CHAPTER IV

OPTIMIZATION OF REACTION SYNTHESIS VIA CONVENTIONAL AND STATISTICAL APPROACHES USING DUAL LIPASES SYSTEM IN FERULATE ESTERS SYNTHESIS

4.1 Introduction

Optimization can be simply defined as "doing the most with the least" (Gomez et al., 2006). Today, there has been an increased interest in a process to obtain maximum production yield with low-cost production system, short reaction period and minimum raw materials requirement. In enzymatic synthesis, optimization can be achieved by understanding the interplay among the reaction parameters.

The conventional method of optimization involves varying one parameter at a time and keeping the other constant (Salis et al., 2003; Mat Radzi et al., 2011). As such, this conventional technique fails to explain interactions amongst these parameters in combination. Considering the industrial demand of optimization process, understanding the interactive effects of each parameter in coherence are essential for achieving maximum product yields.

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A statistical based technique commonly used for this purpose is response surface methodology (RSM). While most researches focused on the syntheses methodology of ferulate esters, few attempts have been made to investigate the relationships between parameters in the maximization of product yields by using RSM and revealed high potential to be employed (Ciftci & Saldana, 2012; Yang et al., 2012; Sun et al., 2013; Sun et al., 2014).

Numerous factors may affect the response of the system studied, and it is necessary to select those parameters with major effects. According to Yang and co-workers (2012), the yield of lipase-catalyzed reactions is significantly dependent on the operating conditions such as reaction temperature, substrates molar ratio and reaction time.

The target of this work was therefore to maximize the conversion of ferulate esters in optimal reaction conditions. Both studies on optimization of reaction synthesis using conventional method and statistical approach were explored. The investigated reaction conditions include reaction time, lipase dosage, mass ratio substrates and reaction temperature.

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4.2 Materials and Methods

4.2.1 Materials

Substrates (ethyl ferulate and olive oil) were obtained from Sigma-Aldrich (St. Louis, USA), solvents and chemicals (toluene, ethanol, acetone and potassium hydroxide) were purchased from Merck, Germany. Commercial lipases of Novozym 435 (immobilized lipase B from *Candida antarctica*) and Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*) were purchased from Sigma-Aldrich (St. Louis, USA). All chemicals were commercially available and of analytical grade unless otherwise specified.

4.2.2 Conventional Analysis Using One Factor At-A-Time Approach

The synthesis procedure has been previously described by Compton et al. (2000) with some modifications. The effects of various parameters were separately evaluated. All transesterification reactions were performed using ratio of 1: 9 Novozym 435-Lipozyme RM IM as an optimal dual lipases system and under shaking condition of 200 rpm unless stated otherwise.

a. Effect of Reaction Time

The transesterification was performed by using ethyl ferulate and olive oil (1: 4, g/g ratio), and100 mg of lipase dosage in 5 mL of toluene. They were placed in a 25 mL

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of screw-capped vial and incubated at 60 °C using a controlled water-bath shaker. The mixture was continuously reacted at various time intervals (4, 8, 12, 16 and 20 hrs).

b. Effect of Lipase Dosage

The transesterification was performed by using different amounts of lipase dosage (60, 70, 80, 90, 100 and 120 mg), and ethyl ferulate and olive oil (1: 4, g/g ratio) in 5 mL of toluene. They were placed in a 25 mL of screw-capped vial and incubated at 60 °C using a controlled water-bath shaker. The mixture was continuously reacted for 12 hrs of reaction time.

c. Effect of Mass Ratio Substrates

The transesterification was performed by using different mass ratios of substrates (ethyl ferulate/ olive oil; 1: 2, 1: 4, 1: 6 and 1: 8) and 80 mg of lipase dosage in 5 mL of toluene. They were placed in a 25 mL of screw-capped vial and incubated at 60 °C using a controlled water-bath shaker. The mixture was continuously reacted for 12 hrs of reaction time.

d. Effect of Reaction Temperature

The transesterification was performed by using ethyl ferulate and olive oil (1: 4, g/g ratio), and 80 mg of lipase dosage in 5 mL of toluene. They were placed in a 25 mL of screw-capped vial and incubated at different reaction temperatures (40, 50, 60, 70 and 80

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°C) using a controlled water-bath shaker. The mixture was continuously reacted for 12 hrs of reaction time.

4.2.3 Statistical Analysis by Response Surface Methodology (RSM)

Further optimization of the experimental conditions of ferulate esters synthesis was achieved by using computer software Design Expert Version 7.1.6 (StatEase Inc., Statistics Made ease, Minneapolis, MN, USA). A four-factor-five-level central composite design (CCD) was employed, requiring 28 experiments including 16 factorial points, 6 axial points and 6 center points. The factors and their selected levels are presented in Table 4.1, which were selected based on the conventional study. Table 4.2 represents the design matrix of the actual experiments carried out for developing the model. Triplicate experiments were set up for each run with all 28 runs performed in random order.

Three main analytical steps: analysis of variance (ANOVA), regression analysis and plotting of response surface were generated using the software Design Expert Version 7.1.6 to establish an optimum condition for the transesterification.

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TABLE 4.1: Summary of Experimental Design of RSM

Study type	Response Surface			**Runs**	28
Initial design	Central Composite			**Blocks**	No block
Design model	Quadratic				
Factor	**Name**	**Units**	**Type**	**Low actual**	**High actual**
A	Reaction time	hrs	Numeric	4	12
B	Lipase dosage	mg	Numeric	60	90
C	Mass ratio substrates	g/g	Numeric	1: 2	1: 6
D	Temperature	°C	Numeric	50	70

TABLE 4.2: Design Matrix of the Actual Level for a Four-Factor-Five-Level Central Composite Rotatable Design (CCRD)

Std	A: Time (hrs)	B: Lipase dosage (mg)	C: Mass ratio (Ethyl ferulate : olive oil) (g/g)	D: Temperature (°C)
1	4 (-1)	60 (-1)	1: 2 (-1)	50 (-1)
2	12 (1)	60 (-1)	1: 2 (-1)	50 (-1)
3	4 (-1)	90 (1)	1: 2 (-1)	50 (-1)
4	12 (1)	90 (1)	1: 2 (-1)	50 (-1)
5	4 (-1)	60 (-1)	1: 6 (1)	50 (-1)
6	12 (1)	60 (-1)	1: 6 (1)	50 (-1)
7	4 (-1)	90 (1)	1: 6 (1)	50 (-1)
8	12 (1)	90 (1)	1: 6 (1)	50 (-1)
9	4 (-1)	60 (-1)	1: 2 (-1)	70 (1)
10	12 (1)	60 (-1)	1: 2 (-1)	70 (1)
11	4 (-1)	90 (1)	1: 2 (-1)	70 (1)
12	12 (1)	90 (1)	1: 2 (-1)	70 (1)

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13	4 (-1)	60 (-1)	1: 6 (1)	70 (1)
14	12 (1)	60 (-1)	1: 6 (1)	70 (1)
15	4 (-1)	90 (1)	1: 6 (1)	70 (1)
16	12 (1)	90 (1)	1: 6 (1)	70 (1)
17	16 (2)	75 (0)	1: 4 (0)	60 (0)
18	8 (0)	45 (-2)	1: 4 (0)	60 (0)
19	8 (0)	105 (2)	1: 4 (0)	60 (0)
20	8 (0)	75 (0)	1: 8 (2)	60 (0)
21	8 (0)	75 (0)	1: 4 (0)	40 (-2)
22	8 (0)	75 (0)	1: 4 (0)	80 (2)
23	8 (0)	75 (0)	1: 4 (0)	60 (0)
24	8 (0)	75 (0)	1: 4 (0)	60 (0)
25	8 (0)	75 (0)	1: 4 (0)	60 (0)
26	8 (0)	75 (0)	1: 4 (0)	60 (0)
27	8 (0)	75 (0)	1: 4 (0)	60 (0)
28	8 (0)	75 (0)	1: 4 (0)	60 (0)

4.2.4 Percentage Conversion of Ferulate Esters

After completion of each assigned period, the reaction was terminated with 7 mL of ethanol:acetone (1: 1 v/v) and the lipase were filtered. The percentage conversion (%) of ferulate esters was measured by titration with 0.3 M KOH in an automatic tittrator (Metrohm, Switzerland). All the samples were assayed in triplicate and the experiment was repeated twice. The percentage conversion of ferulate esters was calculated based on the Equation 4.1.

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Conversion of ferulate esters (%) =

$$\frac{\text{Volume of KOH (without lipases)} - \text{Volume of KOH (with lipases)}}{\text{Volume of KOH (without lipases)}} \times 100$$

(Equation 4.1)

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4.3 Results and Discussion

4.3.1 Study on Individual Effects of Reaction Parameters

4.3.1.1 Effect of Reaction Time

The effect of varying reaction time (4, 8, 12, 16 and 20 hrs) on the synthesis of ferulate esters catalyzed by Novozym 435-Lipozyme RM IM is depicted in Figure 4.1. The reaction time profile is important to determine the shortest time necessary for obtaining good yields and so enhance the viability of the process.

Basically, increasing the reaction times will increase the frequency of collision between enzyme and substrate molecules in the mixture which lead to high formation of interest products. This can be clearly seen from the data as the ferulate esters conversion increased slightly from period of 4 to 12 hrs to give an optimal point of 85.95 %.

However, prolonged the periods was unfavorable as the ferulate esters conversion was drastically decreased to 35.03 % at 16 hrs and further dropped to 28.64 % at 20 hrs. According to Virto & Adlercreuz (2000), the substrates concentration decreased as the reaction progressed and led to a fall in the degree saturation of the lipase with substrates. Extended reaction time, furthermore, may generate excess amount of water and ethanol, as side products of the transesterification, and promote reverse hydrolysis reaction and inhibit the catalytic activity of Novozym 435-Lipozyme RM IM.

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Xin et al. (2009) also reported time course profiles for lipase-catalyzed transesterification of ethyl ferulate with triolein in solvent-free medium, where a long reaction period of 72 hrs was required to reach equilibrium. Thereafter, Yang et al. (2012) reported high conversion of 92.4 % in the transesterification of ethyl ferulate with fish oil after 120 hrs. Better conversion of 98.3 % was achieved by Sun et al. (2013) within 23 hrs using ethyl ferulate and monostearin under optimization using RSM.

By referring to the results, the optimum time to achieve high percentage conversion of ferulate esters was considered shorter than previous works (Compton et al., 2000; Compton & King, 2001; Xin et al., 2009). The short reaction time was promoted by complete solubility of the reaction mixture in the organic solvent (Herbst et al., 2012) and may be due to high purity of ethyl ferulate (98 %) used. Furthermore, the application of a dual lipases system as biocatalyst may be one of the core reasons contributed to the high conversion, in line with previous studies (Lee et al., 2006, Guan et al. 2010). Thus, in the subsequent experiments reaction time of 12 hrs was selected in the synthesis of ferulate esters.

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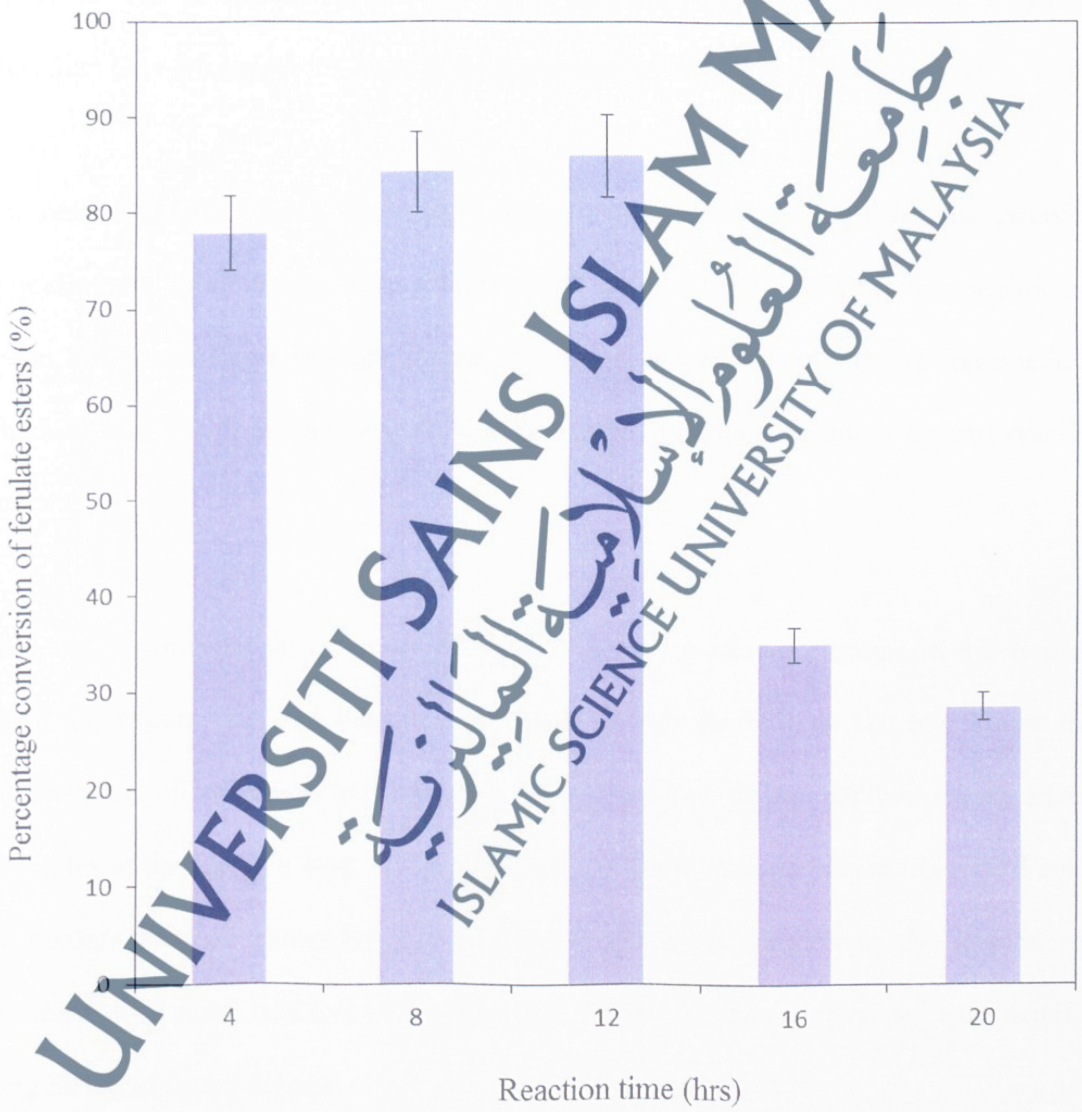
FIGURE 4.1: Effect of Reaction Time on the Conversion of Ferulate Esters. The Reaction Was Carried Out at 60 °C, Ethyl Ferulate and Olive Oil (1: 4, g/g Ratio), 5 mL of Toluene and 100 mg of Immobilized Lipases (Novozym 435-Lipozyme RM IM).



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4.3.1.2 Effect of Lipase Dosage

The amount of enzyme used is a crucial economical factor for successful industrial application, where minimum enzyme concentration with high conversion of products is favourable. Therefore, the effect of lipase dosage was examined. In this experiment, lipase dosage was varied (60 – 120 mg) as shown in Figure 4.2 by keeping 1: 9 w/w of Novozym 435 to Lipozyme RM IM as an optimum dual lipases system. Based on preceding time course profile, results were obtained after 12 hrs of reaction time.

The results show a slightly increase in the percentage conversion of ferulate esters by increasing the lipase dosage from 60 mg (79.19 %) to 80 mg (85.21 %). The addition of lipase in the substrates mixture increased the formation of acyl-enzyme intermediate, which in turn led to an increase in ferulate esters conversion within a certain reaction time.

However, beyond the optimum point, there was no significant increase in the ferulate esters conversion with an increase of the lipase dosage from 80 to 120 mg. Under this circumstance, all substrates are bound to the enzyme and further addition of any lipase molecules in the reaction may cause substrate limitation. Also, it was not practical since the mixture became extremely viscous due to the agglomeration of the lipases and promoted diffusional problems (Sonare et al., 2010). Further parameters were studied using 80 mg of lipase dosage.

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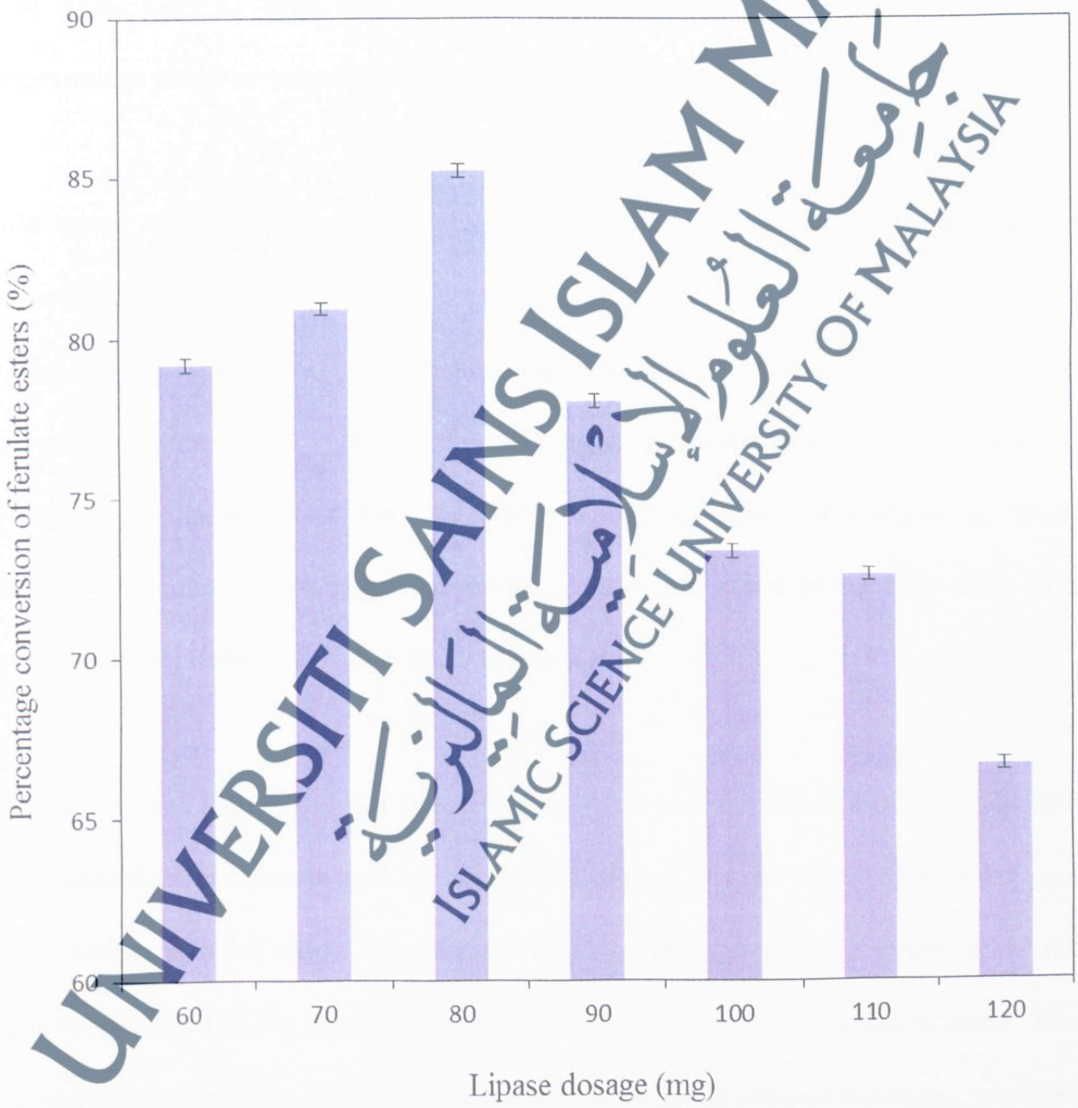
FIGURE 4.2: Effect of Lipase Dosage on the Conversion of Ferulate Esters. The Reaction Mixture Composed of Ethyl Ferulate and Olive Oil (1: 4, g/g Ratio), 5 mL of Toluene and Immobilized Lipases (1: 9 Novozym 435-Lipozyme RM IM). The Experiment Was Conducted at 60 °C for 12 hrs.



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4.3.1.3 Effect of Mass Ratio Substrates

The mass ratio of ethyl ferulate to olive oil is one of the most important parameters affecting the reaction synthesis. Due to the cost limitation, ethyl ferulate was fixed at 1 g whilst olive oil was varied to give varying ratios (1: 2, 1: 4, 1: 6 and 1: 8) as presented in Figure 4.3. The maximum ferulate esters conversion (85.21 %) was observed when mass ratio of ethyl ferulate and olive oil was kept at 1: 4. The substrates concentration above or below this ratio resulted decline in conversion of ferulate esters.

Mole ratios of 1: 1 ethyl ferulate: castor oil (Sun et al., 2014); 1: 1 ethyl ferulate: monostearin (Sun et al., 2013); 1: 2 ethyl ferulate: fish oil (Yang et al., 2012), 1: 2 ethyl ferulate: triolein (Xin et al., 2011) were reported for the synthesis of ferulate esters. According to Sun et al. (2013), the difference substrate ratios in achieving high conversion of ferulate esters were subjected to the fatty acid compositions as feruloyl acceptor. The authors also suggested lower the steric hindrance of the fatty acids on the glycerol backbone may result in high conversion rate.

Ciftci & Saldana (2012) did not report any increase in the synthesis of phenolic lipids from flaxseed oil and ferulic acid in supercritical carbon dioxide ($SCCO_2$) with the use of a high substrate molar ratio. This adverse effect of the increased substrate molar ratio might be attributes to the presence of less feruloyl groups in the reaction mixture. From the stand point of reaction efficiency, 1: 4 was chosen as the optimal substrates mass ratio during the synthesis conditions.

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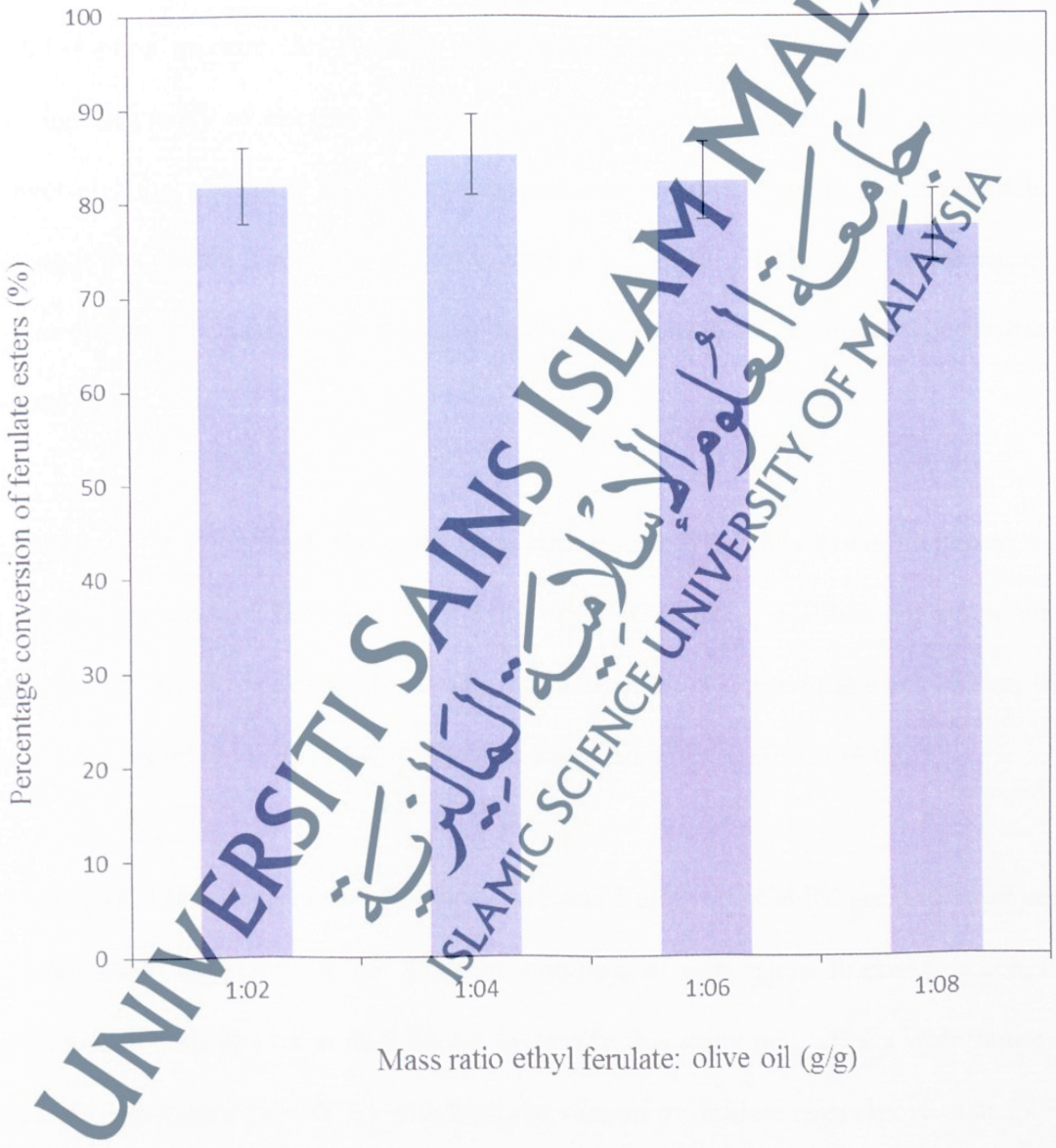
FIGURE 4.3: Effect of Mass Ratio of Ethyl Ferulate to Olive Oil on the Transesterification of Ferulate Esters. The Reaction Was Carried Out at 60 °C for 12 hrs, 80 mg of Immobilized Lipases (1: 9 Novozym 435-Lipozyme RM IM) in 5 mL of Toluene.



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4.3.1.4 Effect of Reaction Temperature

The influence of reaction temperature on transesterification reaction was investigated at five different values (40, 50, 60, 70 and 80 °C). Principally, at lower temperature the reaction rate is limited by mass transport phenomena due to the viscosity of the reaction mixture. An elevated temperature within a certain range could improve collision frequency of enzyme-substrate molecules; thus accelerate initial reaction rate. Conversely, the treatment at high temperature may disrupt enzyme tertiary structure, making it inactivate (Chandel et al., 2011; Kumar & Kanwar, 2011). So, it is necessary to find an optimum temperature of this new dual lipases system for the higher conversion of ferulate esters.

As displayed in Figure 4.4, the percentage conversion of ferulate esters increased with increasing temperature from 40 °C (62.2 %) to 60 °C (85.21 %). When the temperature surpassed 60 °C, the opposite effect was obtained where the percentage conversion was slightly decreased at 70 °C (79.43 %) and decreased sharply at 80 °C (34.66 %).

Kuo et al. (2012) suggested that Novozym 435 and Lipozyme RM IM are optimally used at temperature between 45 to 65 °C in the synthesis of wax esters. In consequence, the application of both lipases as dual lipases system in this study may offer a wide range of potential temperature (50-70 °C) with high conversion of ferulate esters more than 79 %. Still, 60 °C appeared to be the optimum temperature for this dual lipases system.

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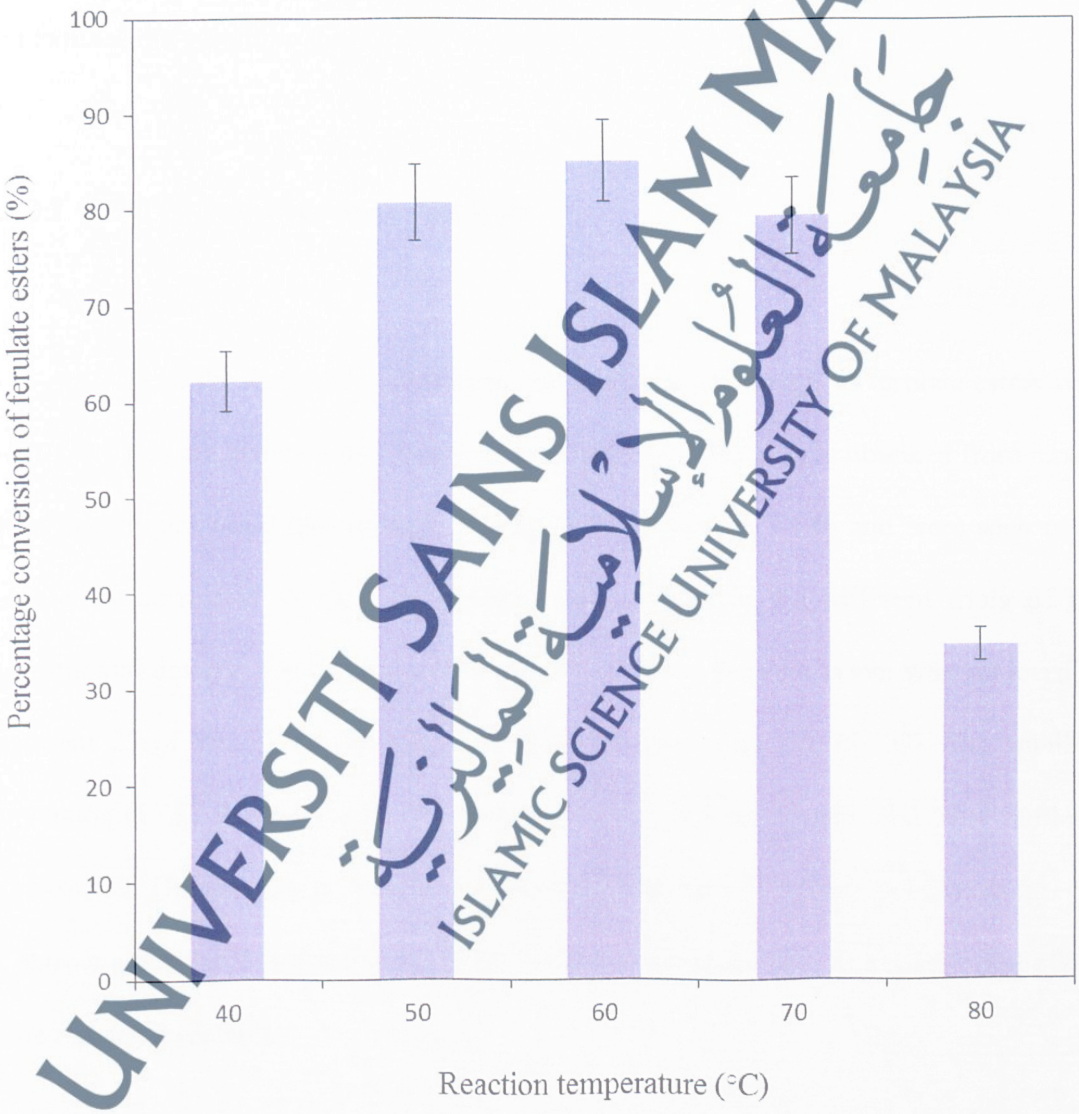
FIGURE 4.4: Effect of Reaction Temperature on the Transesterification of Ferulate Esters. The Reaction Mixture Composed of Ethyl Ferulate and Olive Oil (1: 4, g/g Ratio), 5 mL of Toluene and 80 mg of Immobilized Lipase (1: 9 Novozym 435-Lipozyme RM IM). The Experiment Was Conducted for 12 hrs.



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4.3.2 Study on Interactive Effects of Reaction Parameters and Their Optimization Using RSM

From the preceding section, it was implied that each selected parameter has a considerable effect on the reaction synthesis. Thus, experiments in the following section were performed to have a close look on the combined interactive effects of the parameters and explore the possibility to achieve an improved reaction synthesis.

4.3.2.1 ANOVA and Regression Analysis

All 28 of the designed experiments for enzymatic synthesis of ferulate esters with predicted value are depicted in Table 4.3. The predicted values were obtained from model fitting techniques using the software (Design Expert version 7.1.6) and were seen to be sufficiently correlated to the experimental values. Among the different trials of the experimental designs, the highest conversion (92.4 %) of ferulate esters was achieved in treatment 25 (8 hrs, 75 mg lipases, 1: 4 ethyl ferulate: olive oil, 60 °C). The smallest conversion (55.62 %) was shown in treatment 9 (4 hrs, 60 mg lipases, 1: 2 ethyl ferulate: olive oil, 70 °C). Most of the treatments exhibited percentage conversion between 55– 90 %, showing that Novozym 435-Lipozyme RM IM used presented a good catalyzing effect in the transesterification.

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TABLE 4.3: Experimental Data, Actual and Predicted Values for Four-Factor-Five-Level Response Surface Analysis

Std	A (hrs)	B (mg)	C (g/ g)	D (°C)	Conversion (%)	
					Predicted	Actual
1	4 (-1)	60 (-1)	1: 2 (-1)	50 (-1)	54.07	55.65
2	12 (1)	60 (-1)	1: 2 (-1)	50 (-1)	89.48	87.94
3	4 (-1)	90 (1)	1: 2 (-1)	50 (-1)	64.13	66.37
4	12 (1)	90 (1)	1: 2 (-1)	50 (-1)	87.43	87.65
5	4 (-1)	60 (-1)	1: 6 (1)	50 (-1)	58.91	63.98
6	12 (1)	60 (-1)	1: 6 (1)	50 (-1)	86.99	85.41
7	4 (-1)	90 (1)	1: 6 (1)	50 (-1)	64.67	65.5
8	12 (1)	90 (1)	1: 6 (1)	50 (-1)	80.64	82.04
9	4 (-1)	60 (-1)	1: 2 (-1)	70 (1)	52.96	55.62
10	12 (1)	60 (-1)	1: 2 (-1)	70 (1)	87.21	85.54
11	4 (-1)	90 (1)	1: 2 (-1)	70 (1)	65.05	65.79
12	12 (1)	90 (1)	1: 2 (-1)	70 (1)	87.2	86.19
13	4 (-1)	60 (-1)	1: 6 (1)	70 (1)	59.35	58.29
14	12 (1)	60 (-1)	1: 6 (1)	70 (1)	86.27	88.09
15	4 (-1)	90 (1)	1: 6 (1)	70 (1)	67.15	72.75
16	12 (1)	90 (1)	1: 6 (1)	70 (1)	81.97	79.55
17	16 (2)	75 (0)	1: 4 (0)	60 (0)	85.42	89.42
18	8 (0)	45 (-2)	1: 4 (0)	60 (0)	82.31	81.28

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19	8 (0)	105 (2)	1: 4 (0)	60 (0)	88.06	85.87
20	8 (0)	75 (0)	1: 8 (2)	60 (0)	86.24	83.01
21	8 (0)	75 (0)	1: 4 (0)	40 (-2)	61.33	58.83
22	8 (0)	75 (0)	1: 4 (0)	80 (2)	61.54	60.81
23	8 (0)	75 (0)	1: 4 (0)	60 (0)	88.56	86.55
24	8 (0)	75 (0)	1: 4 (0)	60 (0)	88.56	87.2
25	8 (0)	75 (0)	1: 4 (0)	60 (0)	88.56	92.4
26	8 (0)	75 (0)	1: 4 (0)	60 (0)	88.56	88
27	8 (0)	75 (0)	1: 4 (0)	60 (0)	88.56	89.5
28	8 (0)	75 (0)	1: 4 (0)	60 (0)	88.56	87.7

Multiple regression analysis was further carried out to explain the interactions (linear, two factorial, quadratic, cubic) among the parameters involved and quadratic polynomial model was selected as the most adequate. Finally, a mathematical model was generated based on the data obtained. The purpose of such modeling is to develop the mathematical connections between responses and parameters so the future predictions can be made. According to Santos et al. (2013), positive signal indicate synergic effect in the yield increases and negative signal show antagonist effect. The fitted quadratic model (Equation 4.2) for the transesterification between ethyl ferulate and olive oil catalyzing by Novozym 435-Lipozyme RM IM was obtained as:

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Percentage conversion =

$$88.56 + 12.56\,A + 1.44\,B - 0.1\,C + 0.052\,D - 3.03\,AB - 1.83\,AC - 0.29\,AD - 1.07\,BC + 0.51\,BD + 0.39\,CD - 7.06\,A^2 - 0.84\,B^2 - 0.53\,C^2 - 6.78\,D^2$$

(Equation 4.2)

Where A is reaction time; B is lipase dosage; C is mass ratio substrates; D is reaction temperature

The analysis of variance (ANOVA) results which were achieved in order to determine the significance of the model, each parameter and their interactions on the obtained responses are shown in Table 4.4. P-value was employed as a tool to verify the significance of each coefficient. Low value of probability (<0.05) indicates a high significance of the corresponding coefficient. The model's regression F-value (30.36), with p-value <0.0001, implies that the quadratic polynomial model was significant at 95 % trust level.

It was also observed that reaction time (A) had a significant effect over lipase dosage (B), mass ratio substrates (C) and reaction temperature (D). Besides that, the effects of interaction between reaction time and lipase dosage (AB), and the quadratic terms of reaction time (A^2) and reaction temperature (D^2) were also significant. These results showed that the effects of various factors on the ferulate esters conversion by dual lipases system were not simple linear relationship. The lipase dosage (B), mass ratio substrates (C) and reaction temperature (D), despite not being statically significant, they must remain in the equation to keep the hierarchy, or else the equation loses its robustness

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(Santos et al., 2013). On the contrary, the model also showed statistically insignificant lack of fit, confirming that the model explains very well the experimental data in the chosen intervals.

TABLE 4.4: ANOVA for Joint Test

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	6311.18	14	450.8	30.36	< 0.0001 [a]
A-Reaction time	3784.58	1	3784.58	254.89	< 0.0001 [a]
B-Amount of enzyme	49.59	1	49.59	3.34	0.089 [b]
C-Ratio substrates	0.17	1	0.17	0.011	0.9162 [b]
D-Reaction temperature	0.064	1	0.06	0.0043	0.9486 [b]
AB	146.53	1	146.53	9.87	0.0072 [a]
AC	53.73	1	53.73	3.62	0.0779 [b]
AD	1.33	1	1.33	0.09	0.7688 [b]
BC	18.45	1	18.45	1.24	0.2838 [b]
BD	4.16	1	4.16	0.28	0.6048 [b]
CD	2.42	1	2.42	0.16	0.6926 [b]
A^2	1330.54	1	1330.54	89.61	< 0.0001 [a]
B^2	18.92	1	18.92	1.27	0.2779 [b]
C^2	4.5	1	4.5	0.3	0.5906 [b]
D^2	1226.25	1	1226.25	82.59	< 0.0001 [a]
Residual	207.87	14	14.85		
Lack of Fit	185.3	9	20.59	4.56	0.0547 [b]
Pure Error	22.57	5	4.51		
Cor Total	6519.05	28			

[a] Significant at "*Prob > F*" less than 0.05.
[b] Insignificant at "*Prob > F*" more than 0.05.

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The quality of the model can be checked by the determination coefficient (R^2) as presented in Table 4.5. The obtained value for the determination coefficient (R^2 = 0.9681) indicated that 96.81 % of the experimental response variability can be explained by the previously discussed model (Equation 4.2). In this study, low value of coefficient of variation (CV = 5.068) indicated reproducibility of the model. According to Beg et al. (2003), a model can be considered reasonably reproducible if the CV is not greater than 10 %. The predicted R-squared of 0.807 was in reasonable agreement with adjusted R-squared of 0.936 suggested a satisfactory representation of the model. Adequate Precision was used to measure the signal to noise ratio where a ratio greater than 4 is desirable. A ratio of 19.591 indicated an adequate signal.

TABLE 4.5: R-Squared (R^2) Analysis of Quadratic Model

Standard deviation	4.85
Mean	76.8
CV %	6.068
PRESS	1260.02
R-squared	0.968
Adjusted R-squared	0.936
Predicted R-squared	0.807
Adequate precision	19.591

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4.3.2.2 Response Surface Analysis

Equation 4.2 was then used to facilitate plotting of 3D response surfaces. Two parameters were plotted at any one time on the $\chi 1$ and χ 2 axes, respectively, with other two remaining parameters were fixed at zero level.

a. Interactive Effect of Reaction Time (A) and Lipase Dosage (B)

Figure 4.5 shows the response surface plots as a function of reaction time versus lipase dosage for quadratic model with other parameters fixed at their center points: ratio substrates (1: 4 ethyl ferulate: olive oil) and reaction temperature (60 °C). Increases in percentage conversion with increase reaction time and lipase dosage were noticed from the plot. Two molecules of enzymes acting independently will transform twice as much substrate in a specific time (Dixon & Webb, 1979).

The trend observed was similar to the previous works. Gunawan et al. (2005) predicted an increase in percentage yield with simultaneous increases in incubation time and Lipozyme IM dosage during synthesis of wax ester from palm oil and oleyl alcohol. According to Hamsaveni et al. (2001) in the enzymatic synthesis of isobutyl isobutyrate, it was shown that reaction time and Lipozyme IM-20 interaction were significant and prevalent at high levels of both parameters.

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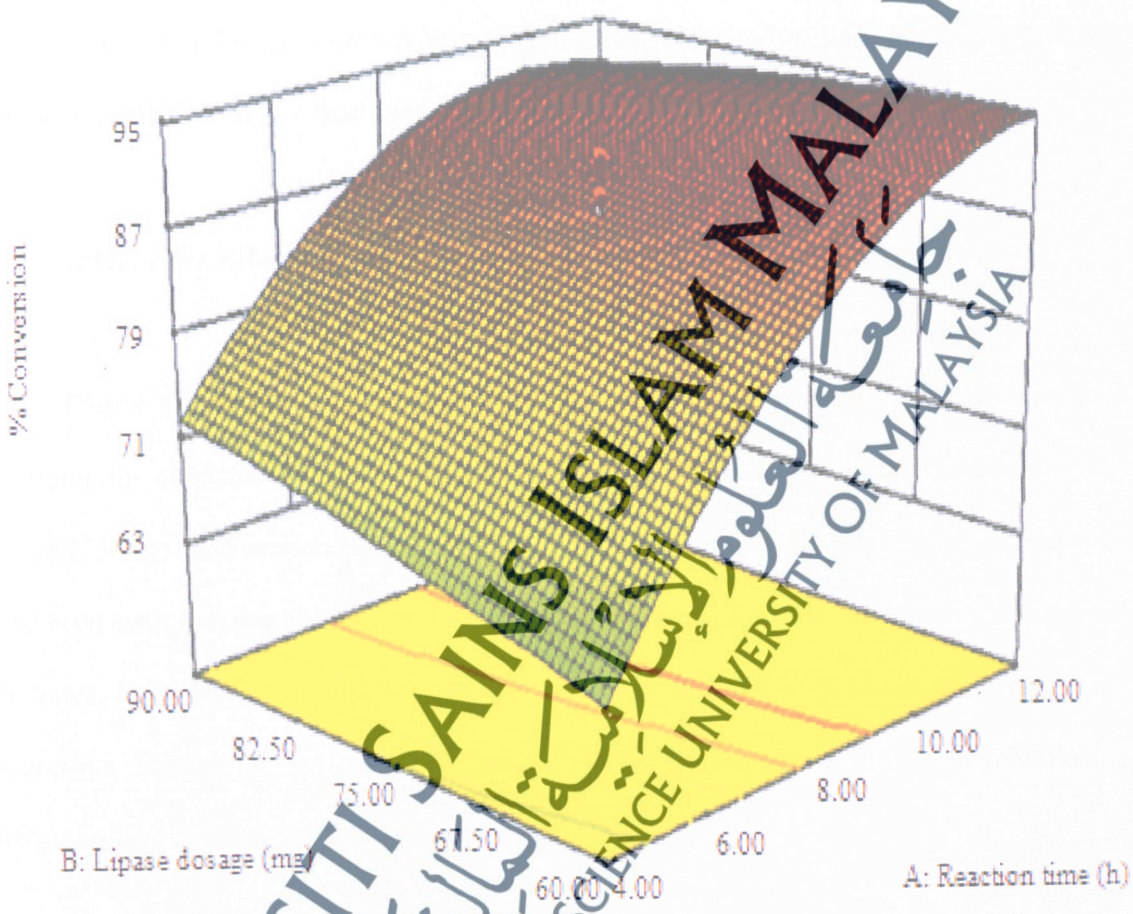
FIGURE 4.5: Response Surface Plot of Reaction Time versus Lipase Dosage in Quadratic Model



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In our study, however, a slight decrease in percentage conversion was detected at highest level of lipase dosage (90 mg) and reaction time (12 hrs). One possible explanation is that too much lipase molecules in a system caused mass transfer limitation due to the agglomeration of the lipases even at longer incubation period. Therefore, minimum lipase dosage (60 mg) and maximum reaction time (12 hrs) appeared to be the most favorable condition for the transesterification reaction.

b. Interactive Effect of Reaction Time (A) and Mass Ratio Substrates (C)

Figure 4.6 depicts the response surface plot of reaction time versus mass ratio substrates for quadratic model with other parameters fixed at their center points: lipase dosage (75 mg) and reaction temperature (60 °C). The interactions show trend similar to those seen with reaction time versus lipase dosage (Figure 4.5). At any given mass ratio substrates, increase in reaction time from 4 to 12 hrs resulted in increase of percentage conversion. Though, at 12 hrs axis, a linear downfall of percentage yield was remarked with increasing amount of substrates ratio.

The first step in the mechanism of lipase-catalyzed reaction is attack of the catalytic serine (Ser^{120}) on the carbonyl carbon of the acyl donor to form the acyl-enzyme intermediate (Carvalho et al., 1997). Longer reaction time may increase the chance of contact between enzyme and substrate, however, further increment of olive oil molecules in this study may distant the active site of the lipases from the limited concentration of ethyl ferulate.

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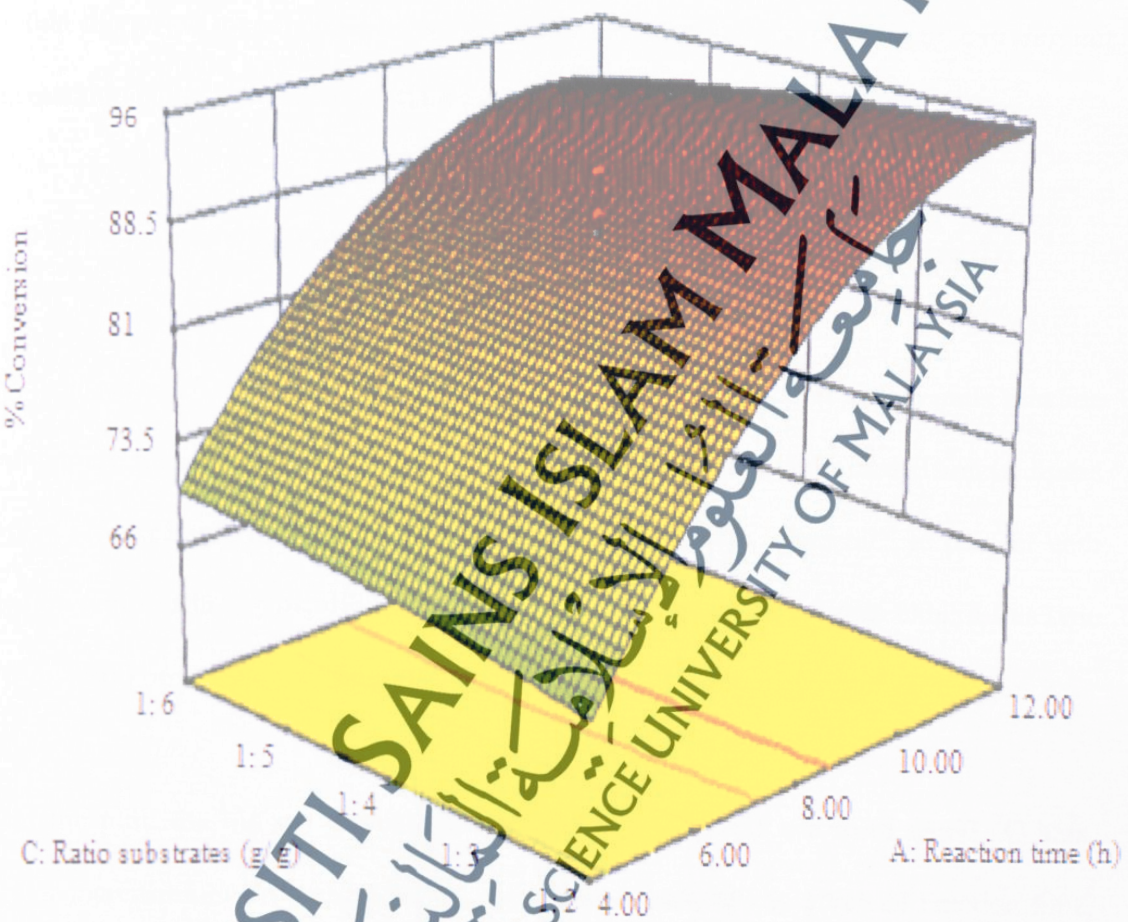
FIGURE 4.6: Response Surface Plot of Reaction Time versus Ratio Substrates in Quadratic Model



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The findings of Yang et al. (2012) have indicated that high viscosity of fish oil in large amounts of application contributes to the decrement of bioconversion of ethyl ferulate. In addition, too much concentration of one substrate also leads to ineffective mixing of reactants (Xin et al., 2011). In this transesterification reaction, maximum conversion (within the tested range) could be achieved at high reaction time (12 hrs) by carrying out the reaction using low substrates ratio (1: 2 ethyl ferulate: olive oil).

c. Interactive Effect of Reaction Time (A) and Reaction Temperature (D)

Figure 4.7 represents the response surface plots of reaction time versus reaction temperature for quadratic model with other parameters fixed at their center points: lipase dosage (75 mg) and mass ratio substrates (1: 4 ethyl ferulate: olive oil). In contact with reaction temperature, generally, many lipases catalysed reaction systems exhibit this type of plot, also known as dome shaped.

From the plot, the highest percentage conversion was clearly observed at 60 °C axis. Further increasing or decreasing the reaction temperature, at any given of reaction time, the percentage conversion started to reduce. Gunawan et al. (2005) have observed a similar trend of reaction time versus reaction temperature profile for synthesis wax esters. The authors explained that this phenomenon was due to the critical temperature which reaction synthesis was drastically decreased beyond the optimum point.

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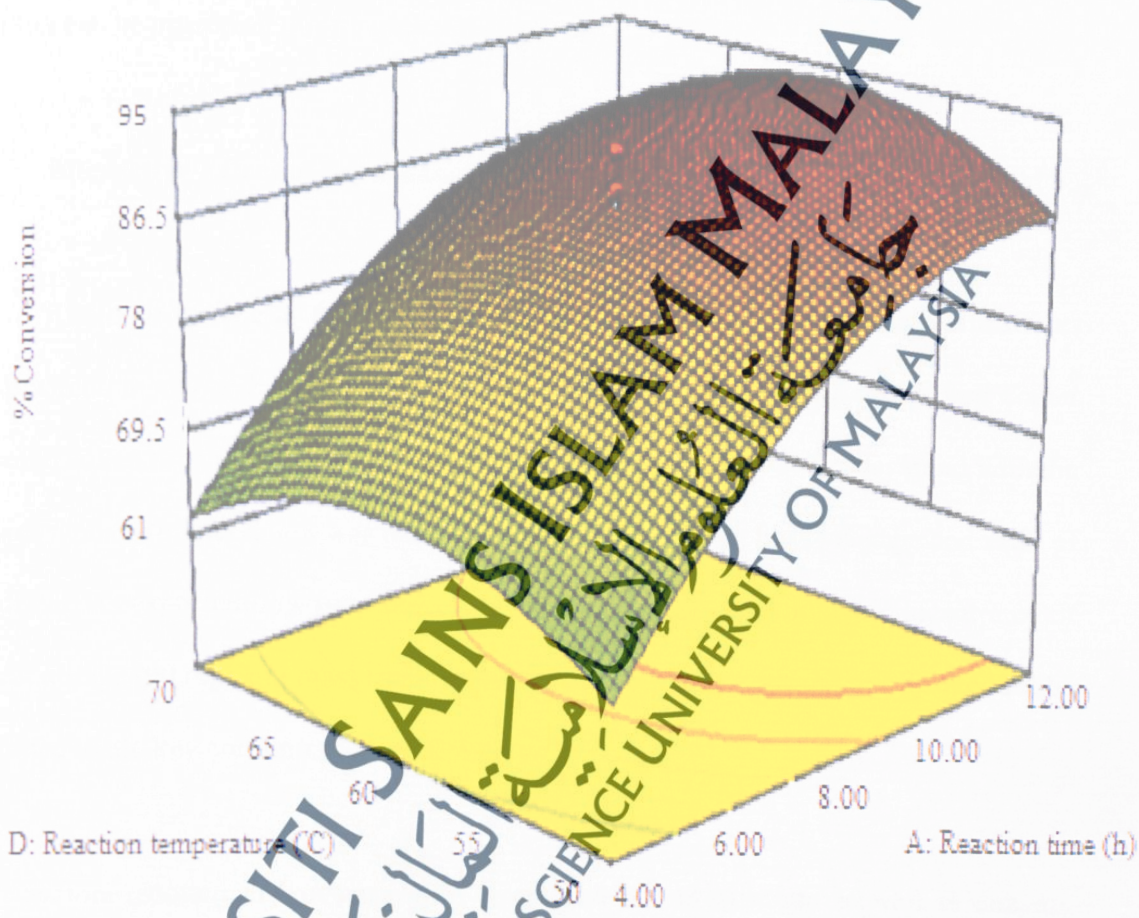
FIGURE 4.7: Response Surface Plot of Reaction Time versus Reaction Temperature in Quadratic Model



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Again, increment of reaction temperature from 4 to 12 hrs shows a positive effect when correlated with any reaction temperatures. In this work, the most conducive conditions were seen at long reaction time (12 hrs) with moderate reaction temperature (60 °C). The use of moderate temperatures is beneficial in that power costs can be reduced and lipase stability can be preserved during prolonged operation system.

d. Interactive Effect of Lipase Dosage (B) and Mass Ratio Substrates (C)

Response surface as the interactions of lipase dosage and mass ratio of substrates for quadratic model is displayed in Figure 4.8 with other parameters fixed at their center points: reaction time (8 hrs) and mass reaction temperature (60 °C). Similar trend with the previous, positive interaction was observed with increasing the lipase dosage and ratio of substrates. As expected, low percentage conversion was indicated at high concentrated of lipases dosage (90 mg) and mass ratio substrates (1: 6 ethyl ferulate: olive oil). It could be attributed to the low concentration of acyl donor.

Most authors reported the presence of a larger amount of substrate as well as enzyme contributed to a high percentage yield of esters due to the high probability of enzyme substrate collision forming an acyl-enzyme intermediate (Jeong et al., 2009; Zhao et al., 2011). Conversely, this linking is ineffective when there are limiting factors involved such as low substrate concentration, presence of activators, inhibitors or mass transfer effect (Gunawan et al., 2005).

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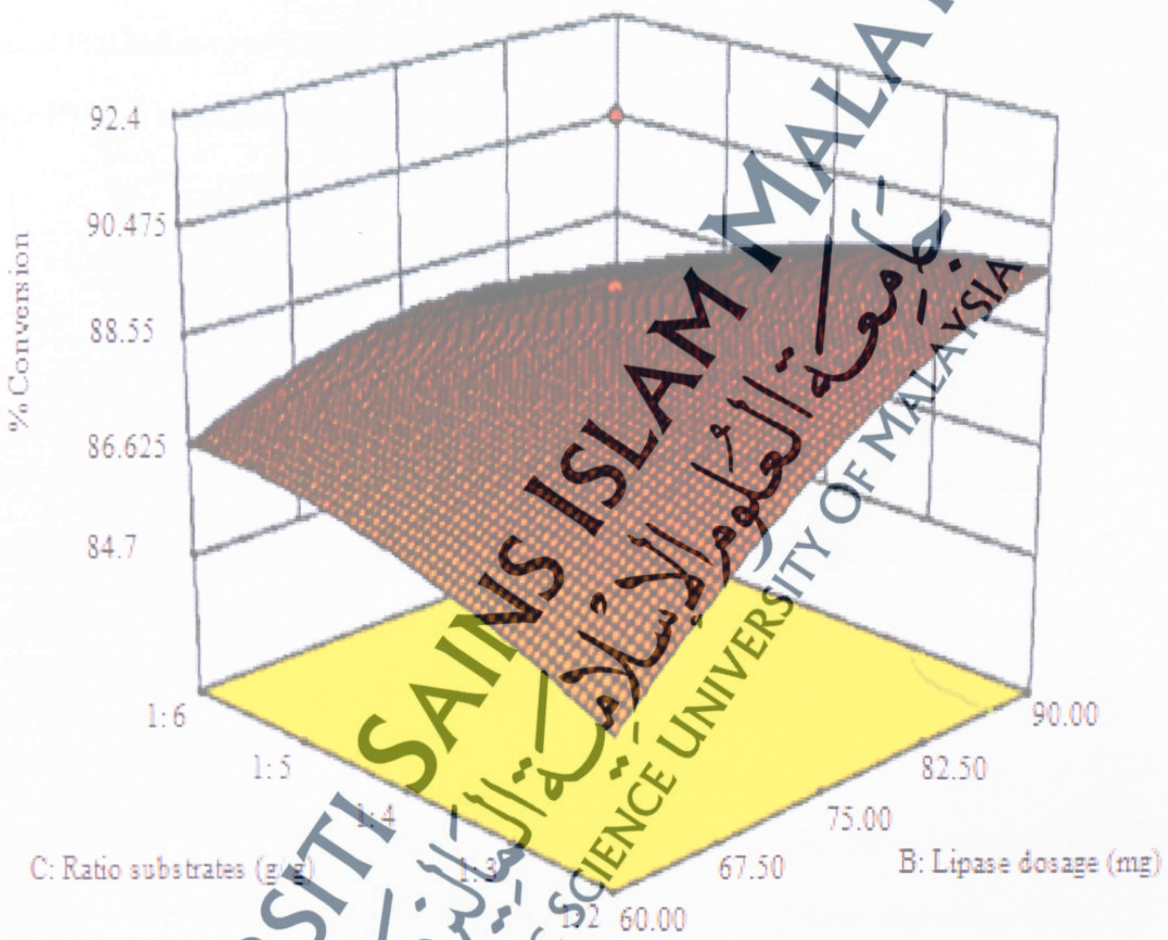
FIGURE 4.8: Response Surface Plot of Lipase Dosage versus Mass Ratio Substrates in Quadratic Model



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On the other hand, Hari Krishna et al. (2001) have successfully synthesized high isoamyl isobutyrate at high substrate levels with low enzyme concentration. The good yields achieved with minimal amount of enzyme suggest an economic advantage since the cost of enzyme usually higher than that of substrate. From the results obtained, it can be deduced that highest percentage conversion of ferulate esters are possible at high lipase dosage (90 mg) by employing low substrates ratio (1: 2 ethyl ferulate: olive oil).

e. Interactive Effect of Lipase Dosage (B) and Reaction Temperature (D)

Figure 4.9 illustrates the response surface plots of lipase dosage versus reaction temperature for quadratic model with other parameters fixed at their center points: reaction time (8 hrs) and mass ratio substrates (1: 4 ethyl ferulate: olive oil). This plot clearly shows that the percentage conversion did not exceed 86 % for reaction temperature lower and upper than 60 °C even by increasing lipase dosage.

At below critical temperature, indeed, the reaction conversion was rather low due to viscosity of reaction system. At this condition, active sites of the lipases difficult to find and attach to the substrates which leads to mass transfer limitation. Beyond the critical temperature, however, the benefits of low viscosity reaction system were offset by operational instability of the lipases. The observations reported here conform to the findings of Zhao et al. (2011) in the synthesis of ascorbyl esters from lard. Therefore, high amount of enzyme (90 mg) and moderate reaction temperature (60 °C) appeared to be the most favorable conditions for the transesterification reaction.

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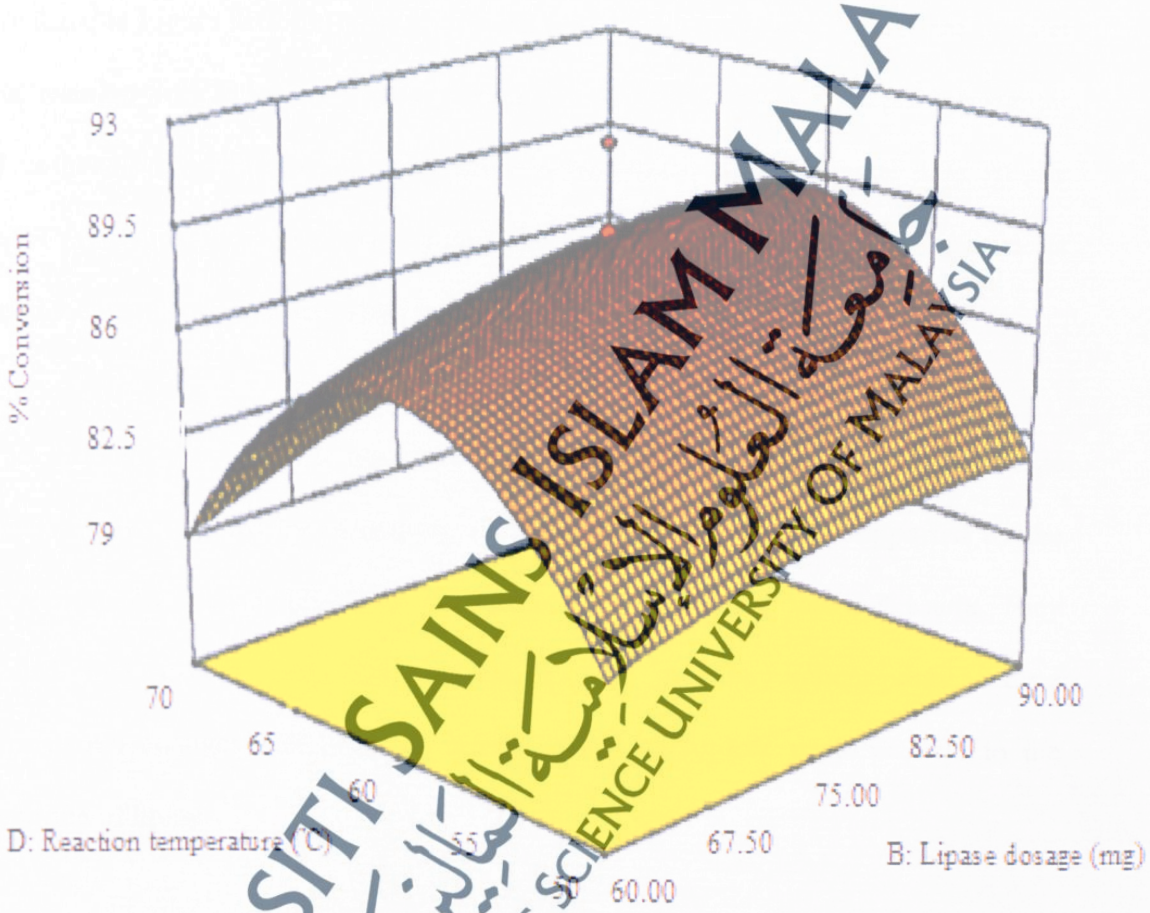
FIGURE 4.9: Response Surface Plot of Lipase Dosage versus Reaction Temperature in Quadratic Model



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f. Interactive Effect of Mass Ratio Substrates (C) and Reaction Temperature (D)

Response surface for the interactions of ratio substrates and reaction temperature is visualized in Figure 4.10 for quadratic model with other parameters fixed at their center points: reaction time (8 hrs) and lipase dosage (75 mg). The interactions portray similar trend to those seen with reaction temperature versus lipase dosage (Figure 4.9). At any given of ratio substrates, percentage conversion increased with reaction temperature at first and the trend was reversed when it was beyond 60 °C.

Besides, through the lowest temperature (50 °C) axis, a slight decrease of percentage conversion was predicted by increasing of ratio substrates. This was supported by the findings from Zhao et al. (2013) in the synthesis of lard-based ascorbyl esters. They suggested that excess lard concentration in the reaction system may induce conformational changes that limit the access of the hydrophilic ascorbic acid to the catalytic site of lipase.

However, different trend was published by Yang et al. (2012) in the enzymatic transesterification of ethyl ferulate with fish oil. The authors claimed that high temperature above 60 °C can reduce the viscosity of the fish oil and consequently lead to high conversion of esters with more than 90 %. From the response surface plotted, thus, low ratio of substrates (1: 2 ethyl ferulate: olive oil) and moderate reaction temperature (60 °C) appeared to be the favorable condition.

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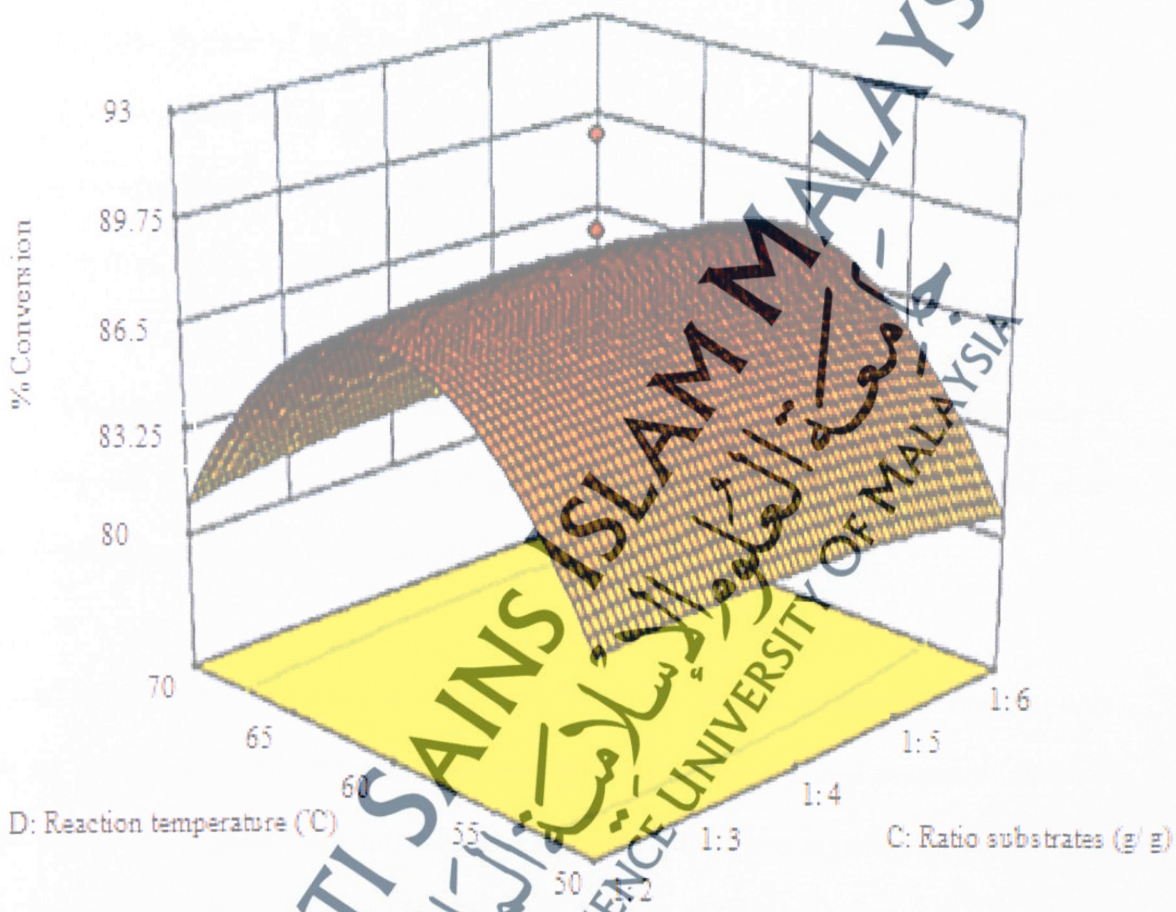
FIGURE 4.10: Response Surface Plot of Mass Ratio Substrates versus Reaction Temperature in Quadratic Model



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4.3.2.3 Optimum Conditions

Within the experimental range studied, the optimum conditions for the conversion of ferulate esters by Novozym 435-Lipozyme RM IM were predicted using numerical optimization feature of the Design Expert. The adequacy of the predicted model was examined by performing three additional independent experiments at the suggested optimum conditions. More than 90 % of percentage conversions ware obtained at optimal conditions as shown in Table 4.6.

The results obtained show an increment about 10 % over our conventional study of varying one parameter at a time. The good agreement between the predicted and actual results demonstrated the validation of the model.

From an economic viewpoint, it is desirable to choose the lowest possible reaction time, lipase dosage, ratio substrates and reaction temperature for practical transestrification of ferulate esters. Significantly, much shorter of reaction time was required for the synthesis of ferulate esters by Novozym 435-Lipozyme RM IM with high percentage conversion as compared to the traditional synthetic methods by using single lipase (Compton et al., 2000; Xin et al., 2009; Yang et al., 2012). This finding can be used for future scaleup synthesis of ferulate esters.

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TABLE 4.6: Optimal Conditions Derived by RSM (Quadratic Model)

No	A (hrs)	B (mg)	C (g/ g)	D (°C)	Conversion (%)	
					Predicted	Actual
1	11.86	60.24	1: 2.07	65.28	92.96	92.56
2	11.72	87.81	1: 2.13	62.27	94.83	94.03
3	11.82	63.82	1: 2.45	64.58	93.80	92.73

4.4 Conclusion

The influence of several parameters on the Novozym 435-Lipozyme RM IM synthesis of ferulate esters were effectively investigated through conventional and statistical analysis. The four factors were reaction time, lipase dosage, mass ratio substrates and reaction temperature where reaction time showed high significant effect among the others. Maximum yield of 94.03 % was obtained at optimum reaction conditions. Therefore, optimization of ferulate esters synthesis by using dual lipases system was successful.

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