CHAPTER III

Enzymatic Acidolysis of Palm Stearin and Oleic Acid by Dual Lipase System via Response Surface Methodology (RSM)

3.1 Introduction

Enzymatic acidolysis has been widely applied into the modification of lipid. The acidolysis process is defined as an exchange of acyl group between an esters and an acid (Rodrigues and Fernandez-Lafuente, 2010). The selective properties, and regiospecificity of the lipase enzyme to cut down fatty acids chains on the triglycerides at *sn*-1,3 positions increased the chance of the desired fatty acids to incorporate into the glycerols and to form a structured lipid. This supported by Xu (2000) which stated that the production of specific structured TAG can be done by using lipases as catalyst in which the lipase regiospecificity was exploited. The lipase regiospecifity have been an advantages in acidolysis process. Among other processes, semi-solid fats can be synthesized by acidolysis reactions catalyzed by lipases, where it can incorporate a desired acyl group onto a specific position of the triacylglycerol (Palla et al., 2012).

Besides many researches have been done, lot of them contribute on altering the TAG of fat and oil by acidolyzed it with medium chain fatty acid (Mounika and Reddy, 2012; Hita et al., 2009; Koh et al., 2008; Namal Senanayake and Shahidi, 2002; Sellappan and Akoh, 2000), polyunsaturated fatty acid (Nagachinta and Akoh, 2012; Tecelão et al., 2010; Hamam and Shahidi, 2007; Kojima et al., 2006; Peng et al., 2002) and monounsaturated fatty acid (Esteban et al., 2011; Robles et al., 2011; Tecelão et al., 2010; Hamam and Shahidi, 2007; Sellappan and Akoh, 2000; Balcao et al., 1998). In fact, there are some modification of fat done as an alternative to hydrogenation products such as acidolysis of sunflower oil with a palmitic-stearic acid mixture, where high incorporation of saturated fatty acid was investigated to obtain structured lipid (SL) (Carrín and Crapiste, 2008).

Thus, modification of fats using monounsaturated fatty acid have been extensively done to produce structured lipid. Oleic acid is one of the monounsaturated fatty acids. The blending of the oleic acid and palm stearin in the acidolysis can produce more amount of oleic acid in the triacylglycerol of palm stearin compared to the native one. The modification of palm stearin in this study has promoted an increment of oleic-oleic-oleic (OOO), palmitic-oleic-oleic (POO), and palmitic-palmitic-oleic (PPO) and reduced the palmitic-palmitic-palmitic (PPP) triglycerides in the structured lipid.

Acidolysis reaction of palm stearin and oleic acid have been optimised using Response Surface Methodology (RSM) by design expert, stat ease, ver 7.0 (DOE) via Central Composite Rotatable Design (CCRD). This RSM technique can explain the influence of

the reaction parameters towards the responses as well as generate the optimum condition of the reaction according to favarouble amount of response (whether it is have to be maximum of yield convertion or minimum value of responses).

Optimization process via RSM have been widely used in many applications and field of study such as synthesis cocoa butter equivalent (Kadivar et al., 2014), synthesis of *trans*-free structured margarine fats analog (Pande and Akoh, 2013), transesterification of lard fo biodiesel production (Huang et al., 2010), synthesis of conjugated linoleic acid (Li et al., 2010), synthesis of medium- and long-chain triglycerols (Koh et al., 2008) and acidolysis of structured phospholipid (Peng et al., 2002).

The formation of structured lipid in this reaction comprises 4 factors; enzyme ratio, enzyme load, substrate molar ratio and reaction time. All these parameters were optimized in order to achieve an optimum reaction conditions as the maximum amount of fatty acid composition (FAC) of oleic acid incorporation with minimum free fatty acid percentage (FFA) can be obtained.

3.2 Materials

Lipozyme TL IM was purchased from Novozymes, Germany. Lipase AK Amano 20 were purchased from Sigma-aldrich, Japan. Palm Stearin was obtained from Sime Darby Jomalina, Malaysia. Oleic acid, n-hexane of GC grade, chloroform of LC grade, acetonitrile, acetone, and sodium methoxide were purchased from Merck, Germany.

3.3 Methods

3.3.1 Acidolysis of Palm Stearin and Oleic Acid

Acidolysis reaction was performed in a shaker water bath at 250 rpm speed and temperature of 60°C according to Kadivar et al., 2014, Nagachinta and Akoh, 2012 and Kamar et al., 2011. The total amount of palm stearin and oleic acid ratio, lipase load, lipase ratio and reaction time were set according to the values generated by Response Surface Methodology (RSM) software-Design of Expert 7.0, as shown in Table 5. Anhydrous sodium sulphate was added to remove the water (if may present) and the reaction were stopped by filtering out the lipases using filter paper Whatman No.1 at 50°C. All blends were made in triplicate. The product obtained was then analyzed for its responses, fatty acid content (FAC) and percentage of free fatty acid (FFA). The result obtained were fitted in the second-order polynomial equation as shown below (gained from the model) to predict the response value and compared with the actual one.

$$Y = b_o + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=j}^{3} \sum_{j=i+1}^{4} b_{ij} x_{ij}$$

Equation 1: 2nd polynomial order equation

3.3.2 Experimental Design via Response Surface Methodology (RSM)

Acidolysis reaction was done within the range given by the design from the response surface methodology (RSM). This multivariate data analysis generated 30 sets of the

experiment to be run based on the five-level factorial experiment, four-factor, central composite rotatable design (CCRD) according to the range of parameters that have been set up. Table 5 shows the corresponding factors to be determined in the reaction and their range.

TABLE 5: The parameter ranges of four factors and responses.

Parameters	Name	Unit	Low Actual
A	Enzyme Ratio	-	5:5, 7:3, 9:1
B	Enzyme Load	%	8, 10, 12
C	Substrate Molar Ratio	-	1:3, 1:5, 1:7
D	Reaction Time	H	3, 5, 7
Response 1	Free Fatty Acid	%	
Response 2	Fatty Acid Composition (oleic acid)	%	

In this table, noted that value for the range of enzyme ratio, 5-9, was for lipase AK where 5:5 indicating 5 ratio lipase AK to 5 ratio of TL IM while 9:1 indicates 9 ratios of lipase AK to 1 ratio of TL IM and it continues to other values. The substrate molar ratio implies the same concept as well where by 3-7 value range was for oleic acid while the palm stearin remains 1 ratio for all values. For example, 1:3 indicates 1 ratio of palm stearin to 3 ratios of oleic acid while 1:7 indicates 1 for palm stearin and 7 for oleic acid ratio. These continue to the other set which were 1:4 and 1:6.

After setting up the range of factors for corresponding reactions, the software has generated about 30 sets of experiments to be run accordingly. These 30 run of experiment were conducted each in triplicate and the products were collected prior to analysis for the response. Table 6 shows the sets of the experiment for this acidolysis reaction.

TABLE 6: The series of experiments generated by RSM.

Run	Enzyme Ratio	Enzyme Load (%)	Sub.Mol. Ratio	Time (h)
1	9	12	3	7
2	11	10	5	5
3	5	12	3	7
4	7	14	5	5
5	7	10	1	5
6	9	8	7	3
7	7	10	9	5
8	7	10	5	5
9	7	10	5	9
10	5	12	7	3
11	9	12	7	7
12	7	10	5	1
13	9	12	7	3
14	5	8	3	7
15	5	12	7	7
16	7	10	5	5
17	7	10	5	5
18	9	12	3	3
19	5	8	3	3
20	7	10	5	5
21	5	8	7	7
22	3	10	5	5
23	5	12	3	3
24	9	8	7	7
25	7	10	5	5
26	5	8	7	3
27	9	8	3	7
28	7	6	5	5
29	9	8	3	3
30	7	10	5	5

3.3.3 Optimization and verification of modified palm stearin using RSM

The effect of various important operating parameters on the synthesis of acidolysis of palm stearin and oleic acid was studied via Central Composite Rotatable Design (CCRD)

of RSM by using the optimization function in the software. The experimental design considered 4 factors which include reaction time, substrate molar ratio, enzyme ratio and enzyme load.

3.3.4 Free Fatty Acid (FFA) Percentage

The free fatty acid percentage was determined by using titration method according to MPOB test method, MPOB p2.5 (Kuntom et al., 2004). Samples of 2.5 g was weighted and dissolved in 6.25 ml of isopropanol. The sample was then heated until clear in crystal and phenolphthalein was added as indicator prior to titration. The sample was titrated with sodium hydroxide (0.2N) until permanent light pink colour appeared. Free fatty acid was calculated as percentage of oleic acid according the following formula;

$$\text{FFA percentage (as oleic acid)} = \frac{V \times 28.2 \times N}{W}$$

Where, V : Volume of NaOH solution used

N : Normality of NaOH solution used

W : Sample weight

Equation 2: Percentage of free fatty acids of oleic acid

3.3.5 Fatty Acids Composition (FAC) using Gas Chromatogrpahy

The fatty acid profile analysis was performed by using gas chromatography (GC). Fatty acid methyl esters (FAMEs) of the samples were prepared according to MPOB Test

Method, MPOB p3.4 (Kuntom et al., 2004). Sample (0.05 g) was dissolved in 0.95 ml hexane and 0.05 ml sodium methoxide (30%). The reaction mixture (in a 1.5 ml microcentrifuge tube) then was shaken for 60 sec by vortex mixer and left if for 60 sec before the sample was added with anhydrous sodium sulphate to remove the water that may present in the sample. The sample then once again was vortex for 60 sec, then continued by centrifugation using microcentrifuge at the rate of 13 ,000 rpm for 1 min. The sample was then filtered and put into vials prior to analysis.

The sample (1 µl) was injected into Agilent Technology 7890A GC System. The detector used was Flame ionization detector (FID). A capillary column HP88 (100 m, 0.25 mm ID, 0.2 µm film thickness) and Helium at a column head pressure of 20 psi were used as the carrier gas. The detector temperature was maintained at 280 °C. The split ratio was 1:50 and the flow rate of helium carrier gas was 1 ml/min. Chromatograms and data obtained from integration of peaks were compared with fatty acid methyl ester standard. The peak areas percentage of FAME was obtained as area percentages by direct normalization. The analysis was performed in triplicate for each sample.

3.4 Results and Discussion

3.4.1 Model Fitting and ANOVA

This reaction comprises of two types of analysis as the responses; fatty acid composition, and percentage of free fatty acid. Fatty acid compositions (FAC) analysis in this

experiment focused on the high amount of oleic acid that may present in the product after the reaction. In contrast, free fatty acid percentage (FFA) was aimed in lower amount in this experiment. Free fatty acid percentage indicates the remaining unbound fatty acids in the final product. The minimum FFA was expected to be in the final product as this FFA if presents in the oil, it can enhance the oxidation process of the product.

Free fatty acids and unsaturated fatty acids were more likely to react with oxygen under the presence of heat, resulting in the formation of hydroperoxides which subsequently break down to form undesirable secondary compounds such as acids, aldehydes and ketones (Koh et al., 2009). Hydroperoxides are very unstable, flavourless and odorless compound which then can promote the secondary oxidation to occur (Kamal-Eldin et al., 2003). Thus, a wide range of polymeric compound are produced and contribute to some undesirable rancid odors in the fat stock. In short, it is important to keep the FFA as low as possible to maintain the oxidative stability of the final product.

Table 7 shows FAC and FFA obtained from the experiment and predicted values generated by RSM. For both responses, it can be seen that there was not much difference between the predicted and the actual amount obtained. This resulted from the variation of parameters value. The analysis from RSM promotes a design of quadratic model as the suggested model for both responses as seen in Table 8. It showed the model fitting and ANOVA analysis of FAC and FFA. From Table 8, it was found that the model F-value was 10.50 and 41.76 for FAC and FFA respectively.

TABLE 7: FAC and FFA obtained for RSM

Run	Enzyme Ratio	Enzyme Load (%)	Sub.Mol. Ratio	Time (h)	FAC (%)		FFA (%)	
					Actual	Predicted	Actual	Predicted
1	9	12	3	7	48.29	47.55	58.66	57.41
2	11	10	5	5	41.23	42.01	67.98	67.69
3	5	12	3	7	55.82	54.99	60.91	60.01
4	7	14	5	5	55.90	55.49	69.03	70.17
5	7	10	1	5	42.94	44.94	39.59	42.09
6	9	8	7	3	38.39	39.35	73.32	74.78
7	7	10	9	5	50.61	50.14	78.51	76.75
8	7	10	5	5	50.04	49.59	68.36	69.23
9	7	10	5	9	55.37	54.00	67.16	69.38
10	5	12	7	3	53.93	53.89	74.45	75.31
11	9	12	7	7	51.25	53.90	74.45	74.88
12	7	10	5	1	40.38	43.29	69.48	68.01
13	9	12	7	3	51.10	48.67	73.32	74.28
14	5	8	3	7	50.74	51.50	60.24	57.97
15	5	12	7	7	57.69	58.39	78.96	77.32
16	7	10	5	5	52.52	49.59	70.01	69.24
17	7	10	5	5	49.82	49.59	68.73	69.23
18	9	12	3	3	43.65	43.81	58.66	57.65
19	5	8	3	3	48.55	46.03	57.08	57.20
20	7	10	5	5	49.50	49.59	68.58	69.23
21	5	8	7	7	51.88	51.85	74.45	76.01
22	3	10	5	5	54.23	54.99	68.88	69.91
23	5	12	3	3	52.17	51.98	59.33	58.85
24	9	8	7	7	48.52	47.04	75.80	74.98
25	7	10	5	5	50.09	49.59	71.82	69.23
26	5	8	7	3	45.81	44.88	74.45	74.40
27	9	8	3	7	43.58	43.75	57.08	56.78
28	7	6	5	5	40.74	42.69	69.03	68.63
29	9	8	3	3	39.92	37.55	57.08	57.42
30	7	10	5	5	45.58	49.59	67.91	69.23

TABLE 8: ANOVA and statistical analysis for FAC and FFA

Response	FAC					FFA				
Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob> F	Sum of Squares	DF	Mean Square	F Value	p-value Prob> F
	quadratic					Quadrati c				
Model	746.91	14	53.35	10.50	< 0.0001[a]	20.26	14	1.44	41.76	< 0.0001[a]
A-Enzyme Ratio	252.79	1	252.79	49.75	< 0.0001[a]	0.01	1	0.01	0.15	0.71[b]
B-Enzyme Load	245.98	1	245.98	48.41	< 0.0001[a]	0.12	1	0.12	3.43	0.08[b]
C-Substrate Molar Ratio	40.53	1	40.53	7.98	0.01[b]	18.64	1	18.64	537.86	< 0.0001[a]
D-Reaction Time	171.76	1	171.76	33.80	< 0.0001[a]	0.17	1	0.17	4.96	0.04[b]
AB	0.10	1	0.10	0.02	0.90[b]	0.01	1	0.01	0.37	0.55[b]
AC	8.70	1	8.70	1.71	0.21[b]	0.00	1	0.00	0.00	0.99[b]
AD	0.53	1	0.53	0.10	0.75[b]	0.01	1	0.01	0.22	0.64[b]
BC	9.35	1	9.35	1.84	0.20[b]	0.01	1	0.01	0.25	0.63[b]
BD	6.06	1	6.06	1.19	0.29[b]	0.01	1	0.01	0.37	0.55[b]
CD	2.23	1	2.23	0.44	0.52[b]	0.05	1	0.05	1.43	0.25[b]
A^2	2.05	1	2.05	0.40	0.54[b]	0.06	1	0.06	1.66	0.22[b]
B^2	0.43	1	0.43	0.09	0.77[b]	0.13	1	0.13	3.80	0.07[b]
C^2	7.20	1	7.20	1.42	0.25[b]	1.01	1	1.01	29.11	< 0.0001[a]
D^2	1.54	1	1.54	0.30	0.59[b]	0.02	1	0.02	0.62	0.44[b]
Residual	76.21	15	5.08			0.52	15	0.03		
Lack of Fit	51.09	10	5.11	1.02	0.53[b]	0.28	10	0.03	0.58	0.79[b]
Pure Error	25.12	5	5.02			0.24	5	0.05		
Cor Total	823.12	29				20.78	29			

R^2 for FAC= 0.91, R^2 for FFA= 0.98

[a] significant at Prob>F less than 0.01

[b] insignificant at Prob>F more than 0.01

There was only 0.01% chances that the model could occur due to noise which implies the quadratic model was suggested significant for both responses based on Table 8. There was 53% and 79% chances that a lack of fit for FAC and FFA respectively, could occur due to noise. Not significant lack of fit is good because we want the model to be fit. The high value of R^2, 0.91 and 0.98, for FAC and FFA respectively indicates that the quadratic model describe a relationship and high correlation between the parameters and the response.

Besides, based on the Table 8, all factors monitored was significant, $p<0.0001$, except for the substrate molar ratio for FAC response. In contrast to FFA response, among those 4 factors determined, only the substrate molar ratio have a significant effect, $p<0.0001$, towards the production of FFA as the final product. Basically, most of the interactions between factors for both responses were found to be insignificant at 99% confidence interval. The final equation derived from the model in terms of coded factors for this reaction as shown in Equation 3 and Equation 4 to predict the expected value.

$$FAC = 49.59 - 3.25A + 3.20B + 1.30C + 2.68D + 0.08AB + 0.74AC + 0.18AD + 0.76BC - 0.62BD + 0.37CD - 0.27A^2 - 0.13B^2 - 0.51C^2 - 0.24D^2 \quad \text{...Equation 3}$$

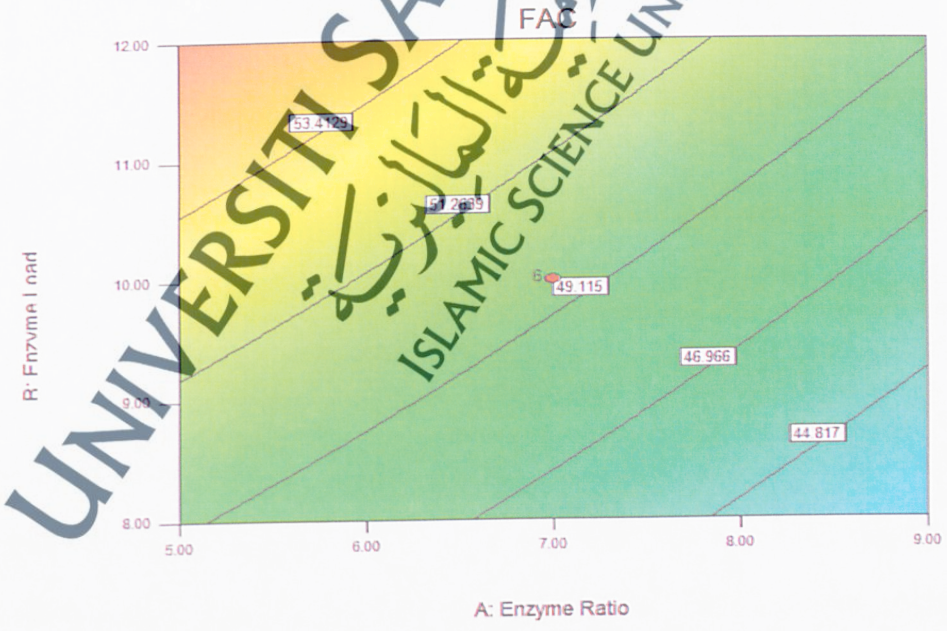
$$FFA = 69.23 - 0.55A + 0.39B + 8.67C + 0.34D - 0.35AB + 0.04AC - 0.35AD - 0.18BC + 0.10BD + 0.21CD - 0.11A^2 + 0.04B^2 - 2.45C^2 - 0.14D^2 \quad \text{...Equation 4}$$

Whereby A was the enzyme ratio, B was the enzyme load, C was the substrate molar ratio and D indicates the time of the reaction.

3.4.2 Effect of parameters on FAC

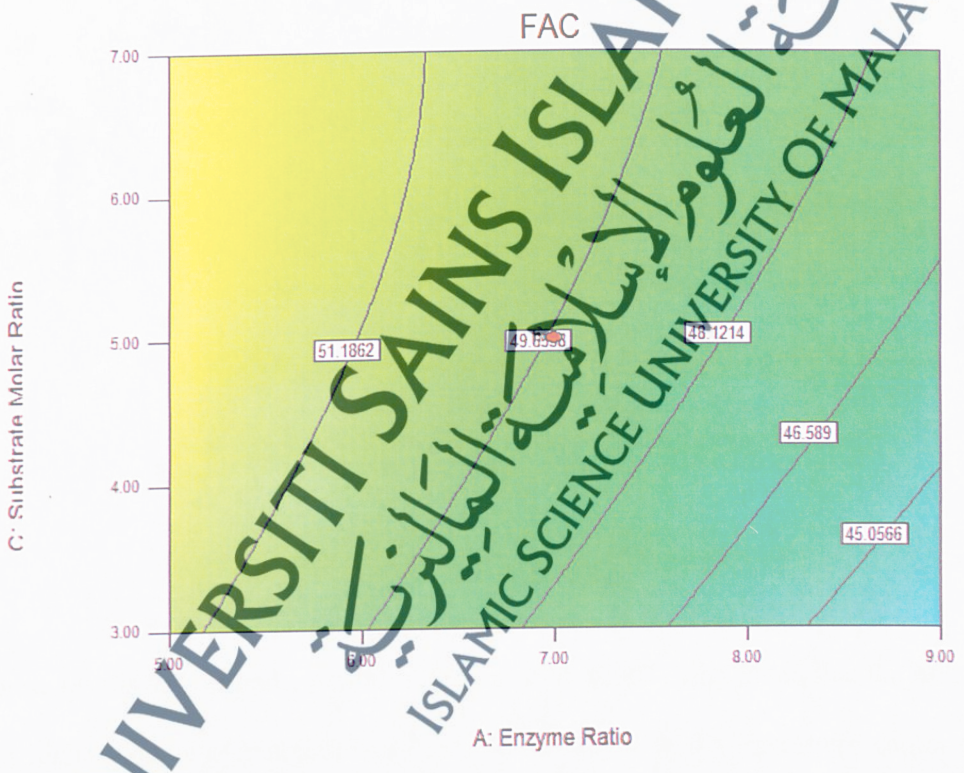
Figure 3 shows that the increase of enzyme load with the lowest of enzyme ratio gives the highest value of FAC or oleic acid. This was in agreement with Namal Senanayake and Shahidi (2002) whereby stated that the amount of incorporation of capric acid increased when increased up to 10% enzyme load. While an increase in enzyme load and enzyme ratio can drop down the FAC value of oleic acid. This indicates that the changes in ratio of lipase AK towards TLIM do not give much to increment of FAC rather than enzyme load however, high ratio of AK in high enzyme load will contribute to lower the FAC value.

FIGURE 3: Effects of enzyme ratio with enzyme load towards FAC



The same pattern result on Figure 3 shown in Figure 4 and Figure 5. The high amount of substrate molar ratio and longer reaction time with low enzyme ratio of AK towards TLIM give the highest value of FAC. The increase in enzyme ratio together prolong the reaction time and added more substrate dropped down the oleic acid value in the final product. This, resulted the same interactions as Figure 3 whereby increase in enzyme ratio may lower the FAC amount, however, increase in other 3 factors, can rise the oleic acid in the final product.

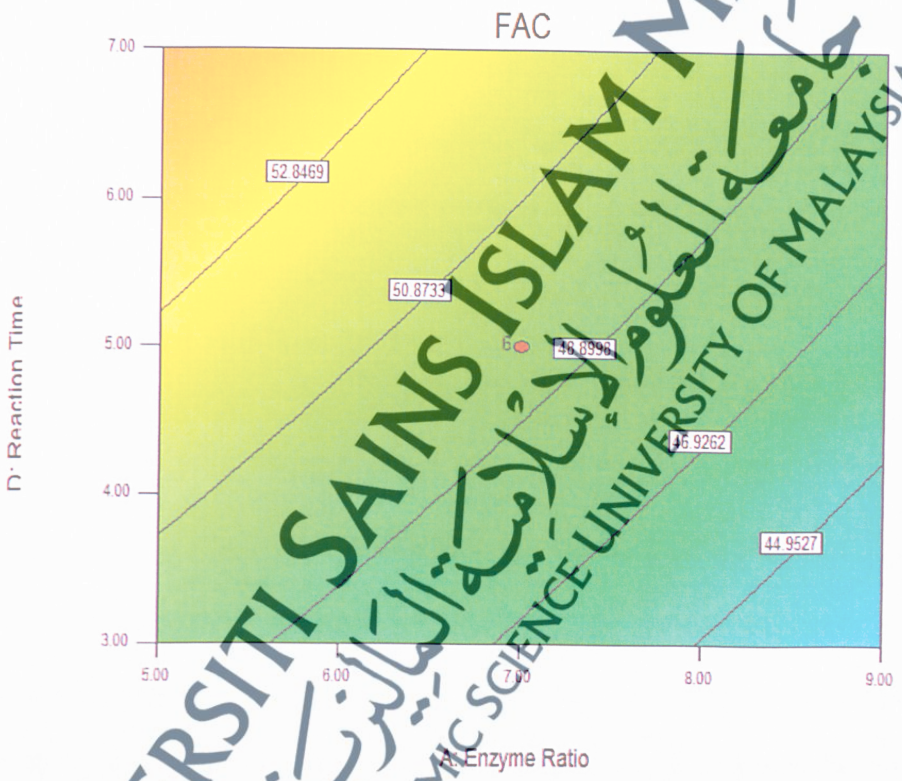
FIGURE 4: Effects of enzyme ratio with substrate molar ratio towards FAC



These pattern in changes of enzyme ratio shows that ratio of AK towards TL IM do give effects towards FAC value when combining with other parameters. The result also shows that high enzyme ratio will contribute to lower the FAC value. Thus, the amount of AK

towards TL IM may have to be in moderate or low so that it will not drop down the FAC value in the final product. Thus, among ratio that have been studied ranging from 5:5, 7:3 and 9:1 (AK to TL IM) a ratio of 5:5 was the best as seen in Figure 3, 4 and 5 whereby it can give the highest value of FAC whenever interact with 3 other factors.

FIGURE 5: Effects of enzyme ratio with reaction time towards FAC



These results are contradictory with Ibrahim et al. (2007) who found that the best ratio to get higher degree of reaction was 7:3, AK to TL IM in the interesterification of palm stearin and coconut oil. These result contradict to each other may due to the different reaction done where Ibrahim et al. (2007) done an esterification between palm stearin and coconut oil while in this research was the acidolysis of palm stearin and oleic acid. Higher

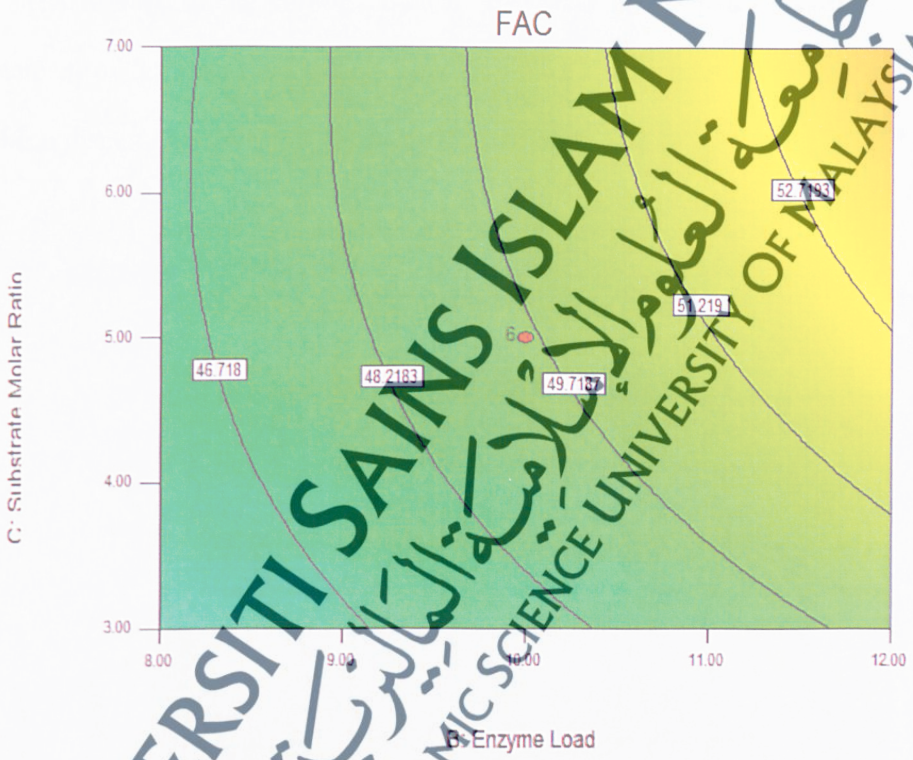
amount of AK than TL IM may needs in the esterification done by Ibrahim et al. (2007) for cutting down triacylglycerols at the *sn*-1,3 position in the palm stearin and coconut oil so that more occurence on rearrangement of fatty acids will happen while in this acidolysis, the TL IM will majorly act as carrier to the lipase AK and enhance the lipase AK activity.

The mode of interaction of dual lipase system implies the idea that the presence of an immobilized enzyme in the reaction can possibly act as a carrier to absorb co-existing free enzyme (Ibrahim et al., 2007). In acidolysis of palm stearin and oleic acid, the same pattern and interactions applied, however, AK focus on cutting down the TAG in palm stearin and more TL IM needed to enhance the activity of AK and act as carrier to the free lipase. The balanced ratio between both lipases was compromising enough to get a maximum amount of FAC of oleic acid within high enzyme load, low substrate molar ratio and in short or longer time reaction. The cooperation between both enzymes enhances the incorporation of oleic acid to be exchanged with the existing fatty acid in the palm stearin, under a supportive condition including an adequate supply of the total enzyme load, the substrate as well as the time reaction. This support the result from Table 8, whereby shows that enzyme ratio gave significant effect towards the FAC value, $p<0.0001$.

Interaction between enzyme load with substrate molar ratio and the reaction time shown in Figure 6 and 7. The graph explains that the higher the enzyme load (12%) together with higher substrate molar ratio (1:7, of palm stearin to oleic acid) and reaction time (7

h) resulted in a higher amount of FAC (above 50% amount of oleic acid may present). This shows that when all parameters were at the maximum value and supply, a greater chance of the exchange and incorporation of oleic acid to the palm stearin occurred, which then produced more FAC of oleic acid.

FIGURE 6: Effects of enzyme load with substrate molar ratio towards FAC



Besides, between these 3 parameters, the high amount of enzyme load (12 %) even with the low substrate molar ratio of oleic acid supply (1:3) as well as less reaction time given (3 h) still can promote high FAC of oleic acid. This result indicates that among these 3 parameters, the total amount of enzyme load plays a crucial role in the reaction. This support by Kadivar et al. (2014) which stated that addition in lipase above 10 wt% does

not increase the amount of stearic-oleic-stearic TAG significantly but might enhance the reaction rate, resulting faster equilibrium. Thus, shorter time needed to complete the reaction under an exact amount of oleic acid supply.

Based on this research result, the cut down of sn-1,3 position at the TAG in palm stearin were enzyme dependent. A high total enzyme with low oleic acid supply in the reaction, gave an advantage to the cutting down of the TAG in the palm stearin so that more replacement of fatty acid can occur. Thus, it shows the amount of the three factors that have been discussed above still produces FAC of oleic acid up to more than 48 %.

FIGURE 7: Effects of enzyme load with reaction time towards FAC

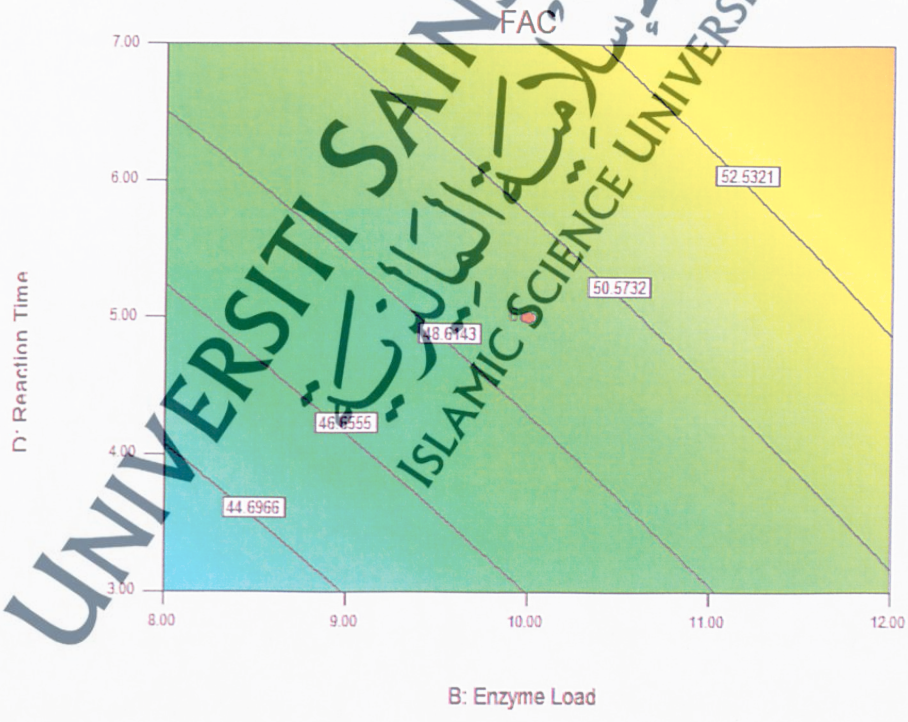
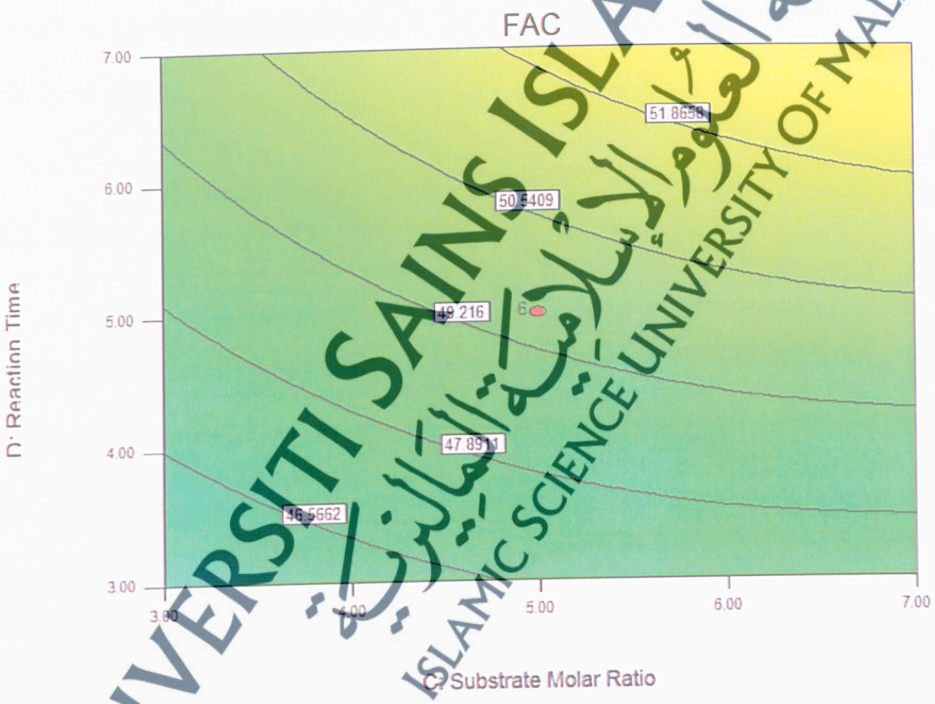


Figure 8 shows that a longer reaction time with a high ratio of oleic acid gives the highest amount of FAC for oleic acid (>50%). Between these 2 factors, however, the time given for the reaction was significant towards producing a high yield of FAC (>49%) even the supply of oleic acid were limited. During the acidolysis, enzymes were added to catalyze the reaction by cutting down the fatty acid chain at a specific sn-1,3 position. The longer the reaction time given, the longer the process took placed and the more chance for oleic acid to exchange.

FIGURE 8: Effect of substrate molar ratio with reaction time towards FAC



A huge difference can be seen whereby the FAC amount was produced less (45-47%), when the reaction time was only 3 hours and a high ratio of oleic acid (1:7) supplied. This contradicts with Wang et al. (2012) in lipase-catalyzed acidolysis of canola oil with

caprylic acid where, the incorporation of caprylic acid was increased by increasing the substrate molar ratio. They found at a 4:1 molar, incorporated caprylic acid was 45.31%.

Thus, for substrate molar ratio, the ratio between palm stearin to oleic acid, 1:3, was sufficient for the reaction to produce an optimum amount of FAC. The excess of free acid as acyl donor may effect other parameters. Kim et al. (2002) state that a high concentration of free acid as acyl donor results in decreased enzyme activity in the acidolysis reaction. Thus, based on the previous paragraphs, among three parameters, high enzyme load gives the best positive effect towards producing high FAC followed by reaction time and enzyme ratio. On the other hand, the substrate molar ratio was best to be fixed at 1:3 ratio.

3.4.3 Effect of parameters on FFA

From the Figure 9, 10 and 11 it shows that there was the same pattern for all 3 graphs. All 3 factors, enzyme ratio, enzyme load and reaction time were oleic acid supply dependant. This happened due to increasing of substrate molar ratio or oleic acid ratio to be exact, resulted on increasing of FFA percentage for all 3 other factors either the factors were in maximum or minimum value itself. The ANOVA analysis explained that only substrate molar ratio was significant, $p<0.0001$, in the model for the interactions between the parameters and the response. As for reaction time, wether it was 3 hours or 7 hours reaction, there is not much differences towards the response. The increase in the substrate molar ratio will increase the FFA no matter how long the reaction time is given. These in

lined with Kadivar et al. (2014), where they stated that higher substrate ratio are not desirable from an economic point of view because they may need an expensive extra separation step. This follows Çiftçi et al. (2009) whereby stated that higher levels substrate mole ratios caused a high level of oil loss during purification.

This result suggests that a longer reaction time on the acidolysis between palm stearin and oleic acid does not increase any undesirable side products. Therefore, a shorter reaction time is sufficient for the acidolysis to complete. In another word, prolong the time does not necessary. Thus, less time is required to perform the whole experiment and more energy can be saved.

FIGURE 9: Effects of substrate molar ratio with enzyme ratio towards FFA

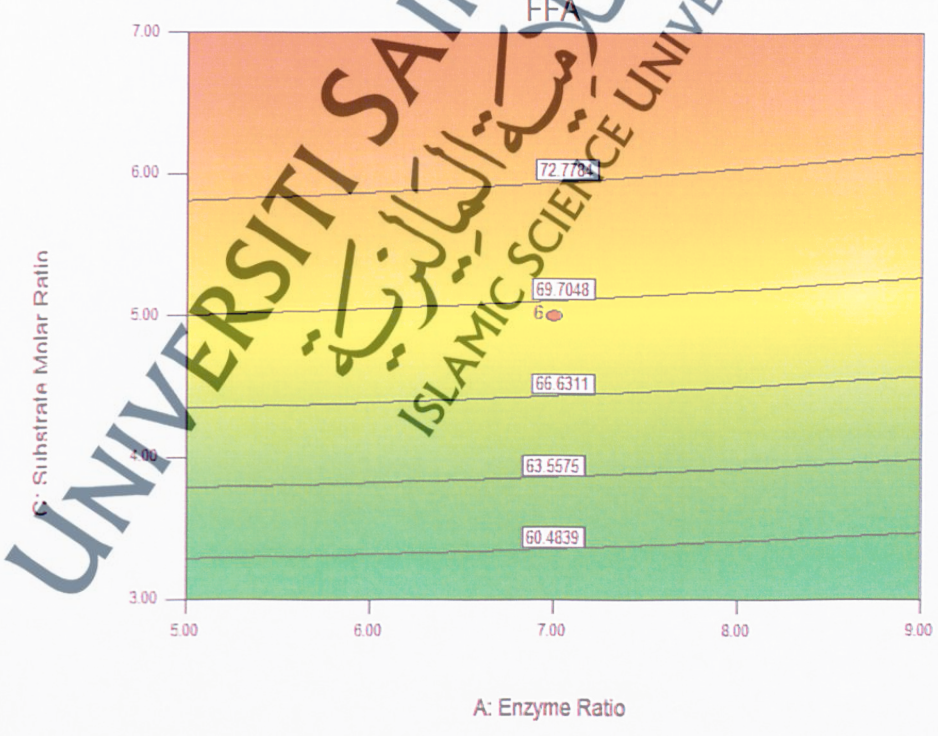


FIGURE 10: Effects of substrate molar ratio with enzyme load towards FFA

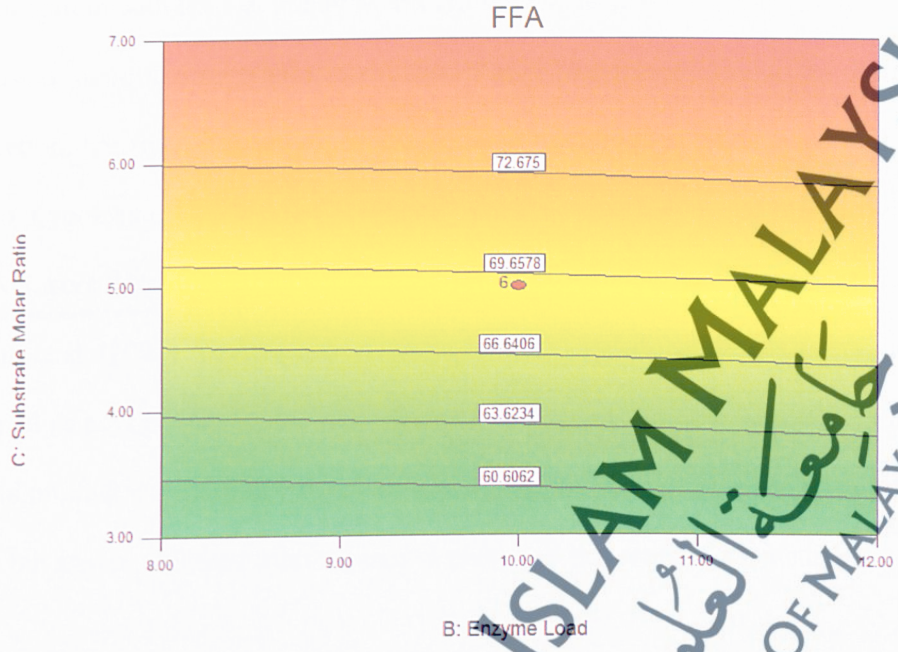
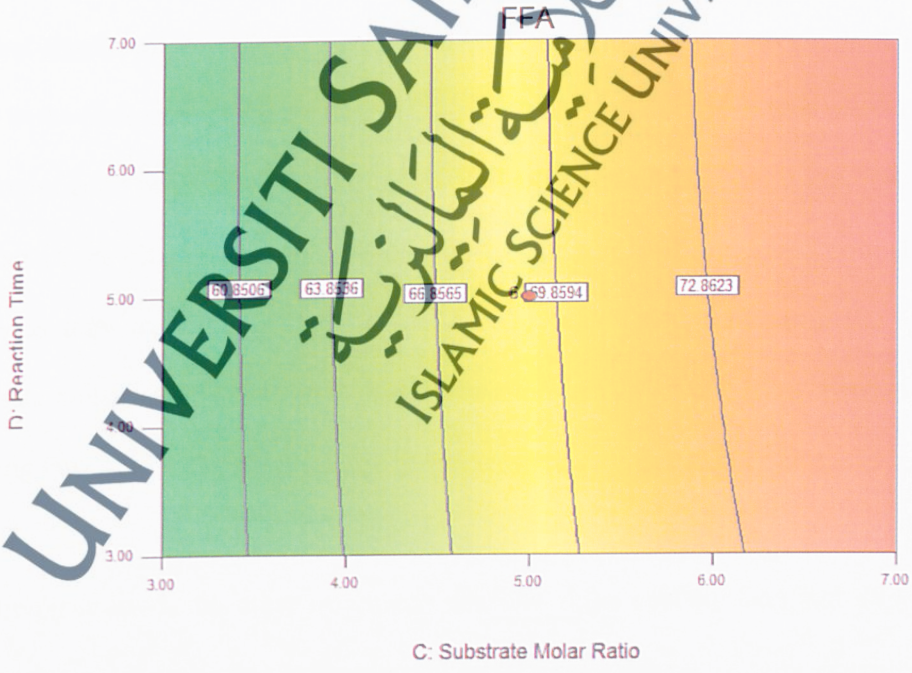


FIGURE 11: Effects of substrate molar ratio with enzyme load towards FFA



Meanwhile, for the enzyme ratio, Figure 9 and enzyme load, Figure 10 both show the similar pattern with the reaction time, Figure 11. The increase of FFA was affected by the increase of oleic acid supplied either with higher or low enzyme load to the reaction. FFA production, however, influenced by the increase or change in the enzyme ratio of lipase AK to Lypozyme TL IM. This happened possibly due to the enzyme activity in the reaction were decreased when congested with a bulk of oleic acid in the blends, as stated by Kim et al. (2002). These group of researchers also added that the high concentration of free acid as acyl donor resulted in a decrease in enzyme activity. Therefore, the FFA as the end product were not only resulted from the exchanged and rearrangement of the fatty acids, but also contributed from the excess oleic acid that remained to be unbound.

As reported in other previous response for the RSM (FAC) the results obtained in this experiment are also showing that the substrate molar ratio is the only parameter which was not significant towards the interactions between all four parameters in producing high FAC in the oleic enriched palm stearin. The oleic acid ratio signifies how much oleic acid was supplied to be exchanged with the present fatty acid in the TAG chain. Thus, higher number in the oleic acid ratio indicates more oleic acid being supplied through out the reactions may resulting in high unbound oleic acid. Oleic acid or in this case, the substrate molar ratio of oleic acid should be in an exact amount to be exchanged with the existing fatty acid in the palm stearin TAG to reduce the formation of FFA.

On the other hand, the other parameters reaction time, enzyme load and enzyme ratio when interact to each other with constant (either one of the 3 factors) or with constant

substrate molar ratio resulted in a little difference in the percentage of FFA. As illustrated in Figure 12 and 13, the increase in the reaction time had increased a small amount of FFA percentage. However, the increment was still within a narrow difference range (67-70%).

A similar pattern of the result was also obtained in the enzyme load and enzyme ratio interaction in Figure 14. The higher load of the enzyme did increase the FFA percentage while the ratio of AK to TL IM should be retained in a balanced amount. For this specific experiment, ratio 5:5 is enough to reduce the FFA content, providing a strong evidence that the ratio does not significant in the formation of more FFA. All graphs in Figure 6 explains the ANOVA and supports only substrate molar ratio gives effects on the formation of high FFA percentage.

FIGURE 12: Effects of reaction time with enzyme load towards FFA

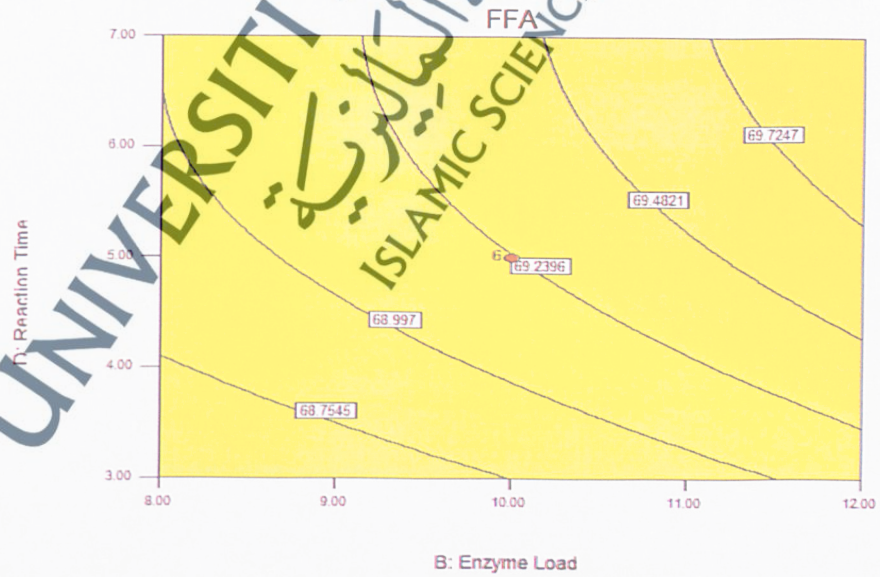


FIGURE 13: Effects of reaction time with enzyme ratio towards FFA

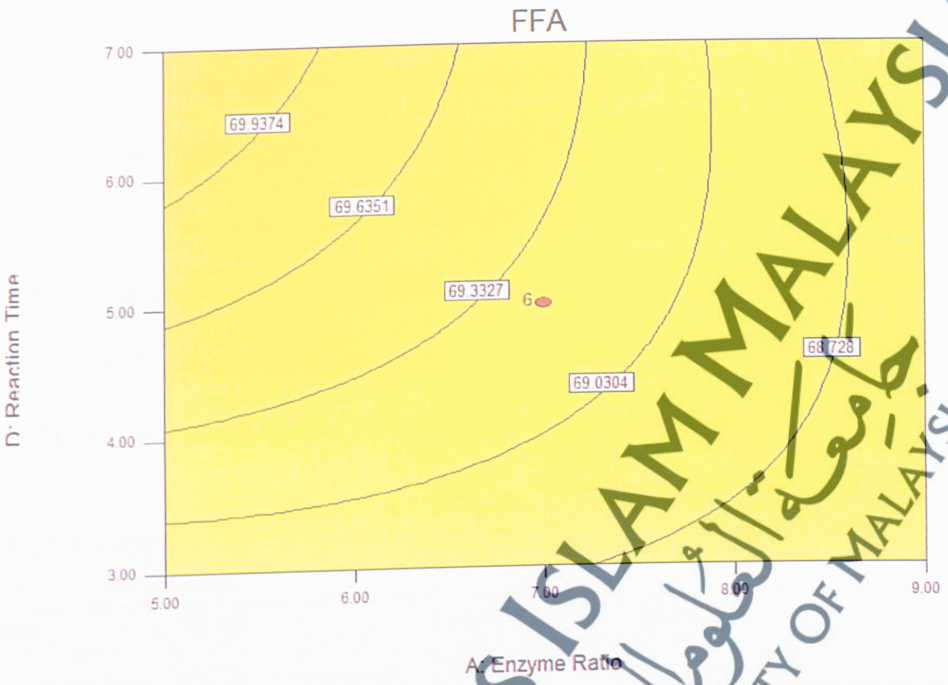
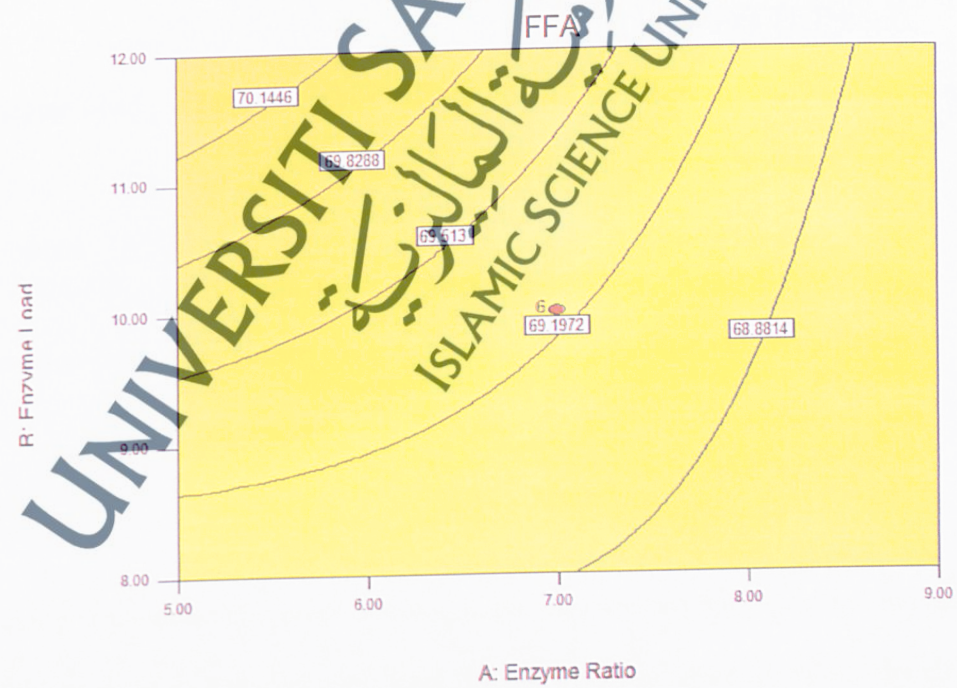


FIGURE 14: Effects of enzyme load and enzyme ratio towards FFA



3.4.4 Optimization and Verification

Optimization process was done by setting the optimum value for each corresponding factor in the reaction as seen in Table 9, with goals to have a maximum value of FAC or oleic acid being incorporated in the modified palm stearin besides having a lower amount of FFA that resulted from the reaction itself. All goals in each factor were justified based on the ANOVA and regression analysis that have been done previously towards the FAC and FFA percentage and also based on the economic perspectives.

TABLE 9: Determining the value of each corresponding factors for optimization and verification.

Name	Goal	Lower Limit	Upper Limit	Reason
Enzyme Ratio	Minimize	5	7	Balanced amount lipase AK to Lypozyme TL IM gives higher FAC. Reduce the cost, where by lipase AK cost more than Lypozyme TL IM.
Enzyme Load	Target = 10.00	8	12	High enzyme load can promote high FAC yet still want to reduce the cost.
Substrate Molar Ratio	in range	3	7	Reduce the FFA %
Reaction Time	minimize	3	7	Short time is sufficient
FAC	maximize	38.39	57.69	
FFA	minimize	39.59	78.96	

Looking at the economic point of view, in the industry, the enzymatic interesterification should be done in a shorter time (less reaction time) in order to reduce the electricity

supply and usage as well as man power. In addition, the less reaction time taken can also reduce the production cost. Even though lipase AK is a little bit more expensive than Lypozyme TL IM, it has a significant role for the reaction where it provides a good result for the combination.

The optimization condition was done in a series of experiments for verification. The actual factors value generated by the optimization function in RSM shown in Table 10. From this table, the predicted and the actual result of the FFA and FAC, both have a range of difference. The actual value from the experiment was lower as compared with the predicted from the model for both responses. This may due to some outliers or possible errors that occurred during the 30 sets of the experiment before. However, the consistency of the result remains good and does not have much dispersion between the data, as shown from the standard deviation for actual value of FAC, 0.53, while the actual FFA% obtained, 0.94.

From the result obtained, however, the lower amount of actual FFA as compared with predicted is favarable since the optimum condition was aimed to reduce the amount of FFA. The lower actual amount of FAC compared to predicted were in acceptable range as a high value of FAC determine the amount of oleic acid present in the TAG or amount of how many oleic acid have been incorporated. It should not exceed 50% of the total fatty acid composition of the whole product as we want to retain the structure of modified palm stearin to be in solid state, yet not as hard as the refined, bleached and deodorized (RBD) palm stearin before blend.

TABLE 10: The predicted and actual value from both responses under optimum condition.

Run	enz. Ratio	enz. load (%)	sub.mol ratio	reaction time (h)	FAC (%)		FFA (%)	
					Actual	Predicted	Actual	Predicted
1	5	10	3	3	44.88	49.26	53.016	57.98
2	5	10	3.04	3	45.65	49.28	51.888	58.22
3	5	10	3.11	3	44.79	49.32	53.016	58.72
4	5	10	3	3.21	44.76	49.52	51.888	58.06
5	5	10	3	3.32	45.86	49.65	50.76	58.10
Std. Dev	-	-	-	-	0.53	0.17	0.94	0.29

3.5 Conclusion

As for this objective, the optimization was successfully done for all four factors which are, enzyme ratio, enzyme load, substrate molar ratio and reaction time. The substrate molar ratio was found to have a significant effect towards the percentage of FFA thus making up the selection goes for a lower substrate molar ratio for optimization. Shorter time reaction given was enough to complete the reaction to achieve a maximum value range of FAC and minimum value range of FFA. As compared to enzyme load, the higher enzyme load to the blends, the higher FAC value obtained, thus resulting in a fixed amount, 10%, for selection in the optimization process. Meanwhile, for the enzyme ratio, cooperation between lipases, lipase AK and Lypozyme TL IM showed a balanced amount from both results in the higher value of FAC. Therefore, the optimum condition for acidolysis of palm stearin and oleic acid was 5:5 lipase AK to Lypozyme TL IM, 10% enzyme load, 1:3 of palm stearin to oleic acid molar ratio, and 3 hours time reaction were

taken. This hereby concludes that quadratic model derived from the RSM can be applied to predict FFA and FAC under any given condition within the experimental values.