# Optimization of Oleyl Ester using Statistical Approach of Response Surface Methodology

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***Abstract*—**Oleyl oleate was synthesized by green process of lipase-catalyzed esterification reaction between oleic acid and oleyl alcohol. Dual enzymes system consisting of Novozym 435 and Lipozyme TL IM were used due to its specificity towards an ester bond. The reaction was optimized by statistical approach of RSM with four important parameters such as reaction time, reaction temperature, amount of enzymes and molar ratio of substrates. 97.52 % of oleyl oleate was achieved at the most optimum condition (58.67 min, 59.68 °C, 0.37 g enzymes and 2.88 molar ratio of substrates). Regression analysis shows all parameters were significant (Prob P < 0.05) except for the reaction temperature.

***Keywords*—**Enzymatic esterification, Dual Enzymes System, Oleyl oleate, Response surface methodology.

## I. Introduction

Nowadays, lot of cosmetic products have been produced throughout the world. In Malaysia as well, cosmetic industry has begun to develop. Wax esters are used as one of the main ingredients in the production of cosmetic products. This is due to some special characteristics of this type of ester such as non-toxic, excellent moisturizing behavior at interfaces and good fat soluble properties [1]. Other than cosmetics, wax esters are also used in other areas such as pharmaceutical [2], medical, food and lubricant industries [3]. Naturally, wax esters can be extracted from animals and plant materials such as sperm whale, beeswax and jojoba oil. Nevertheless, they are too expensive and often in short supply for commercialization. Therefore, the scientists come out with the synthesis of a synthetic product, which having a similar characteristics with natural resources [4].

According to Basri et al. [1], there are two methods that can be used to produce wax esters, which are chemical method and enzymatic catalyzed method. The chemical reaction between an organic acid with an alcohol will produce wax esters, with the presence of an acid catalyst. Wax esters which are obtained from chemical method are cheap but the esters are not natural. This method requires high-temperature process. It can damage the esters and lead to undesired side reactions [5-9]. Enzymatic catalyzed method only requires moderate temperature and pressure, which can be carried out at mild reaction condition [1]. In this method, enzymes were used as biocatalyst and the esters produced can be considered close to 'natural' [2]

Oleyl oleate can be considered as a synthetic analogue of jojoba oil. It is a liquid wax ester that having a long chain alkyl carbon. High molecular weight esters are from long-chain alcohols and long-chain acids which typically referred as waxes. Lipase catalyzed production of this ester was carried out using oleic acid and oleyl alcohol in a solvent-free system as starting materials.

In order to obtain a high yield of ester, Response surface methodology (RSM) can be used to optimize the reaction. This statistical approach is said to be the most effective technique for the complex chemical process investigation. RSM has been applied to optimize the enzymatic synthesis of esters and biodiesel, and successfully applied in many researches [10]. In this research, there are 4 parameters were studied which are reaction time, reaction temperature, molar ratio of substrates and amount of enzymes.

## II. Material and Methods

### A. Materials

Substrate (oleic acid and oleyl alcohol) were obtained from Sigma-Adrich (St. Louis, USA); chemicals (ethanol, acethone, hexane) were purchased rom Merck, Germany. Commercial lipases of Novozym 435 and Lipozyme TLIM were procured from Novo, Malaysia. All chemicals were commercially available and of analytical grade unless otherwise specified.

### B. Enzymatic Synthesis of Oleyl Oleate

The reaction mixtures consisted of oleic acid (2.0 mmol), oleyl alcohol (4.0 mmol) and immobilized enzyme (Novozym 435:Lipozyme TLIM, 0.15 g:0.15 g). The mixture was incubated at 37 °C for 1 hour with continuous shaking at 150 rpm. Then, the reaction was terminated by adding 7.0 mL of (ethanol : acetone, 1:1 v/v). The remaining fatty acid in the

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reaction mixture was determined by titration with 0.1 M NaOH using an automatic titrator (Metrohm, Switzerland) to an end point of 10.0.

The percentage conversion (%) of oleyl ester was calculated by determining the remaining fatty acid in the reaction mixture by titration with 0.1 M NaOH. The activity of lipase for each reaction was expressed of converted oleic acid in percent as reported in Mat Radzi et al. [2].

### *C. Optimization Study of Reaction Synthesis*

The reaction synthesis was carried out in a small scale using screw-capped vial. Response surface methodology (RSM) was employed to optimize the reaction with four important parameters such as reaction time, reaction temperature, amount of enzymes and molar ratio of substrates. The analysis was carried out using computer software of Design Expert Version 7.1.6 (Stat-Ease Inc., Statistics Made ease, Minneapolis, MN, USA).

A four-factor Central Composite Rotatable Design (CCRD) was employed in this study and the design summary is presented in Table 1. The matrix consisted of 30 experiments with 8 fact points, 12 axial points, and 10 center points. All the experiments were carried out in screw-capped vials at various reaction temperatures from 40-60 °C, reaction time from 5-60 minutes and molar ratio of substrates (oleyl alcohol : oleic acid) from 1 : 1 to 3 : 1. Effect of dual enzymes system (Novozym 435 : Lipozyme TL IM; 1 : 1 w/w) was evaluated with various amounts from 0.1-0.5 g. At the end of the reaction period, the sample was taken out and terminated with 7 mL of ethanol : acetone (1:1 v/v), before being titrated with 0.1 M NaOH. The percentage of conversion of oleyl oletae was calculated based on the remaining unreacted fatty acid in the reaction mixture as mentioned earlier.

TABLE I
SUMMARY OF THE EXPERIMENTAL DESIGN OF RSM

| | | | | |
|---|---|---|---|---|
| **Study Type** : **Response Surface** | | | **Experiments: 30** | |
| **Design** : **CCRD** | | | | |
| **Response** **Name** | | | **Unit** | **Value of** |
| **$Y_1$** **Oleyl oleate** | | | **Conversion (%)** | **Conversion (%)** |
| | | | **Coded Values of Variables** | |
| **Factors** | **Name** | **Unit** | **-1** | **1** |
| A | Reaction time | min | 5 | 60 |
| B | Reaction Temperature | °C | 40 | 60 |
| C | Amount of enzyme | g | 0.1 | 0.5 |
| D | Substrate ratio | ratio | 1 | 3 |

## III. RESULTS AND DISCUSSION

### *A. Enzymatic Synthesis of Oleyl Oleate*

Esterification reaction of oleyl oleate between oleic acid and oleyl alcohol was carried out using consortium enzymes, Novozym 435, an immobilized lipase from *Candida antartica* and Lipozyme TL IM from *Thermomyces lanuginosus*, in the presence of n-hexane as solvent in order to dissolve the medium as well as to shift the equilibrium for synthetic process. Salis et al. [11] stated that among the organic solvents, n-hexane can be removed easily due to its low boling point (50-70 °C).

The catalytic mechanism involves the reaction between oleic acid and oleyl alcohol. Oleic acid acts as an acyl donor, combines with enzyme at first to form acyl-enzyme intermediate which is thermodynamically unstable. While, oleyl alcohol acts as a neuclophile as well as an acyl acceptor, attacks this intermediate structure and combines with oleic acid to form oleyl oleate, which is a wax ester. The enzyme will be out and reacts with another unreacted fatty acid. The loss of enantioselectivity because of reversibility of the reaction may be avoided since no net amount of water is formed.

A new approach of dual enzymes system as also known as consortium enzymes was used in this study. Combination between specific enzymes (Lipozyme TL IM) with non-specific enzymes (Novozym 435) was believed to promote better catalytic efficiency towards an efficient esterification reaction. Furthermore, a synergistic interaction among enzymes will improve the thermostability, thus increase the acyl group migration. These were believed to improve the production of ester.

### *B. Optimization Study on Reaction Synthesis*

1. Analysis of Variance (ANOVA)

The ANOVA indicated that the model was adequate to represent the actual relationship between the significant variables and the response. Design matrix for enzymatic synthesis of oleyl oleate is shown in Table II. Using the software (Design Expert 7.1.6), the predicted values of parameters were obtained from model fitting techniques and were seen to be almost correlated to the observed values. Fitting the data to the various models (linear, two factorial, quadratic and cubic) and their subsequent ANOVA showed that the synthesis of oleyl oleate was suitably described with quadratic polynomial model.

At 0.03 % confidence level, the computed value of 8.72 implies that the model was significant due to the higher actual value than the tabulated value as shown in Table III. The "Lack of Fit F-Value" of 78.13 implies that the lack of fit was significant relative to pure error. There was only a 0.01 % chance that a "Lack of Fit F-Value" this large could occur because of noise. The significant lack of fit is bad unfortunately because we would like the model to fit [4].

TABLE II
DESIGN MATRIX OF THE ACTUAL EXPERIMENTS

| Standard | A (min) | B (°C) | C (g) | D (ratio) | Actual (%) | Predicted (%) |
|---|---|---|---|---|---|---|
| 1 | 5.0 | 40 | 0.1 | 1 | 91.03 | 90.27 |
| 2 | 60.0 | 40 | 0.1 | 1 | 92.06 | 93.14 |
| 3 | 5.0 | 60 | 0.1 | 1 | 91.11 | 90.21 |
| 4 | 60.0 | 60 | 0.1 | 1 | 92.47 | 93.05 |
| 5 | 5.0 | 40 | 0.5 | 1 | 92.06 | 92.86 |
| 6 | 60.0 | 40 | 0.5 | 1 | 93.37 | 92.81 |
| 7 | 5.0 | 60 | 0.5 | 1 | 92.56 | 93.42 |
| 8 | 60.0 | 60 | 0.5 | 1 | 93.68 | 93.34 |
| 9 | 5.0 | 40 | 0.1 | 3 | 87.37 | 88.27 |
| 10 | 60.0 | 40 | 0.1 | 3 | 95.52 | 94.86 |
| 11 | 5.0 | 60 | 0.1 | 3 | 88.30 | 89.07 |
| 12 | 60.0 | 60 | 0.1 | 3 | 95.88 | 95.63 |
| 13 | 5.0 | 40 | 0.5 | 3 | 93.16 | 92.79 |
| 14 | 60.0 | 40 | 0.5 | 3 | 95.00 | 96.46 |
| 15 | 5.0 | 60 | 0.5 | 3 | 94.74 | 94.21 |
| 16 | 60.0 | 60 | 0.5 | 3 | 96.88 | 97.85 |
| 17 | 87.5 | 50 | 0.3 | 2 | 95.52 | 94.76 |
| 18 | 32.5 | 30 | 0.3 | 2 | 95.74 | 95.18 |
| 19 | 32.5 | 70 | 0.3 | 2 | 96.70 | 96.50 |
| 20 | 32.5 | 50 | 0.7 | 2 | 95.10 | 94.34 |
| 21 | 32.5 | 50 | 0.3 | 4 | 94.79 | 94.03 |
| 22 | 32.5 | 50 | 0.3 | 2 | 94.90 | 94.61 |
| 23 | 32.5 | 50 | 0.3 | 2 | 94.59 | 94.61 |
| 24 | 32.5 | 50 | 0.3 | 2 | 94.54 | 94.61 |
| 25 | 32.5 | 50 | 0.3 | 2 | 94.60 | 94.61 |
| 26 | 32.5 | 50 | 0.3 | 2 | 94.48 | 94.61 |
| 27 | 32.5 | 50 | 0.3 | 2 | 94.54 | 94.61 |

TABLE III
ANOVA FOR THE QUADRATIC MODEL

| Sources | Sum of Squares | Degree of Freedom | Mean Square | F-Value | Prob > F |
|---|---|---|---|---|---|
| Model | 124.98 | 14 | 8.93 | 8.72 | 0.0003* |
| Residual | 12.28 | 12 | 1.02 | | |
| Lack of Fit | 12.17 | 7 | 1.74 | 78.13 | < 0.0001* |
| Pure Error | 0.11 | 5 | 0.022 | | |
| Total | 137.27 | 26 | | | |

2. Regression Analysis

The precision of a model can be checked by the determination coefficient, "R-Squared". High coefficient of determination was derived from quadratic model which is 0.9105, showed the real relationship among the parameters studied in each reaction is therefore presented by the model. The higher value of "R-Squared" was obtained by Güvenç et al. in 2007 which was 0.9970 and 0.9811 by Yi et al. [12]. The "Predicted R-Squared" of 0.0577 was not as close to the "Adjusted R-Squared" of 0.8061 as one might normally expect. This may indicated a large block effect or a possible problem with the model and/or data. Several things need to be considered were model reduction, response tranformation, outliers, etc. "Adequate Precision" measures the signal to noise ratio. It compares the range of predicted values at the design points to the average prediction error. A ratio greater than 4 was desirable. A ratio of 12.704 indicated an adequate signal. This model can be used to the design space.

Regression analysis of each set data then generated corresponding sets of coefficients for developing model equation as shown in Table III. The effect of parameters; reaction time (A), amount of enzymes (C) and molar ratio of substrates (D) were positive in quadratic model which indicated positive influences of the percentage yield of the product. Meanwhile, negative values of coefficients estimated negative influences of the parameters on the reaction. Values of "Prob > F" less than 0.0500 indicate model terms are significant. From the results obtained, we can see that several factors give postive influence such as reaction time (A), amount of enzymes (C), molar ratio of substrates (D), reaction time-amount of enzymes (AC), reaction time-molar ratio of substrates (AD), reaction time-reaction time ($A^2$) and amount of enzymes-amount of enzymes ($C^2$). On the contarary, terms and interactions of all variables were significant. All the terms were included in the respective equation in (1), in terms of coded factors:

$$\textit{Oleyl oleate (\%)} = 94.61 + 1.63A + 0.33B + 1.20C + 0.63D - 0.01AB - 0.73AC + 0.93AD + 0.16BC + 0.22BD + 0.48CD - 0.78A^2 + 0.31B^2 - 0.67C^2 - 0.46D^2 \quad (1)$$

TABLE IV
REGRESSION ANALYSIS FOR THE QUADRATIC MODEL

| Factor | Coefficient Estimate | Prob > F |
|---|---|---|
| Intercept | 94.61 | |
| A | 1.63 | < 0.0001* |
| B | 0.33 | 0.1338 |
| C | 1.20 | 0.0004* |
| D | 0.63 | 0.0260* |
| AB | -0.01 | 0.9749 |
| AC | -0.73 | 0.0135* |
| AD | 0.93 | 0.0032* |
| BC | 0.16 | 0.5499 |
| BD | 0.22 | 0.4106 |
| CD | 0.48 | 0.0810 |
| $A^2$ | -0.78 | 0.0133* |
| $B^2$ | 0.31 | 0.1477 |
| $C^2$ | -0.67 | 0.0281* |
| $D^2$ | -0.46 | 0.1127 |

3. Mutual Effects of Reaction Time and Reaction Temperature (AB)

Fig. 1 shows the response surface plots as a function of reaction time versus reaction temperature in quadratic model. Response surface plots for the interaction between reaction time and reaction temperature was generated with amount of enzymes (0.3 g) and molar ratio of substrates (mmol oleyl alcohol : mmol oleic acid; 2:1) fixed at their center point. By referring to Table 4, we can see that the mutual effects of reaction time and reaction temperature (AB) is not significant.

From Fig. 1, reaction time seems to show a better influence on the yield compared to the reaction temperature. The increased in reaction time from 5-60 min at any given reaction temperature from 40-60 °C, led to higher yields. It shows that the percentage conversion increased with increasing the reaction time at the same temperature. Gunawan et al. (2005) reported that the increase in percentage yield is a clear indication of the conformational change indicating greater unfolding of the enzyme at 50 °C.

Reaction temperature can be said as not significant (B, P = 0.1338) compared to reaction time (A, P = < 0.0001). From the results obtained, the percentage conversion was 96.09 % as we increased the reaction temperature from 40-60 °C at fixed reaction time (60 min), while as we decreased the reaction time from 60-5 min at fixed reaction temperature (40 °C), the percentage conversion was 92.17 %.

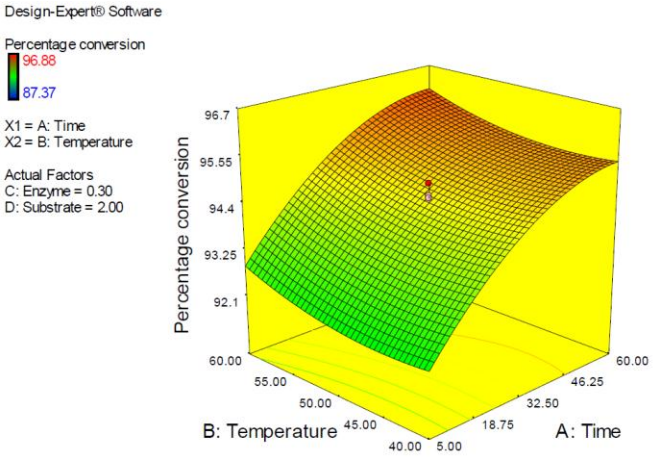


Fig.1 Response Surface Plots of Reaction Time versus Reaction Temperature (AB) in Quadratic Model

4. Mutual Effects of Reaction Time and Amount of Enzymes (AC)

The response surface plots as a function of reaction time versus amount of enzymes in quadratic model is represented in Fig. 2. Response surface plots for the interaction between reaction time and amount of enzymes was generated with reaction temperature (50 °C) and molar ratio of substrates (mmol oleyl alcohol : mmol oleic acid; 2:1) fixed at their center point. We can see that the mutual effects of reaction time and amount of enzymes (AC) is significant by referring to Table IV.

From Fig. 2, the increased in reaction time from 5-60 min as well as the increased in amount of enzymes from 0.1-0.5 g led to higher yields. A linear increase in the production of oleyl oleate with increase in reaction time and amount of enzymes has been observed. The rate increased proportionally with enzymes loading. The trend is similar as reported by Gunawan et al. [10] in their lipase-catalyzed synthesis of palm-based wax ester. Enzymes contribute to the increasing of product's yield by increasing the formation of acyl-enzyme complexes as well as by increasing the reaction time.

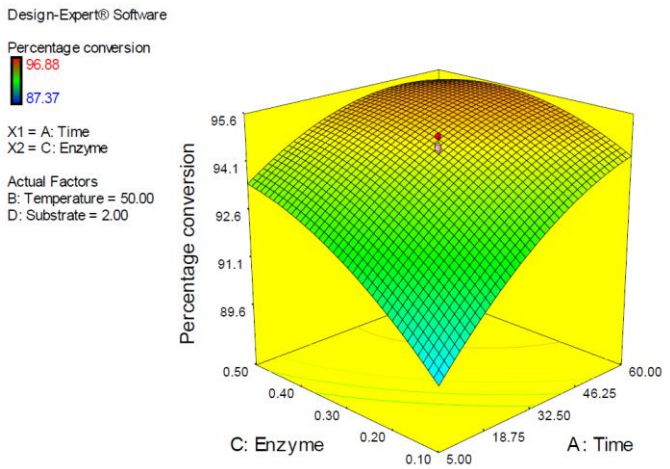


Fig.2 Response Surface Plots of Reaction Time versus Enzyme (AC) in Quadratic Model

Both parameters, reaction time and amount of enzymes, are significant (A, P = < 0.0001, C, P = 0.0004). From the results obtained, the percentage conversion was 95.27 % as we increased the reaction time from 5-60 min at fixed amount of enzymes (0.5 g), while as we decreased the amount of enzymes from 0.5-0.1 g at fixed reaction time (5 min), the percentage conversion was 89.63 %.

5.Mutual Effects of Reaction Time and Molar Ratio of Substrates (AD)

The response surface plots as a function of reaction time versus molar ratio of substrates in quadratic model is shown in Fig. 3. Response surface plots for the interaction between reaction time and molar ratio of substrates was generated with reaction temperature (50 °C) and amount of enzymes (0.3 g) fixed at their center point. By referring to Table IV, we can see that the mutual effects of reaction time and molar ratio of substrates (AD) is significant.

From Fig. 3, the increased in reaction time from 5-60 min, and the decreased in molar ratio of substrates (mmol oleyl alcohol : mmol oleic acid) from 1:1 to 3:1 favored maximal yields. In other word, the longer the reaction time and the smaller the molar ratio of substrates exhibit a similar tendency. Regarding to Güvenç et al. [13], higher levels of equilibrium conversion usually led by higher concentration of alcohol (nucleophile/acyl acceptor) because of the availability of exess nucleophile for acyl transfer. However, excess alcohol above critical level of alcohol-acid ratio may result in lower initial reaction rates which could be an indication of binding by alcohol, due to a strong effect inhibition on enzyme activity.

Reaction time and molar ratio of substrates, both parameters are significant (A, P = < 0.0001, D, P = 0.0260) but reaction time shows a better influence on the yield as compared to molar ratio of substrates. It can be seen from Fig. 3 that the percentage conversion was 92.05 % by increasing molar ratio of substrates (mmol oleyl alcohol : mmol oleic acid) from 3:1

to 1:1 at fixed reaction time (5 min). In contrary as we increased the reaction time from 5-60 min at fixed molar ratio of substrates (mmol oleyl alcohol : mmol oleic acid; 3 : 1), the percentage coversion was 96.55 %.

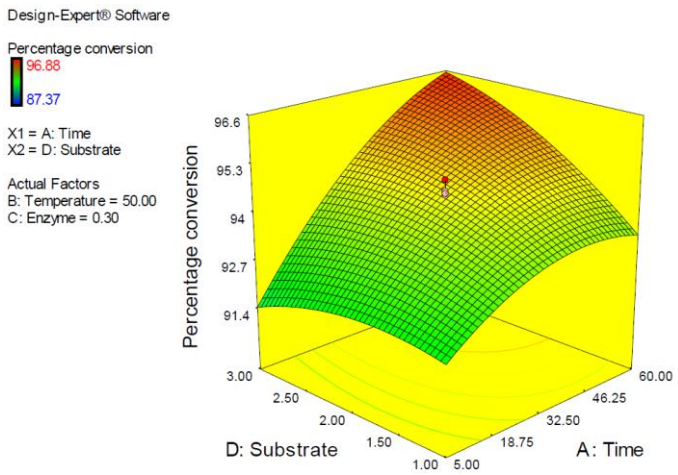


Fig.3 Response Surface Plots of Reaction Time versus Substrate (AD) in Quadratic Model

6. Optimum Condition

Within the experimental range study, optimal conditions for the enzymatic synthesis of oleyl oleate were predicted using the optimization function of the Design Expert 7.1.6. These are presented in Table 5 along with their predicted values. High percentage conversion of >90 % was achieved at these optimal conditions.

TABLE V
OPTIMUM CONDITIONS DERIVED BY RSM

| No | A (min) | B (°C) | C (g) | D (ratio) | Predicted (%) |
|---|---|---|---|---|---|
| 1 | 58.69 | 59.68 | 0.37 | 2.88 | 97.52 |
| 2 | 50.18 | 60 | 0.45 | 2.83 | 97.45 |
| 3 | 58.31 | 55.88 | 0.48 | 2.98 | 97.34 |
| 4 | 54.08 | 57.65 | 0.46 | 2.84 | 97.29 |
| 5 | 51.84 | 56.24 | 0.48 | 2.97 | 97.22 |

All the optimal conditions can be used in order to prodece high percentage yield of oleyl oleate. However, among the various optimal conditions, the highest percentage yield (97.52 %) was from the condition of 58.69 min, 59.68 °C, 0.37 g dual enzymes and substartes molar ratio 2.88:1.00. This was because the use of small amount of enzymes compared to others. Condition no.5 leads to the highest cost in the production of oleyl oleate due to the excess of enzymes and substrates.

## IV. CONCLUSION

Enzymatic synthesis of oleyl oleate was successfully carried out between oleic acid and oleyl alcohol. A new approach of dual lipases system has been proved to optimize the reaction performance by combining immobilized lipases from *Candida antartica* and *Thermomyces lanuginosus*. Response surface methodology (RSM) was used to evaluate the important parameters and revealed good correspondence between experimental and predicted values.

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