced precancerous and cancerous lesions and acceleration of aging (*1, 4*). Besides, FA also has well-known antioxidative activities, as exemplified by the deterioration inhibition effects of ferulate esters in food frying (*5*). Increasing attention has been paid to expanding the applications of FA in the food, health, cosmetics, and pharmaceutical industries.

However, FA is less effective in hydrophobic media as a result of its low solubility therein. Grafting of alkyl chains and other lipophilic moieties alike (partially deacylated triacylglycerols) to an FA molecule is an efficient way of solving this problem (*6–12*). It is well-known that feruloylated monoacyl- and diacylglycerols (FMAG and FDAG) are potential UV absorbers and lipophilic antioxidants, which significantly reduce peroxides and hexanal production and lower rancid odor intensity in oils (*2, 13*).

Phenolic glycerides are conventionally prepared by chemical means, which suffers from the heat sensitivity and oxidation susceptibility of phenolic acids; in addition, the use of high temperatures frequently causes a dark color, burnt taste, and high energy consumption. To overcome these disadvantages, the use of enzymes has opened new avenues for producing phenolic glycerides under mild conditions. Recently, successful transesterifications and esterifications of ethyl ferulate (EF) and FA via enzymatic routes have been reported. Compton et al. (*10*) first reported the esterifications of FA to produce ethyl and octyl ferulate and the transesterification of EF with triolein to produce feruloyl-monooleoylglycerol (FMOG) and feruloyl-dioleoylglycerol (FDOG), using Novozym 435 as the catalysts in both cases. Laszlo et al. (*11, 12*) reported solvent-free preparation of FMAG and FDAG by transesterification of EF with vegetable oil (TAG), monoacylglycerols (MAG), and diacylglycerols (DAG) catalyzed by Novozym 435 in batch and packed-bed reactors. Sabally et al. (*14*) reported the enzymatic transesterification of FA with trilinolein and trilinolenin in organic solvents.

However, the above-cited reports regarding the enzymatic preparation of feruloyl-substituted structured lipids (e.g., FMAG and FDAG), which have superiority over chemical preparation, show disadvantages including low yields, long reaction time,

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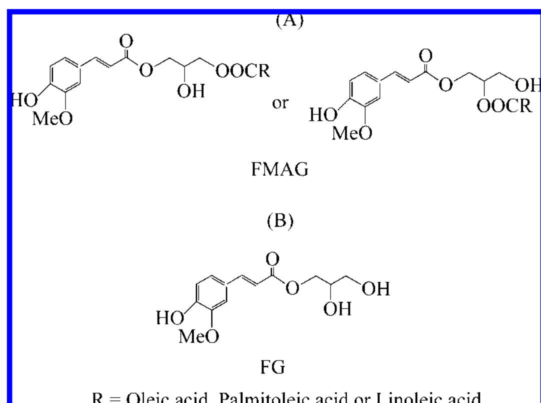


**Figure 1.** Chemical structures of FMAG and FG.

solvent requirements, etc. Recently we have reported a new solvent-free enzymatic route for preparation of FMAG (**Figure 1A**) and FDAG, consisting of two consecutive enzymatic steps, namely, transesterification of EF and excessive glycerol to produce glyceryl ferulate (FG) (**Figure 1B**) followed by esterification of FG with oleic acid (*15*). This route can produce higher yields of FMAG and FDAG in a shorter time.

Our purposes in this study were to better understand the relationship between the factors (reaction time, temperature, oleic acid/(FG + glycerol) mass ratio, and enzyme load) and the responses (percent molar conversion of FG and yield of FMAG) in the esterification step and to determine the optimum conditions for FMAG preparation using Box−Behnken design and response surface methodology (RSM). Enzyme screening and the effect of glycerol on the initial reaction rate were also investigated.

## MATERIALS AND METHODS

**Materials.** Immobilized lipase Lipozyme RM IM (from *Rhizomucor miehei*, RML), Lipozyme TL IM (from *Thermomyces lanuginose*, TLL), and Novozym 435 (lipase B from *Candida antarctica*, CAL) were purchased from Novozymes A/S (Bagsvaerd, Denmark). Lipase (5000 units/g) powder from *Candida lipolytica* was purchased from Xueyan Enzyme Company (Wuxi, China). Pancreatin (Porcine pancreas) powder was purchased from Sigma Corporation (USA). EF (purity >99%) was from Suzhou Changtong Chemical Co., Ltd. (Suzhou, China). Glycerol (purity >99%) and oleic acid (purity >65%) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Oleic acid was fractionated before use to a purity of >90% (GC results: oleic acid 91.2 ± 1.0%, linoleic acid 5.7 ± 1.2%, palmitoleic acid 2.1 ± 0.6%, other fatty acids 1.0 ± 0.5%). Methanol and glacial acetic acid were of HPLC purity. All other reagents were of analytical grade.

**Transesterification.** Transesterification was conducted following the method we have reported (*16*): 1.5 mmol of EF was reacted with 15 mmol of glycerol and 170 mg of Novozym 435 at 60 °C and 1.33 × $10^3$ Pa for 10 h in 25 mL round-bottom flasks on a vacuum-rotary evaporator rotating at 115 rpm.

**Preparation of FG.** FG was purified using the method we have reported (*16*); at the end of transesterification of EF and glycerol, Novozym 435 was removed by filtration. The remaining mixture was separated by normal pressure chromatography using a C18 column (30 μm, 400 × 30 mm) to give purified FG. Elution was achieved successively with 90% (v/v), 80% (v/v), 70% (v/v), and 60% (v/v) aqueous methanol solution at a constant flow rate of 2 mL/min. Products were detected at 325 nm. The fractions containing the desired products were pooled and concentrated to dryness under reduced pressure. The structure of the isolated compounds was examined by $^1$H NMR (*16*), and the result showed that the products consisted of 97.2 ± 1.0% of 1-glyceryl ferulate (1-FG) and 2.8 ± 0.5% of 2-glyceryl ferulate (2-FG).

**Esterification.** Esterification was performed in 25 mL round-bottom flasks on a vacuum-rotary evaporator rotating at 115 rpm using the following conditions: 0.30 g (FG + glycerol, glycerol solution of FG obtained after transesterification) or 0.075 g chromatography purified FG, 2.22 g oleic acid, 0.25 g Novozym 435, 60 °C, 1.33 × $10^3$ Pa.

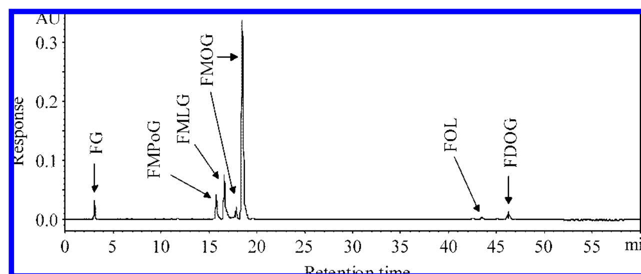


**Figure 2.** HPLC analysis of the Novozym 435-catalyzed esterification of FG with oleic acid. Esterification of 1.93 g oleic acid with 0.32 g (FG + glycerol) was catalyzed with 315 mg Novozym 435 at 60 °C, 1.33 × $10^3$ Pa for 1.5 h in 25 mL round-bottom flasks on a rotary evaporator rotating at 115 rpm. FG (glyceryl ferulate), FMPoG (feruloyl-monopalmitoleoylglycerol), FMLG (feruloyl-monolinoleoylglycerol), FMOG (feruloyl-monooleoylglycerol), FOL (feruloyl-oleoyl-linoleoyl-glycerol), FDOG (feruloyl-dioleoylglycerol).

**Analysis.** The reactants and products were analyzed by a Waters 2996 HPLC system using a Sunfire C18 reverse phase column (Waters Corporation, Milford, Massachusetts) (5 μm, 150 × 4.6 mm) eluted with a binary gradient of solvent A (397 mL of water and 3 mL of glacial acetic acid) and solvent B (400 mL of methanol) at a flow rate of 1 mL/min. The elution sequence consisted, consecutively, of a linear gradient from 50% (v/v) B to 90% B (v/v) in 10 min, then to 100% B in 30 min, followed by an isocratic flow of 100% B for 20 min, all at 35 °C. The eluate was monitored at 325 nm. The chromatogram of the reaction mixture indicating the feruloyl species composition is presented in **Figure 2**.

Based on the HPLC-UV analysis, area percentage on a feruloyl basis was used for the calculation of the conversion of FG ($C_{FG}$) and yields of FMAG ($Y_{MAG}$) and FDAG ($Y_{DAG}$): $C_{FG} = 1 - $ FG% (mol/mol), $Y_{MAG}$ = FMPoG% (mol/mol) + FMLG% (mol/mol) + FMOG% (mol/mol), $Y_{DAG}$ = FOL% (mol/mol) + FDOG% (mol/mol).

**Experimental Design.** A three-level-four-factor Box−Behnken design was employed in this study. The variables and their levels selected for the study of FMAG preparation were reaction time (1, 1.5, and 2 h), temperature (55, 60, and 65 °C), enzyme load (8, 11, and 14%; relative to the weight of total substrates), and oleic acid/(FG + glycerol) (6:1, 7.5:1, and 9:1; w/w). **Table 1** shows the independent factors ($X_i$), levels, and experimental design using coded and uncoded parameters.

**Statistical Analysis.** Box−Behnken design for four independent variables was used to obtain the combination of values that optimizes the responses within the region of the three-dimensional (3D) observation space, which allows one to design a minimum number of experimental runs. The model evaluates the effects of each independent variable on responses. $3^k$ factorial tests allow efficient estimation of a second order polynomial regression. The mathematical relationship relating the variables to the responses can be calculated by the quadratic polynomial equation:

$$Y = \beta_o + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad (1)$$

where $Y$ is one of the two responses, $X_i$ and $X_j$ represent the independent variables, $\beta_o$ is the constant, $\beta_i$ is the linear term coefficient, $\beta_{ii}$ is the quadratic term coefficient, and $\beta_{ij}$ is the cross term coefficient. The experiments were carried out for analysis using Design Expert (State-Ease Inc. Statistics Made Easy, Minneapolis, Minnesota Ver. 5.0.7.1997). For the present study, a total of 29 tests were necessary to estimate the coefficients.

## RESULTS AND DISCUSSION

**Enzyme Screening.** Novozym 435 (*Candida antarctica*), Lipozyme RM IM (*Rhizomucor miehei*), Lipozyme TL IM

**Table 1.** Experimental Design and Results of Conversion of FG and Yield of FMAG as Affected by Reaction Temperature, Enzyme Load, Substrate Ratio, and Reaction Time

| treament no.[a] | reaction temperature $X_1$ (°C) | enzyme load[b] $X_2$ (%) | substrate ratio[c] $X_3$ (w/w) | reaction time $X_4$ (h) | conversion of FG (%) | yield of FMAG (%) |
|---|---|---|---|---|---|---|
| 1 | 0 (60)[d] | −1 (8) | −1 (6:1) | 0 (1.5) | 86.4 ± 0.7 | 79.3 ± 0.8 |
| 2 | 0 (60) | 1 (14) | 0 (7.5:1) | 1 (2) | 95.0 ± 1.1 | 80.1 ± 0.5 |
| 3 | 0 (60) | 0 (11) | 0 (7.5:1) | 0 (1.5) | 95.3 ± 0.5 | 84.9 ± 0.8 |
| 4 | 0 (60) | 1 (14) | 1 (9:1) | 0 (1.5) | 91.6 ± 1.1 | 79.2 ± 0.8 |
| 5 | −1 (55) | 0 (11) | −1 (6:1) | 0 (1.5) | 86.9 ± 0.6 | 81.9 ± 0.8 |
| 6 | 0 (60) | 0 (11) | −1 (6:1) | −1 (1) | 84.7 ± 1.7 | 78.3 ± 1.1 |
| 7 | −1 (55) | 0 (11) | 0 (7.5:1) | 1 (2) | 92.0 ± 0.2 | 86.9 ± 0.4 |
| 8 | 0 (60) | −1 (8) | 0 (7.5:1) | 1 (2) | 93.1 ± 1.1 | 87.0 ± 0.9 |
| 9 | 0 (60) | 0 (11) | 1 (9:1) | 1 (2) | 95.3 ± 1.0 | 83.9 ± 0.9 |
| 10 | −1 (55) | 0 (11) | 1 (9:1) | 0 (1.5) | 89.1 ± 0.9 | 83.7 ± 1.7 |
| 11 | 0 (60) | 0 (11) | 1 (9:1) | −1 (1) | 91.7 ± 0.7 | 85.9 ± 0.3 |
| 12 | 0 (60) | −1 (8) | 0 (7.5:1) | −1 (1) | 87.4 ± 1.3 | 80.4 ± 1.4 |
| 13 | 0 (60) | 0 (11) | 0 (7.5:1) | 0 (1.5) | 95.6 ± 0.4 | 85.6 ± 0.3 |
| 14 | −1 (55) | −1 (8) | 0 (7.5:1) | 0 (1.5) | 87.0 ± 0.3 | 80.1 ± 0.6 |
| 15 | 0 (60) | 0 (11) | 0 (7.5:1) | 0 (1.5) | 96.3 ± 0.5 | 86.0 ± 0.6 |
| 16 | 1 (65) | −1 (8) | 0 (7.5:1) | 0 (1.5) | 93.1 ± 1.3 | 80.1 ± 1.2 |
| 17 | 0 (60) | −1 (8) | 1 (9:1) | 0 (1.5) | 95.2 ± 0.3 | 87.8 ± 0.6 |
| 18 | 0 (60) | 1 (14) | −1 (6:1) | 0 (1.5) | 92.6 ± 0.5 | 84.1 ± 0.2 |
| 19 | 0 (60) | 0 (11) | 0 (7.5:1) | 0 (1.5) | 95.4 ± 0.8 | 85.1 ± 1.0 |
| 20 | 1 (65) | 0 (11) | 0 (7.5:1) | −1 (1) | 91.1 ± 1.2 | 81.5 ± 0.8 |
| 21 | 0 (60) | 0 (11) | −1 (6:1) | 1 (2) | 92.1 ± 0.1 | 85.5 ± 0.4 |
| 22 | 0 (60) | 0 (11) | 0 (7.5:1) | 0 (1.5) | 96.3 ± 0.5 | 85.0 ± 0.5 |
| 23 | 1 (65) | 0 (11) | 0 (7.5:1) | 1 (2) | 93.1 ± 1.1 | 75.6 ± 0.4 |
| 24 | −1 (55) | 0 (11) | 0 (7.5:1) | −1 (1) | 84.2 ± 0.1 | 78.1 ± 0.4 |
| 25 | 0 (60) | 1 (14) | 0 (7.5:1) | −1 (1) | 92.6 ± 0.7 | 86.1 ± 0.8 |
| 26 | 1 (65) | 1 (14) | 0 (7.5:1) | 0 (1.5) | 94.3 ± 1.3 | 75.6 ± 0.7 |
| 27 | −1 (55) | 1 (14) | 0 (7.5:1) | 0 (1.5) | 90.6 ± 0.5 | 83.7 ± 0.8 |
| 28 | 1 (65) | 0 (11) | −1 (6:1) | 0 (1.5) | 88.5 ± 0.5 | 75.2 ± 1.5 |
| 29 | 1 (65) | 0 (11) | 1 (9:1) | 0 (1.5) | 93.8 ± 1.6 | 78.0 ± 1.5 |

[a] Numbers were run in random order. [b] Enzyme load (%, relative to the weight of total substrates). [c] Oleic acid/(FG + glycerol) mass ratio. [d] Numbers in parentheses represent actual experimental amounts.

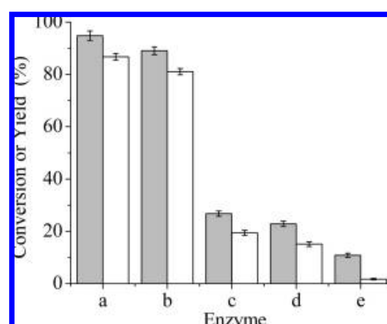


**Figure 3.** Esterification of FG and oleic acid using various lipases at 60 °C, 1.33 × $10^3$ Pa, enzyme load (relative to the weight of total substrates) 11%, and oleic acid/(FG + glycerol) 7.5:1 (w/w) for 1.5 h. Reactions were performed in 25 mL round-bottom flasks on a rotary evaporator rotating at 115 rpm. Conversion of FG (■), Yield of FMAG (□). Novozym 435 (**a**), Lipozyme RM IM (**b**), Lipozyme TL IM (**c**), *Candida lipolytica* lipase (**d**), Pancreatin (**e**).

(*Thermomyces lanuginosa*), Pancreatin (Porcine pancreas), and a lipase from *Candida lipolytica* were screened for their abilities to incorporate oleic acid into FG. The conversion of FG and the yield of FMAG attained with various lipases were in the order of Novozym 435 > Lipozyme RM IM > Lipozyme TL IM > *Candida lipolytica* lipase > Pancreatin (**Figure 3**). Novozym 435 gave the highest conversion of FG (94.8 ± 1.2%) and greatest yield of FMAG (85.7 ± 0.7%) and was thus chosen for subsequent experiments. Similar findings that Novozym 435 gave the highest reaction rate in transesterifications or esterifications of EF and FA were also reported by Compton et al. (*10*).

**The Effect of Glycerol on the Initial Reaction Rate of Esterification.** Considering that FG is more water soluble and is solid at the reaction temperature used, it is predictable that the inclusion of glycerol may enhance the product formation rate. The transesterification product was approximately composed of 75% glycerol and 25% FG. During the early phase of the esterification (1.5 h), it was observed that the FMAG formation rate (2.59 × $10^{-7}$ mol/min) with glycerol in the reaction mixture was approximately 4-fold faster than that in the absence of glycerol (6.86 × $10^{-8}$ mol/min). Similar results that glycerol can enhance reaction rate have also been reported (*13, 17, 18*). Thus, esterification of FG with oleic acid in the following experiments was performed without removing the residual glycerol in the transesterification product.

**Selection of Levels of Independent Variables and Experimental Design.** The effects of the four independent variables on the conversion of FG and yield of FMAG are shown in **Figure 4**. As the reaction time increased, the conversion of FG showed increasing patterns, whereas the conversion of FG showed increasing−decreasing patterns with other variables increasing. And the yield of FMAG showed increasing-decreasing patterns with the four variables increasing. The maximum yield of FMAG (86.0 ± 0.6%) was obtained at 60 °C, 11% enzyme load, oleic acid/(FG + glycerol) 7.5:1 (w/w), and 1.5 h. Therefore the lower, middle, and upper levels of the four independent factors were chosen according to **Table 1**.

**Model Fitting.** RSM is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and the observed results. Modeling of factors and responses was performed by RSM to predict the highest possible conversion of FG and yield of FMAG. The results obtained for the models are listed in **Table 1**. Duplicate experiments were carried out at all design points. The greatest

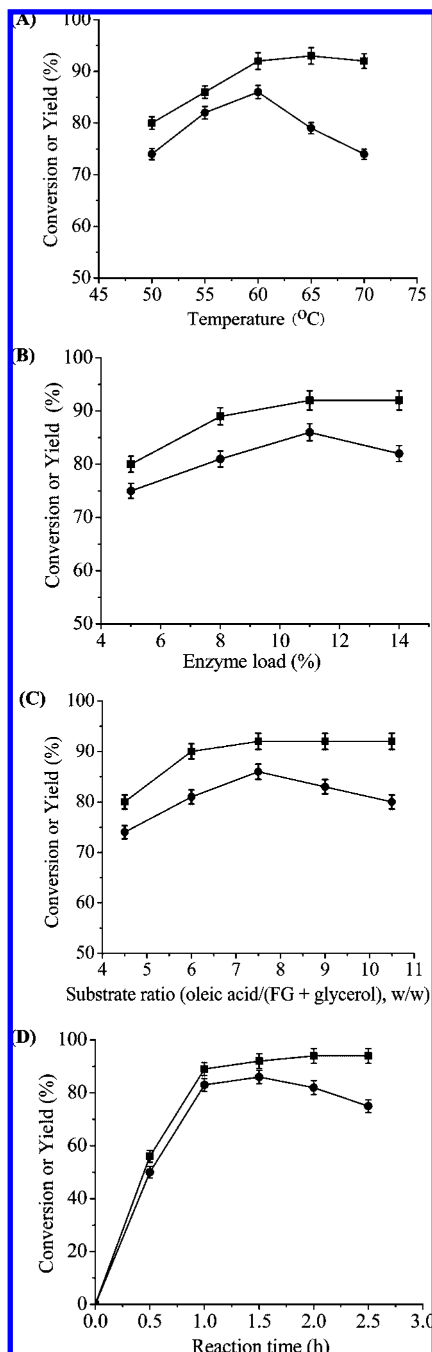


**Figure 4.** Effects of the temperature, enzyme load (relative to the weight of total substrates), substrate ratio (oleic acid/(FG + glycerol), w/w), and reaction time on the conversion of FG (■) and yield of FMAG (●). **A**: enzyme load (relative to the weight of total substrates) 11%, oleic acid/(FG + glycerol) 7.5:1 (w/w), 1.5 h; **B**: 60 °C, oleic acid/(FG + glycerol) 7.5:1 (w/w), 1.5 h; **C**: 60 °C, enzyme load (relative to the weight of total substrates) 11%, 1.5 h; **D**: 60 °C, oleic acid/(FG + glycerol) 7.5:1 (w/w), enzyme load 11% (relative to the weight of total substrates).

yield of FMAG (87.8 ± 0.6%) was related with treatment at 60 °C, enzyme load 8%, oleic acid/(FG + glycerol) 9:1 (w/w), and 1.5 h, and the lowest yield of FMAG (only 75.2 ± 1.5%) was with treatment at 65 °C, 11%, 6:1, and 1.5 h, respectively; both were among all of the treatments tested.

The data were analyzed by employing a multiple regression technique to develop response surface models. Both a linear model and a second-order model were tested, using an F-test at the 95% confidence level. The following two second-order models satisfactorily explained the conversion of FG and the yield of FMAG with nonsignificant lack of fit (**Table 2**). $C_{FG}$ and $Y_{FMAG}$ are the predicted values for the conversion of FG (%) and yield of FMAG (%), respectively, and $X_1$, $X_2$, $X_3$, and $X_4$ are the coded variables as described in **Table 1**.

**Table 2.** Regression Analysis of Variance for Response Surface Quadratic Model (ANOVA) Pertaining to the Predicted Conversion of FG and the Yield of FMAG

| source | sum of squares | degree of freedom | mean square | F-value | $P > F$[a] |
|---|---|---|---|---|---|
| | | Conversion of FG (%) | | | |
| model | 348.49 | 14 | 24.89 | 45.40 | <0.0001 |
| residual | 7.68 | 14 | 0.55 | | |
| lack of fit | 6.70 | 10 | 0.67 | 2.74 | 0.1721 |
| pure error | 0.98 | 4 | 0.24 | | |
| total | 356.17 | 28 | | | |
| | coefficient of variation = 0.81%, $R^2$ = 0.9785 | | | | |
| | | Yield of FMAG (%) | | | |
| model | 387.71 | 14 | 27.69 | 45.58 | <0.0001 |
| residual | 8.51 | 14 | 0.61 | | |
| lack of fit | 7.71 | 10 | 0.77 | 3.88 | 0.1018 |
| pure error | 0.80 | 4 | 0.20 | | |
| total | 396.22 | 28 | | | |
| | coefficient of variation = 0.95%, $R^2$ = 0.9785 | | | | |

[a] $P < 0.05$ indicates statistical significance.

$$C_{FG}\,(\%) = 95.79 + 2.02X_1 + 1.21X_2 + 2.13X_3 + 2.41X_4 - 3.32X_1^2 - 1.42X_2^2 - 2.08X_3^2 - 2.26X_4^2 - 1.43X_1X_4 - 2.46X_2X_3 - 0.84X_2X_4 - 0.94X_3X_4 \quad (2)$$

$$Y_{FMAG}\,(\%) = 85.35 - 2.36X_1 - 0.48X_2 + 1.17X_3 + 0.73X_4 - 4.20X_1^2 - 1.32X_2^2 - 1.41X_3^2 - 0.60X_4^2 - 2.03X_1X_2 - 3.65X_1X_4 - 3.36X_2X_3 - 3.16X_2X_4 - 2.30X_3X_4 \quad (3)$$

The relationships between reaction factors and responses can be better understood by examining the planned series of 2D contour plots (**Figures 5** and **6**) generated from the predicted model.

**Figures 5A** and **6A** show the effect of temperature, reaction time, and their mutual interaction on conversion of FG and yield of FMAG. The maximum conversion of FG appears in the temperature range 59–63 °C and time range of 1.5–1.9 h, whereas the maximum yield of FMAG appears in the temperature range 55–59 °C and time range of 1.8–2.0 h. The decrease of yield of FMAG at higher temperature (>60 °C) could be attributable to more FDAG produced favored by enhanced acyl migration. Similar results that high temperature increased the acyl migration rate were also reported (*19*).

**Figures 5B** and **6B** show the effect of varying the enzyme load and substrate ratio on esterification. Lower enzyme loads (e.g., 8%) and higher substrate ratios (e.g., 9:1) result in a maximum yield of FMAG up to 87.8 ± 0.6%. However, the maximum conversion of FG appears in substrate ratios between 7.5 and 8.3 and enzyme loads between 11.0 and 12.5%.

**Figures 5C** and **6C** show the effect of temperature, reaction time, and their mutual interaction on esterification. Higher enzyme loads and longer reaction times convert more FG to FMAG. However, if enzyme loads are greater than 12.5%, a long reaction time (>1.8 h) would not further increase the yield of FMAG, which is the result of more fatty acids incorporated to FMAG form FDAG. This result showed that higher enzyme

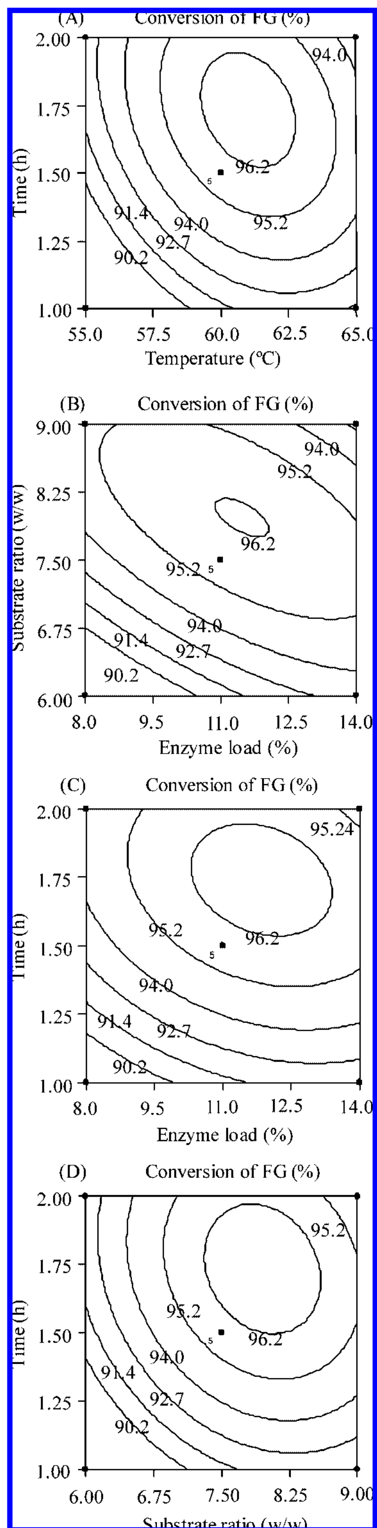


**Figure 5.** Response contour plots between any two parameters for conversion of FG: (**A**) reaction temperature and time combined with the enzyme load (relative to the weight of total substrates) and oleic acid/(FG + glycerol) (w/w) ratio fixed at 11% and 7.5:1, respectively; (**B**) enzyme load (relative to the weight of total substrates) and substrate ratio (oleic acid/(FG + glycerol), w/w) at 60 °C for 1.5 h; (**C**) enzyme load (relative to the weight of total substrates) and reaction time combined with the oleic acid/(FG + glycerol) (w/w) ratio and reaction temperature fixed at 7.5:1 and 60 °C, respectively; (**D**) substrate ratio (oleic acid/(FG + glycerol), w/w) and reaction time combined with the enzyme load (relative to the weight of total substrates) and reaction temperature fixed at 11% and 60 °C, respectively.

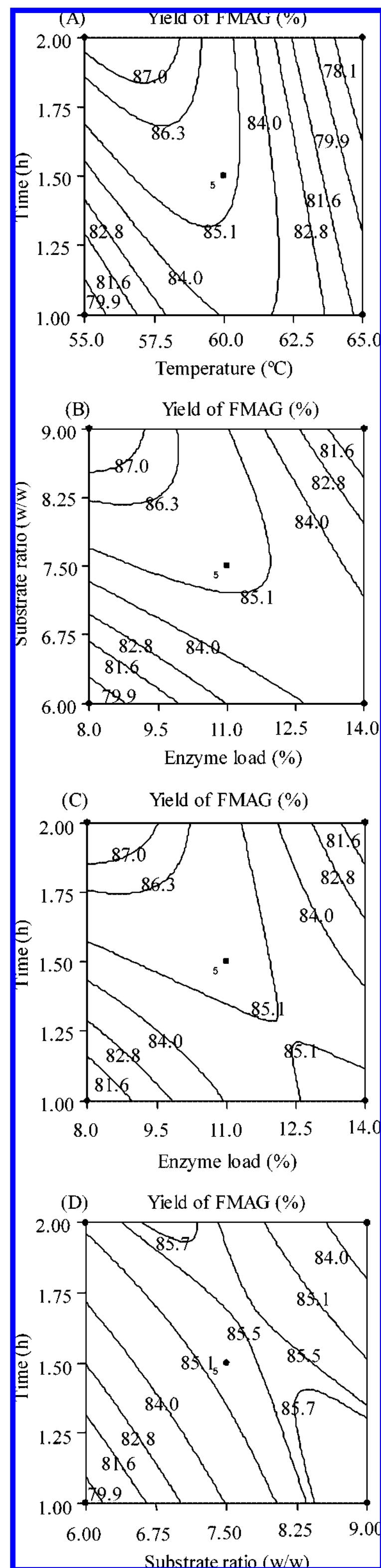


**Figure 6.** Response contour plots between any two parameters for yield of FMAG: (**A**) reaction temperature and time combined with the enzyme load (relative to the weight of total substrates) and oleic acid/(FG + glycerol) (w/w) ratio fixed at 11% and 7.5:1, respectively; (**B**) enzyme load (relative to the weight of total substrates) and substrate ratio (oleic acid/(FG + glycerol), w/w) at 60 °C for 1.5 h; (**C**) enzyme load (relative to the weight of total substrates) and reaction time combined with the oleic acid/(FG + glycerol) (w/w) ratio and reaction temperature fixed at 7.5:1 and 60 °C, respectively; (**D**) substrate ratio (oleic acid/(FG + glycerol), w/w) and reaction time combined with the enzyme load (relative to the weight of total substrates) and reaction temperature fixed at 11% and 60 °C, respectively.

loads and longer reaction time can enhance acyl migration, which is similar to the findings of Guo et al. (*19*).

**Figures 5D** and **6D**, the contour plot drawn for the interaction of reaction time with substrate ratio, show the similar result of the interaction of enzyme load and reaction time on conversion of FG. Maximum yield of FMAG appears at either longer reaction times (1.9–2.0 h) and lower substrate ratios (6.75–7.50) or at shorter reaction times (1–1.5 h) and higher substrate ratios (8.3–9.0). The decrease in the yield of FMAG along with prolonged reaction times (>1.5 h) using higher substrate ratios (8.3–9.0) is attributable to the formation of FDAG (up to 9.0 ± 0.8%), which is in agreement with the results of Xu et al. (*20*).

**Attaining Optimum Conditions.** Besides the exhibition of the effects of the variables on the responses, the contour plots indicate several desirable combinations of the variables that give high conversion of FG and yield of FMAG, which provides options when practical aspects are considered in real commercialization of the process. We suggest, according to optimization by the contour plots, the following set of conditions: temperature 60 °C, enzyme load 8.2%, oleic acid/(FG + glycerol) ratio 8.65:1 (w/w), and reaction time 1.8 h. Under the suggested conditions, the conversion of FG and yield of FMAG are estimated to be 96.1% and 87.8%, respectively.

**Model Verification.** Experiments were done at the predicted optimum conditions to validate the RSM model, and the conversion of FG and yield of FMAG obtained were 96.7 ± 1.0% and 87.6 ± 1.2%, respectively. Good agreements between the observed and predicted values indicate the validation of the model. Moreover, it should be noted that the conversion of FG and yield of FMAG are even higher and the reaction time is much shorter, as compared with that reported (*10–12, 14*).

In conclusion, the preparation of FMAG by solvent-free esterification of FG and oleic acid was successfully achieved using Novozym 435 as a biocatalyst, and RSM was used to model and optimize the incorporation of oleic acid into FG. The preparation conditions were then optimized as follows: temperature, 60 °C; enzyme load, 8.2%; substrate ratio, 8.65:1 (oleic acid/(FG + glycerol), w/w); and reaction time, 1.8 h. Under the optimized conditions, the conversion of FG and the yield of FMAG reached 96.7 ± 1.0% and 87.6 ± 1.2%, respectively.

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