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Optimization of Biocatalytic Synthesis of a Nutraceutical Ester

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Abstract— Glycerol linoleate, an ester with nutraceutical value, was enzymatically synthesized by using immobilized lipase. A simplex lattice mixture design was used for screening of the enzymes. Novozym 435 was found to be the best enzyme for the synthesis of glycerol ester. The effects of three reaction parameters including time, temperature and enzyme amount were studied with response surface methodology (RSM) using a central composite rotatable design (CCRD). A maximum ester conversion of 73.2% was obtained at 65oC and 478 min using 66 mg enzyme. The good correlation between the predicted and actual response showed that the developed model could efficiently predict the conversion yield.

Keywords— Enzymatic synthesis; Esterification; Response surface methodology; Glycerol ester

I. INTRODUCTION

Glycerol esters of fatty acids are widely used as synthetic emulsifiers, in the food, cosmetic, pharmaceutical, and chemical industries [1]. Glycerolysis of fats and oils and direct esterification of glycerol with fatty acids are the two most common commercial processes for the preparation of these compounds [2]. Both reactions lead to a mixture of mono-, di-, and triacylglycerols, which can be separated by molecular distillation. Depending on the presence and type of catalyst and the reaction conditions (such as temperature and molar ratio of substrates), the proportions of glycerol esters are different [1]. The direct esterification may be catalyzed either by acids (e.g., p-toluenesulfonic acid) or bases (e.g., sodium hydroxide) at high temperatures (>150°C) [2]. This is followed by phase separation, neutralization of the catalyst, steam refining, filtration, deodorization and decoloration [1].

Lipase, which breaks down fats into monoacylglycerols and fatty acids, can be used to catalyze the esterification of glycerol with fatty acids. Compared to conventional chemical esterification, enzyme catalyzed synthesis has numerous advantages including milder reaction conditions, low energy requirements, less undesirable side reactions, ease of product isolation, and catalyst reusability [3]. Enzymatic synthesis of pure 1,3-Diacylglycerols by direct esterification of glycerol with some saturated fatty acids including caprylic, capric, lauric, palmitic and stearic acids in solvent free system has been reported by Zhong et al. [4]. Xia et al. [5] have also investigated the enzymatic synthesis of partial glycerol caprate from capric acid and glycerol by using lipase from Candida antarctica in a solvent-free microaqueous media.

In this study, enzymatic esterification of conjugated linoleic acid (CLA) with glycerol by immobilized lipase from Candida antarctica has been performed. CLA is a nutraceutical compound, i.e., food or food product that provides either therapeutic or prophylactic medicinal benefits in addition to nutritional value [6]. The use of CLA as a nutraceutical compound is associated with nutritional and physiological benefits and therapeutic promises such as antiatherogenic effects, protection against certain types of cancer, beneficial effects in the cardiovascular system and enhancement of immunological functions [7]. The esterified form of CLA has greater oxidative stability in comparison to the free acid form. Enzymatic synthesis of glycerol ester from CLA in a solvent-free reaction in a packed-bed reactor containing immobilized lipase from Mucor miehei has been previously done [7]. Reaction parameters were optimized by one variable at a time (OVAT) method which is not only time consuming but also ignores the interactions between the parameters [8]. In the present study, the reaction conditions have been optimized by response surface methodology (RSM). Compared to classical statistical techniques, RSM as a fast and economical statistical technique can explore the experimental space by requiring a small number of experiments [3].

The objective of the present study was to model the lipase-catalyzed esterification reaction between conjugated linoleic acid and glycerol. The effects of reaction parameters (temperature, time and amount of enzyme) on the degree of esterification were evaluated by RSM, and optimal reaction conditions were proposed.

II. MATERIALS AND METHODS

A. Materials

Novozym® 435 (*Candida antarctica* lipase B immobilized on macroporous acrylic resin, activity of 10,000 PLU/g), Lipozyme® TLIM (lipase from *Thermomyces lanuginose* immobilized on silica gel, activity of 250 IUN/g), Lipozyme® RMIM (lipase from *Rhizomucor Miehei* immobilized on anionic exchange resin, activity of 6 BAUN/g), were purchased from NOVO Nordisk A/S (Bagsvaerd, Denmark). Conjugated linoleic acid was purchased from Sigma-Aldrich, USA. Glycerol was obtained from Merck Co. (Darmstadt, Germany). All other chemicals used were of analytical grade.

B. Lipase-catalyzed esterification reaction

Glycerol (0.0921g) and conjugated linoleic acid (0.8414g) with 1:3 molar ratio were mixed in 30mL closed vials. Different amounts of the enzyme were subsequently added.

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Then, 5ml of hexane was added in the vials which acted as the solvent for the reaction. The samples and controls (samples without enzyme) were placed in the water bath shaker at 200 rpm at different temperatures and time periods. All the experiments were performed in triplicate.

C. Screening of the Enzymes

A simplex lattice mixture design was used in this study to optimize the ratio of three commercial immobilized enzymes including Novozym 435, Lipozyme RMIM and Lipozyme TLIM in enzymatic esterification of CLA with glycerol. The reaction system consisted of 1.0 mmol of glycerol, 3 mmol of CLA, 5 mL of hexane and 200 mg of immobilized enzymes. The reaction mixture was reacted for 120 min at 50.0^{o}C and 200 rpm.

For k factors the lattice describes all experimental points having the factor levels 0, 1/m, 2/m,…, (d-1)/m or 1. A set of 14 experiments was performed; six single factor experiments, four binary systems, and four ternary systems (where for three of them one factor was at 2/3 and the others at 1/6, and for one experiment all factors were at 1/3). In mixture designs, the purpose of experimental design is to model the mixture surface with a form of polynomial equation so that the response for any mixture of components can be predicted. Composition of various runs of mixture design for the synthesis of ester is presented in Table 1.

TABLE I
COMPOSITION OF VARIOUS EXPERIMENTS OF MIXTURE DESIGN FOR THE SCREENING OF ENZYME

Exp.	Novozym 435 (mg)	Lipozyme TLIM (mg)	Lipozyme RMIM (mg)	Conversion (%)
1	0.00	100.00	100.00	25.39
2	100.00	100.00	0.00	31.73
3	0.00	200.00	0.00	21.96
4	200.00	0.00	0.00	39.11
5	100.00	100.00	0.00	34.20
6	100.00	0.00	100.00	29.59
7	0.00	0.00	200.00	21.96
8	0.00	0.00	200.00	24.29
9	133.33	33.33	33.33	32.76
10	33.33	33.33	133.33	30.53
11	200.00	0.00	0.00	40.15
12	66.67	66.67	66.67	28.30
13	0.00	200.00	0.00	22.00
14	33.33	133.33	33.33	31.39

D. Analysis and characterization

The esterification reaction was terminated by dilution with 5 mL of ethanol: acetone (50:50 v/v). The enzyme was removed by filtration and the remaining free acid in the reaction mixture was determined by titration with 0.1M NaOH using phenolphthalein as the indicator [9]. The percentage of conversion was calculated by using the values obtained for the controls and test samples as follows:

$$\text{Conversion}\,(\%) = \left(1 - \frac{A}{B}\right) \times 100 \qquad (1)$$

The product was analyzed by Fourier transform-infrared (FT-IR) spectrophotometer (1725X, Perkin Elmer, USA). Formation of the ester was also confirmed by gas chromatography/mass spectroscopy (GC/MS) analysis using an Agilent (model GC 7890A; model MS 5975C; Agilent Technologies Inc., Palo Alto, Ca) instrument with a HP-5MS column (0.25 mm × 30 m, 0.25 μm). The carrier gas was helium, and the total gas flow rate was 20 ml min-1. The injection mode was splitless and the injector temperature was set at 300oC. The oven temperature was maintained at 180oC for 5 min, elevated to 320oC at a rate of 20oCmin-1 and held for 15 min. The spectra were scanned within the range m/z 40-900.

E. Design of experiments, statistical analysis and optimization

A software package of Design Expert® Version 7.1.1 (State-Ease Inc., Statistics Made Easy, Minneapolis, MN, USA) was used for statistical analysis. To obtain a proper model for optimization, a three-factor-five-level central composite rotatable design (CCRD) was employed, requiring 20 experiments. The experimental design comprised of 8 factorial points, 6 axial points and 6 center points. The parameters and their corresponding ranges selected for the synthesis of the ester were: temperature (35-65◦C); time (30-480 min) and enzyme amount (20-400 mg). Table 2 presents the composition of various runs of the CCRD for the synthesis of glycerol ester.

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TABLE II
COMPOSITION OF VARIOUS EXPERIMENTS OF THE CCRD FOR THE SYNTHESIS OF GLYCEROL LINOLEATE

Exp.	Temperature (°C)	Time (min)	Enzyme amount (mg)	Conversion (%)
1	35.00	255.00	210.00	32.79
2	41.08	121.21	97.03	21.23
3	41.08	388.79	322.97	39.24
4	41.08	121.21	322.97	33.45
5	41.08	388.79	97.03	30.08
6	50.00	30.00	210.00	21.49
7	50.00	255.00	20.00	18.85
8	50.00	255.00	400.00	46.68
9	50.00	255.00	210.00	28.50
10	50.00	255.00	210.00	23.10
11	50.00	255.00	210.00	27.70
12	50.00	255.00	210.00	26.80
13	50.00	255.00	210.00	27.50
14	50.00	255.00	210.00	27.75
15	50.00	480.00	210.00	39.56
16	58.92	121.21	322.97	41.25
17	58.92	388.79	322.97	48.24
18	58.92	388.79	97.03	45.49
19	58.92	121.21	97.03	24.29
20	65.00	255.00	210.00	59.97

A second order polynomial equation was developed to study the effects of the variables on the esterification yield as follows:

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^{\ 2} + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij} + e \qquad (2)$$

where y is the dependent variable (percentage of yield) to be modelled, xi and xj are the independent variables (factors), b0, bi, bii and bij are the regression coefficients of model and e is the error of model. The fit of the model was evaluated by analysis of variance (ANOVA) and regression analysis. The significance of each term in the model was estimated by testing the null hypothesis. The model was simplified by elimination of statistically insignificant terms. The optimal condition for the synthesis of ester was obtained by the software's numerical optimization function.

III. RESULTS AND DISCUSSION

A. Identification of the product

Through the FT-IR spectrum, the functional groups present in the ester were determined. The absorption band at 1709.61 cm^{-1} for C=O and at 1240.41 cm^{-1} corresponded to the C-O stretching vibrations, confirmed the presence of ester bond in the samples. By using GC-MS, the formation of the ester was confirmed. The molecular ion of di- and tri-glyceride was barely detectable. In fact, the esters of higher alcohols show much weaker molecular ion peaks [10]. However, an ion peak at m/z = 429 was observed due to the formation of $[C_{25}H_{48}O_5+H]^+$, which confirms the formation of glycerol dilinoleate. In addition, an ion peak at m/z = 475 was observed due to the formation of $[C_{27}H_{55}O_6]^+$, which confirms the formation of glycerol trilinoleate. At the optimum condition, the relative percentage of mono- (retention time= 11.56 min), di- (retention time= 12.58 min), and tri- (retention time= 14.09 min) glyceride was 31.5, 58.8, and 9.7 %, respectively.

B. Model fitting and ANOVA

1) *Enzyme screening*: The simplex lattice design was used in this study to optimize the ratio of enzymes mixture. A quadratic Scheffe model was used to produce the best experimental model. Mathematical model relating the ester conversion to the parameters is as follows:

Conversion (%) = + 39.5 A + 24.3 B + 24.2C (3)

where A is Novozym 435,; B Lipozyme TLIM, and C Lipozyme RMIM.

The ANOVA for the model is shown in Table 3. The computed F-value of the model (32.57) showed that the model was significant at 5% confidence level. The very small P-value (<0.0001) and a suitable coefficient of determination (R^2 = 0.8555) also show that the model is highly significant and can present the actual relationship between the response and

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the parameters. The P-value less than 0.05 indicate that model terms are significant. The "lack of fit F-value" is 5.50 which indicated that lack of fit was not significant relative to pure error [11]. In this case, linear mixture components are significant model terms. The 2D response surface as a function of the enzyme mixture composition is shown in Figure 1. The most effective enzyme composition can be estimated from the response surface. The highest ester conversion (40.15%) was observed in high composition of Novozym 435 (200 mg). It means that Novozym 435 alone is the best enzyme for the synthesis of the ester.

TABLE 3
ANALYSIS OF VARIANCE FOR ENZYME SCREENING

Source	Sum of Squares	Degree of Freedom	Mean Square	F-value	Prob > F
Model	396.55	2	198.28	32.57	< 0.0001
Linear Mixture	396.55	2	198.28	32.57	< 0.0001
Residual	66.97	11	6.09		
Lack of Fit	60.67	7	8.67	5.50	0.0594
Pure Error	6.31	4	1.58		
Cor Total	463.53	13			

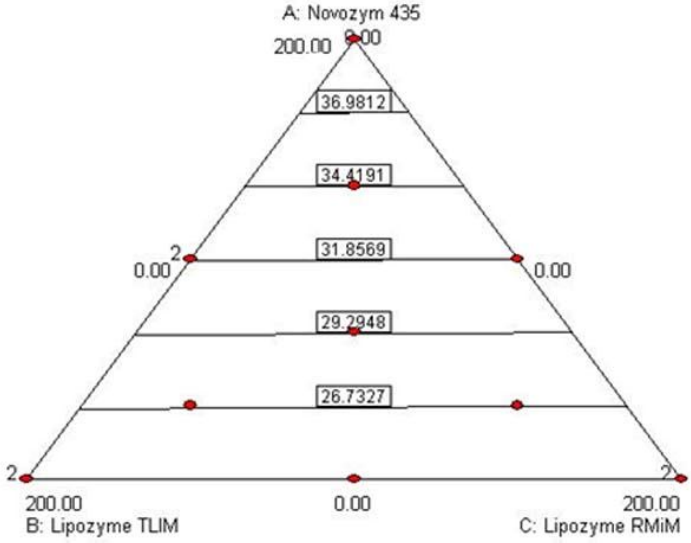

Fig. 1 Contour plot of mixture design for lipase-catalyzed synthesis of glycerol linoleate. The numbers inside the contour plots indicate conversion (%) at given reaction conditions

2) *Synthesis of the glycerol ester:* From the ANOVA table (Table 4), the model F-value is 33.47, which implies that the model is significant. There is only 0.05% chance that a "Model F-value" this large could occur due to noise. The R^2 is 0.9228 which shows that the modified quadratic polynomial model is significant and suitable to represent the actual relationship between the response and the variables. A linear distribution shown in Figure 2(a) is indicative of the good correlation between experimental and predicted conversions. Normal probability plot is presented in Figure 2 (b). The plot indicates that the difference between actual and predicted values (residuals) follows a normal distribution. The P-value less than 0.05 shows that model terms are significant. In this case, A, B, C and A2 are significant model terms. The "Lack of fit F-value" 4.48 is not significant relative to pure error. The term BC was considered in the model to make the lack of fit insignificant. The final equation in terms of coded factors is as follows:

Conversion (%)= + 28.74 + 5.93A + 5.36B + 6.19C + 6.38A2 - 2.16 BC (4)

where A is temperature; B is time; C is enzyme amount

Equation (4) was used then to study the effect of parameters on the conversion of ester.

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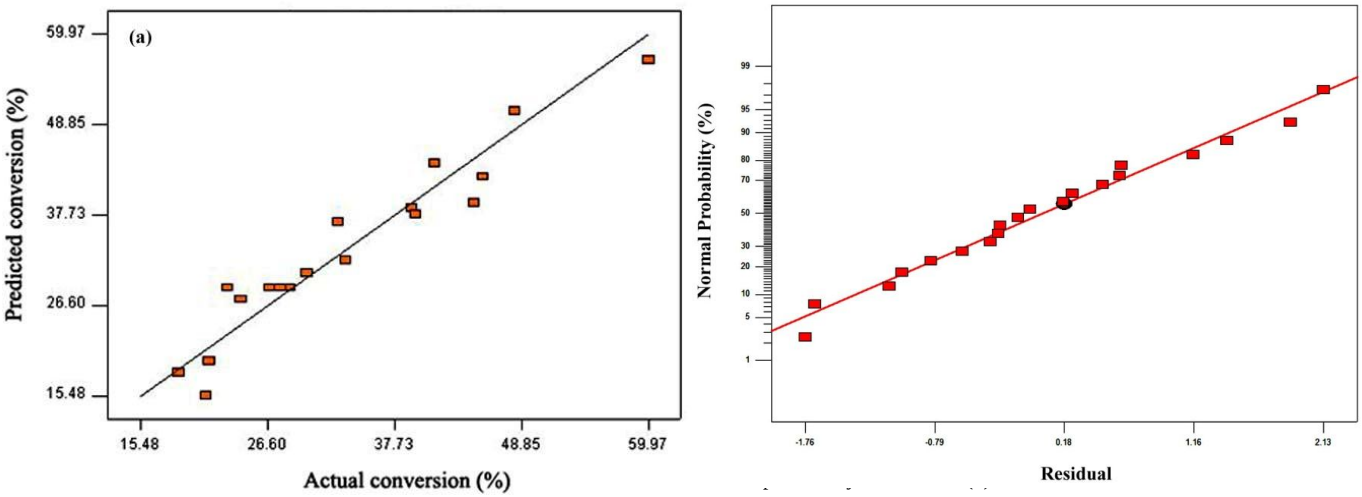

TABLE 4
ANALYSIS OF VARIANCE FOR THE SYNTHESIS OF ESTER

Source	Sum of Squares	Degree of Freedom	Mean Square	F-Value	Prob > F
Model	2030.53	5	406.11	33.47	<0.0001
A- Temperature	480.19	1	480.19	39.58	<0.0001
B- Time	392.56	1	392.56	32.36	<0.0001
C- Enzyme amount	523.21	1	523.21	43.13	<0.0001
A^2	597.28	1	579.28	49.29	<0.0001
BC	37.28	1	37.28	3.07	0.1015
Residual	169.85	14	12.12		
Lack of Fit	151.12	9	16.79	4.48	0.0566
Pure Error	18.73	5	3.75		
Cor Total	2200.38	19			

C. Effect of reaction parameters

The effect of the three independent variables on the synthesis of glycerol ester is shown in Figure 3. The percentage of conversion increased with increase in time from 30 to 480 min at the center point of other variables, 50oC and 210 mg of enzyme (Figure 3(a)). Optimizing the reaction time is crucial to the esterification process [12]. When the reaction time is too short, the esterification will not come to completion. On the other hand, prolonging reaction time will decrease the percentage conversion as the volume of water produced will increase and hydrolysis of ester will occur [13]. There are several methods to remove water from the reaction mixture including air drying, vaporization, vacuum evaporation and addition of molecular sieves that can be considered in future studies. From Figure 3(b), it is obvious that increase in temperature leads to increase in the percentage conversion of ester. When the temperature is too low, the enzyme is inactive. The increament of temperature will increase the rate of esterification as it provides more energy to promote the collisions between substrate and enzyme. Increase in temperature can reduce mixture viscosity, enhance mutual solubility and improve diffusion process of substrates which will reduce mass transfer limitations and favouring interactions between enzyme molecules and substrates [14]. However, when the temperature is too high, thermal deactivation of enzyme will occur [15]. Candida antarctica lipase B has a good thermoresistance and Novoyzm 435 has been known as heat-tolerant enzyme which maintains its activity even at 90^{o}C [16]. Figure 3(c) shows that the percentage conversion increases with increase in amount of enzyme. The highest conversion (46.62%) at this condition was achieved with 400 mg enzyme. When the amount of enzyme is low, the number of substrate molecules is higher than the enzyme molecules present in the reaction mixture. Therefore, substrate molecules have to compete for the limited active site present on the enzyme. Thus the rate of reaction would be lower compared to reaction where higher amount of enzyme is used. On the other hand, excess of enzyme in reaction mixture will decrease the rate of reaction. This is because excess enzyme prevents the active sites of enzyme from exposing to the substrate and also causes internal diffusional limitations within the heterogenous catalyst [16]. Besides, this might be due to the enzyme agglomeration and possible diffusion problems [14].

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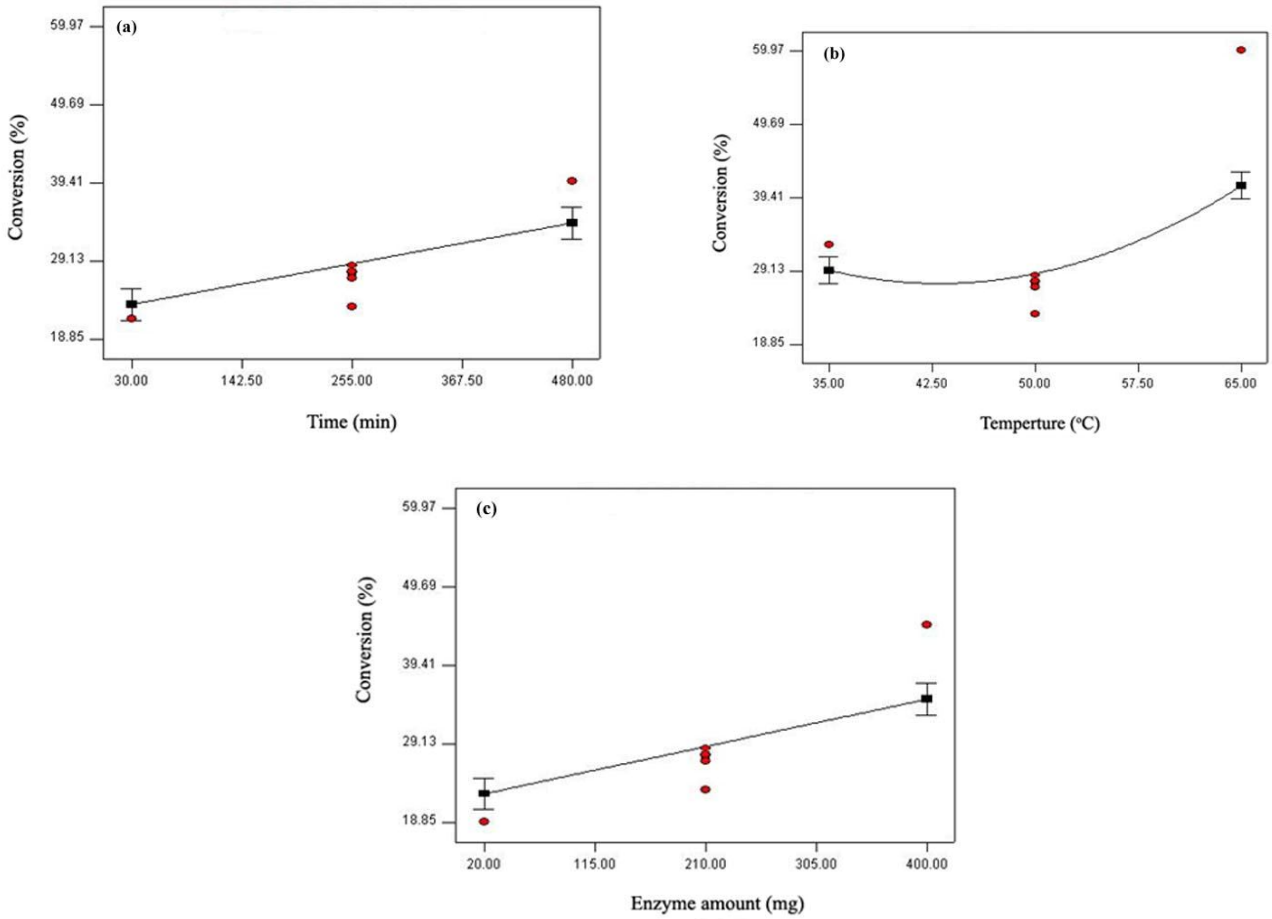

Fig. 3 Effect of individual parameters, time (a), temperature (b), and enzyme amount (c), on the synthesis of glycerol linoleate. One parameter is varied whilst the others are kept constant at their center points.

The relationships between reaction parameters and the response can be better understood by examining the 3D and contour plots generated from the predictive model [17]. The interactive effect of time and enzyme amount is presented in Figure 4. The temperature is constant at 50°C. As it can be seen, the percentage conversion increases when the reaction time and enzyme amount increase. Maximum conversion is observed at 480 min using 400 mg of enzyme.

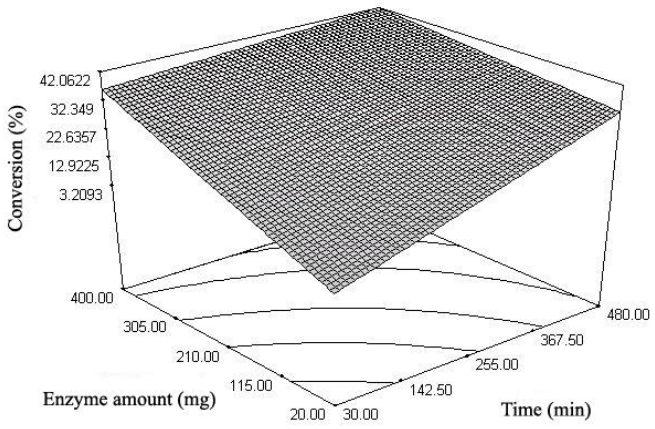

Fig. 4 Response surface plot showing effect of enzyme amount, time and their interaction on the esterification reaction

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D. Optimum condition

The optimum conditions can be used for future upscale synthesis of the ester. The optimum combination of parameters can be determined on the basis of the ridge maximum analysis and the canonical analysis using the optimization function of the software [18]. The experiment with desirability value of 1 was selected. The desirability function is given in the following equation:

$$D = (d_1 \times d_2 \times \dots d_n)^{1/n} = (\prod_{i=1}^{n} d_i)^{1/n} \quad (5)$$

where n is the number of responses in the measure and di is the desirable ranges for each response.

The maximum conversion (70.0 %) for the synthesis of glycerol linoleate was predicted at the condition of 64.9°C, 400 mg enzyme and 478.5 min. The actual experimental value obtained was 73.2% with 3.2% deviation.

IV. CONCLUSIONS

The modeling of immobilized *Candida antarctica* lipase-catalyzed esterification reaction to synthesize glycerol linoleate was successfully performed using response surface methodology. The Model F-value was 33.47 which implied that the model was significant. The value of R^2 was 0.9228 which indicated a good fit of the model. The effect of three main reaction parameters was evaluated by the generated model. Under the optimal conditions of 65°C, 478 min and 66 mg enzyme, an ester yield of 73.2% could be achieved. This study serves as another example for the application of RSM for improvement of an enzymatic esterification process.

REFERENCES

[1] A. Macierzanka, and H. Szelag, "Esterification kinetics of glycerol with fatty acids in the presence of zinc carboxylates: Preparation of modified acylglycerol emulsifiers," *Ind. Eng. Chem. Res.*, vol. 43, pp. 7744-7753, 2004.

[2] G.L. Hasenhuettl, and R.W. Hartel, *Food Emulsifiers and Their Applications*, 2nd ed., Springer Science+Business Media, LCC, New York, USA, 2008.

[3] A.Lee, N. Chaibakhsh, M.B. Abdul Rahman, M. Basri, and B.A. Tejo, "Optimized enzymatic synthesis of levulinate ester in solvent-free system," *Ind. Crop. Prod.*, vol. 32, pp. 246-251, 2010.

[4] N. Zhong, Zh. Gui, L. Xu, J. Huang, K. Hu, Y. Gao, X. Zhang, Zh. Xu, J. Su, and B. Li, "Solvent-free enzymatic synthesis of 1, 3-Diacylglycerols by direct esterification of glycerol with saturated fatty acids," *Lipids in Health and Disease*, vol. 12, pp. 65-71. 2013.

[5] Y.M. Xia, Y. Fang, K.C. Zhang, G.Y. Shi, J.J. Brown, "Enzymatic synthesis of partial glycerol caprate in solvent-free media," *J. Mol.Catal. B: Enzym.*, vol. 23, pp. 3-8, 2003.

[6] A. Philippaerts, J. Van Aelst, and B. Sels, "Conjugated linoleic acids and conjugated vegetable oils: From nutraceutical to biopolymer," *Eur. J. Lipid Sci. Tech.*, vol. 115, pp. 717–720, 2013.

[7] J.A. Arcos, H.S. Garcia, C.G. Hill, Jr., "Continuous enzymatic esterification of glycerol with (poly)unsaturated fatty acids in a packed-bed reactor," *Biotechnol. Bioeng.*, vol. 68, pp. 563-570, 2000.

[8] R.S. Rao, C.G. Kumar, R.S. Prakasham, and P.J. Hobbs, "The Taguchi methodology as a statistical tool for biotechnological applications: a critical appraisal," *Biotechnol. J.*, vol. 3, pp. 510-523, 2008.

[9] M.B. Abdul Rahman, U.H. Zaidan, M. Basri, M.Z. Hussein, R.N.Z.A. Rahman, and A.B. Salleh, "Enzymatic synthesis of methyl adipate ester using lipase from *Candida rugosa* immobilized on Mg, Zn and Ni of layered double hydroxides (LDHs)," *J. Mol.Catal. B: Enzym.*, vol. 50, pp. 33-39, 2008.

[10] D.L. Pavia, G.M. Lampman, and G.S. Kriz, *Introduction to spectroscopy*, 4th ed., Brooks/Cole Cengage Learning, Belmont, Australia, 2009.

[11] E.R. Gunawan, M. Basri, M.B. Abdul Rahman, A.B. Salleh, and R.N.Z.A. Rahman, "Study on response surface methodology (RSM) of lipase-catalyzed synthesis of palm-based wax ester," *Enzyme Microb. Tech.*, vol. 37, no. 7, pp. 739–744, 2005.

[12] P.S. Keng, M. Basri, M.B. Abdul Rahman, A.B. Salleh, R.N.Z.A. Rahman, and A. Ariff, "Optimization of palm based wax esters production using statistical experimenal designs," *J. Oleo Sci.*, vol. 54, pp. 519–528, 2005.

[13] E.L. Soo, A.B. Salleh, M. Basri, R.N.Z.A. Rahman, and K. Kamaruddin, "Response surface methodological study on lipase-catalyzed synthesis of amino acid surfactants," *Process Biochem.*, vol. 39, no. 11, pp. 1511–1518, 2004.

[14] Z.Q. Duan, W. Du, and D.H. Liu, "Novozym 435-catalyzed 1,3-diacylglycerol preparation via esterification in t-butanol system," *Process Biochem.*, vol. 45, pp. 1923-1927, 2010.

[15] M.B. Abdul Rahman, N. Chaibakhsh, M. Basri, R.N.Z.R.A. Rahman, A.B. Salleh, and S.M. Radzi, "Modeling and optimization of lipase-catalyzed synthesis of dilauryl adipate ester by response surface methodology," *J. Chem. Technol. Biot.*, vol. 83, pp. 1534–1540, 2008.

[16] D.W. Park, S.J. Haam, I.S. Ahn, T.G. Lee, H.S. Kim, and W.S. Kim, "Enzymatic esterification of β-methylglucoside with acrylic/methacrylic acid in organic solvents," *J. Biotechnol.*, vol. 107, pp. 151-160, 2004.

[17] R.H. Myers, D.C. Montgomery, and Anderson-Cook, C.M., *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*, 3rd ed., John Wiley & sons, New Jersey, USA, 2009.

[18] Y.K. Twu, I.L. Shih, Y.H. Yen, Y.F. Ling, and C.J. Shieh, "Optimization of lipase-catalyzed synthesis of octyl hydroxyphenylpropionate by response surface methodology," *J. Agr. Food Chem.*, vol. 53, pp. 1012–1016, 2005.

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