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Original Article

Process optimization using response surface design for diacylglycerol synthesis from palm fatty acid distillate by enzymatic esterification

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Abstract

Diacylglycerols (DAG) were synthesized by lipase-catalyzed esterification of glycerol with palm fatty acid distillate (PFAD). Lipase from *Rhizomucor miehei* was used and the reaction was carried out at 45°C. After 24 h, the reaction products were sampled and the DAG content was determined using high performance thin layer chromatography. A response surface methodology (RSM) was used to study the effect of substrate molar ratio (0.75 to 2.25 mol), enzyme concentration (2.0 to 3.0 wt%) and the amount of molecular sieve (25 to 35 wt%) on the DAG yield. An analysis of variance (ANOVA) showed that 84% (R^2 = 0.84) of the observed variation was explained by the polynomial model. The optimum conditions obtained from the response surface analysis were 1.23 mol for the molar ratio between fatty acid distillate and glycerol, 31.1 wt% molecular sieve and 2.32 wt% enzyme. Alkaline neutralization of the esterification products yielded neutralization residues containing approximately 70% DAG.

Keywords: diacylglycerols, palm fatty acid distillate, response surface methodology, lipase, esterification

1. Introduction

Diacylglycerol (DAG) is a glyceride in which the glycerol is esterified with two fatty acids. DAG has two isoforms, 1,2-(or 2,3)-DAG and 1,3-DAG, with a natural isomeric ratio of approximately 3:7 (Kristensen *et al*., 2005a). The 1,2-(or 2,3)-DAG in edible oil is largely converted to the 1,3-isoform by migration of the acyl group during high temperature processing because of its stable form (Matsuo, 2004). DAG has been widely used as an emulsifier in the food industry. Recently, it has been found that 1,3-DAG has a positive effect on human health by reducing fat accumulation (Nagao *et al*., 2000; Murase *et al*., 2001) and preventing obesity (Maki *et al*., 2002). General cooking oil, triacylglycerol (TAG) oil, normally only contains up to 10 wt% DAG. Conversely, DAG oil contains more than 80 wt% DAG (Kristensen *et al*., 2005a; Lo *et al*., 2008). The results from a previous work reported that the thermal stability of DAG oil was not significant different from that of TAG oil (Shimizu *et al*., 2004). Therefore, DAG has a potential to be used as cooking oil in addition to being used as an emulsifier.

Several methods for synthesis of DAG, e.g. glycerolysis, hydrolysis and interesterification, using either separation or a combination of such methods, have been reported (Myrnes *et al*., 1995; Plou *et al*., 1996; Gunstone, 1999; Shimada *et al*., 1999; Weber and Mukherjee, 2004; Kristensen *et al*., 2005a, 2005b; Fregolente *et al*., 2008; Tangkam *et al*., 2008). Another method which is called esterification, uses the reaction of free fatty acids (FFA) with glycerol, and has also been studied using lipase (Berger *et al*., 1992; Li and

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Ward, 1993; Lo *et al*., 2004a, 2004b; Negesha *et al*., 2004; Watanabe *et al*., 2005; Chong *et al*., 2007; Tangkam *et al*., 2008). The fatty acid distillate of edible oils is a cheap by-product of the deodorization process of vegetable oils, which contain lipid components such as FFA, acylglycerols, vitamin E and phytosterols. The fatty acid distillate is generally used as a raw material for the production of soaps and extraction of vitamin E and phytosterols (Lo *et al*., 2004a). Therefore, these products can add value from several different methods for multi-propose products. Palm fatty acid distillate (PFAD), a co-product of the palm oil refining process, is obtained at a rate of about 4% of the total palm oil (Chaiyaso *et al*., 2006). It contains approximately 90% free fatty acids. Thus, it is suitable for use as free fatty acid source for the esterification reaction.

Most DAG synthesis processes are enzymatic catalysis reactions. The enzymatic process advantages over chemical processes since it uses low temperatures, ambient pressures and is able to prevent undesirable by-products (Myrnes *et al*., 1995; Lo *et al*., 2004b; Fregolente *et al*., 2008). Lipases (E.C.3.1.1.3) are enzymes that are used to catalyze hydrolysis and the synthesis of acylglycerols. Regiospecificity of lipases can be classified into non-specific and 1,3-specific lipases. Non-specific lipases can remove an acyl moiety from the *sn*-2 position of the TAG molecule, preferably forming 1,3-DAG or 1(3)-MAG. Therefore, any acyl moiety of TAG molecules can be transesterified to any position in the glycerol. With 1,3-specific lipases, only acyl moiety in the *sn*-1 and *sn*-3 positions of TAG molecules can be transesterified to the *sn*-1 and *sn*-3 positions of the glycerol (Adamczak and Bednarski, 2004; Kristensen *et al*., 2005a). There are many additional factors that can affect DAG synthesis. The effect of a water removal agent, temperature, enzyme specificity, enzyme carrier, enzyme source, enzyme concentration, time and substrate molar ratio have also been reported (Beger *et al*., 1992; Li and ward, 1993; Lo *et al*., 2004a, 2004b; Kristensen *et al*., 2005a, 2005b; Chaiyaso *et al*., 2006; Chong *et al*., 2007; Fregolente *et al*., 2008; Tangkam *et al*., 2008). However, there are few researches that have studied the factors that affect DAG synthesis from PFAD by lipase-catalyzed esterification. Therefore, the objective of this research is to study the factors, substrate molar ratio, enzyme concentration, and amount of molecular sieve, which can affect DAG synthesis from PFAD's using lipase-catalyzed esterification. In order to study the effects of independent variables on DAG production, a central composite design (CCD) with response surface methodology (RSM) was used (Hu, 1999; Montgomery, 2001). A model equation that would predict and determine the optimum conditions for DAG yield was then developed.

2. Materials and Methods

2.1 Materials

Tert-butyl methyl ether (*t*-BuOMe), and molecular sieves 3A (faintly brown and small rod) were products of Fluka (Buchs, Switzerland). Lipase from *Rhizomucor miehei* (23300 LU/G), siliga gel 60, and glycerol were products of Sigma-Aldrich (St. Louis, MO, USA). Analytical and high-performance liquid chromatography (HPLC) grade hexane, diethyl ether, methanol, chloroform, isopropanol, *glacial acetic* acid *and* phosphoric acid were purchased from RCI Lab scan Co., LTD (Bangkok, Thailand). Methyl acetate was a product of CARLO ERBA (Milano, Italy). Potassium *chloride,* cupric acetate and lithium chloride (LiCl) were products of Ajax (New South Wales, Australia). Mono-, Di-, and Tri- olein were products of Restek Corporation (Pennsylvania, USA). HPTLC glass plate siliga gels were purchased from Merck (Darmstadt, Germany). Palm fatty acid distillate (PFAD) was kindly provided by Patum Vegetable Oil Co., LTD (Pathum Thani, Thailand). The crude PFAD mixture was partially purified by dissolving 200 g in 500 ml n-hexane at 45°C, followed by cooling to 4°C until it changed to a solid state. The solid phase was washed several times with chilled n-hexane (8°C), and dried in a desiccator for 1 day (Chaiyaso *et al*., 2006). The original crude PFAD contains approximately 2.1% MAG, 6.8% DAG, 3.5% TAG and 88.2% FFA. After being partially purified, the composition of PFAD was 93.5% FFA, 1.6% MAG and 3.5% DAG. TAG was not detected in purified PFAD.

2.2 Enzyme activity

A spectrophotometric assay with *p-nitrophenyl palmitate* (Sigma-Aldrich, St. Louis, MO, USA) as substrate was measured to determine the lipase activity. A volume of 150 µl lipase was dissolved in 4.35 ml phosphate buffer (50 mM, pH 7). Then the enzyme solution was added to 600 µl of 25 mM, *p-nitrophenyl palmitate in absolute ethanol*. The reaction was carried out at various temperatures (37, 45, 55, 65, 75 and 85°C) for 15 min. After adding Na_2CO_3 1.5 ml (100 mM), the mixture was filtrated at 4°C. The absorbance of the liberated *p-nitrophenol was finally measured* at 420 nm, where one unit (U) was defined as the amount of enzyme that liberated 1 µg *p-nitrophenol* per minute under the test conditions (Becker *et al*., 1997). The highest activity of the enzyme was obtained at 45°C for 467 U/ml. Therefore, the reaction temperature of 45°C was selected for further study of the esterification process.

2.3 Selection of appropriate extraction conditions

Before RSM was applied, approximate conditions for DAG production, namely the amount of molecular sieves, enzyme concentration and substrate molar ratio, were determined by varying one factor at a time while keeping the others constant (Figure 1).

The initial step of the preliminary experiment was to select an appropriate amount of molecular sieves. Four different amounts of molecular sieves (10, 20, 30 and 40 wt%) were examined. The other two factors, enzyme concentration

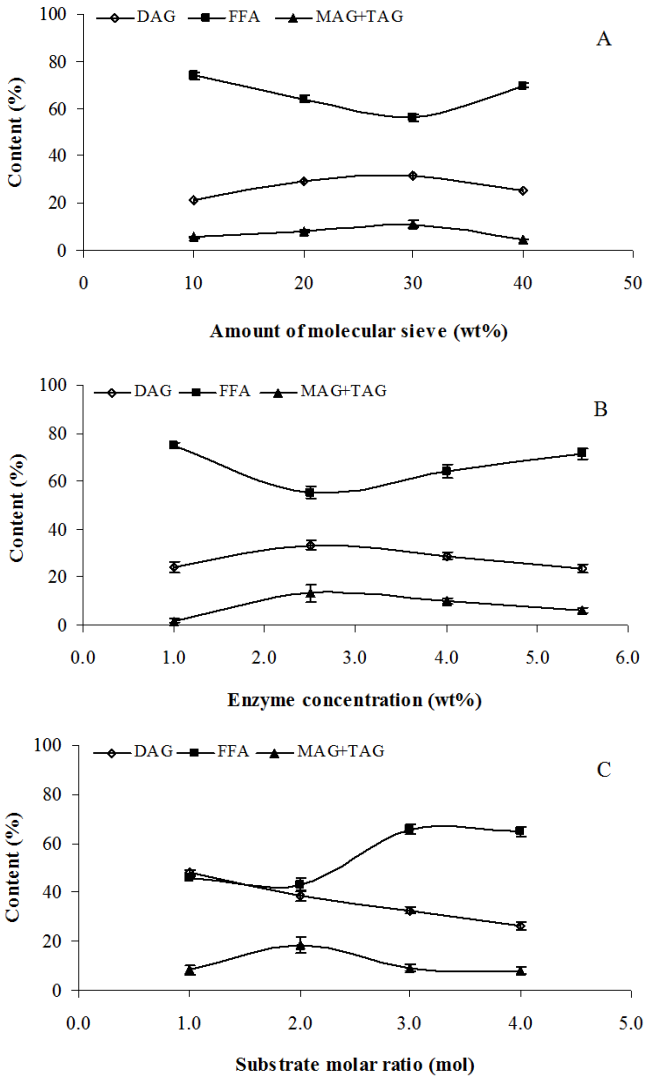

Figure 1. Effect of molecular sieves (A), enzyme concentration (B) and substrate molar ratio (C) on diacylglycerol production.

and substrate molar ratio, were kept constant at 2.5 wt% and 3 mol, respectively. The 0.92 g (1 mol) of glycerol was absorbed on siliga gel (1 g) and then mixed with 7.5 g (3 mol) of purified PFAD dissolved in 20 ml *t*-BuOMe. The water activity of the reactants was controlled at 0.11 by incubation with saturated aqueous salt solution (LiCl) for 2 days at room temperature. The molecular sieves at various amounts (2.36, 4.72, 7.08, 9.45 g) were added and the reaction was started by adding lipase. The reaction was carried out at 45°C on a shaker at 110 rpm for 24 h. The reaction products were stored at -20°C until being analyzed.

Based on the degree of DAG synthesis, the optimum amount of the molecular sieves was chosen. The second step of the preliminary experiment was to determine the enzyme concentration. The DAG was produced using the optimum amount of the molecular sieves chosen in the previous step. The enzyme concentrations varied from 1 to 5.5 wt% while holding the substrate molar ratio constant at 3. The final step of the preliminary experiment was to select an appropriate substrate molar ratio for synthesis of DAG. Using the amount of molecular sieves and enzyme concentration from the previous steps, DAG was synthesized under various substrate molar ratios ranging from 1 to 4. Based on these results the three levels (lower, middle, upper) of each process variable were determined for RSM.

2.4 Experimental design and statistical analysis

A three-factor and three-level central composite design with 17 individual design points was adopted for this study (Myers and Montgomery, 1995; Montgomery, 2001). The independent variables (X_i) and their levels are presented in Table 1. To avoid bias, 17 runs were performed in a totally random order. The independent variables, or factors studied, were the substrate molar ratio (fatty acid distillate/glycerol; X_1) the concentration of enzyme (wt%; X_2) and amount of molecular sieves (wt%; X_3). The response or dependent variable (Y) studied was DAG conversion (%). Duplicate experiments were carried out at all design points.

The effect of these independent variables X_1, X_2 and X_3 on the response Y was investigated using the second-order polynomial regression equation with backward elimination. This equation, derived using RSM for the evaluation of the response variables, is as follows:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum \sum_{i<j=1}^{3} b_{ij} X_i X_J \qquad (1)$$

where b_o, b_i, b_{ii} and b_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively. The X_1, X_2 and X_3 are uncoded values for independent variables. An analysis of variance (ANOVA) was performed to determine the lack of fit and the effect of linear, quadratic and interaction terms on DAG production. The analysis of data

Table 1. Variables (factors) used for central composite design

Independent variables	Symbols	Coded-variable levels		
		-1	0	1
Substrate molar ratio (mol)	X_1	0.75	1.50	2.25
Enzyme concentration (wt%)	X_2	2.0	2.5	3.0
Amount of molecular sieves (wt%)	X_3	25	30	35

and the optimizing process were generated using Minitab statistical software v.15 (Minitab Inc., USA). Model verification was carried out using a combination of variables at different levels within the experimental range.

2.5 Esterification

The water activity of the purified PFAD and glycerol absorbed on the siliga gel were controlled at 0.11 by incubation with saturated aqueous salt solution (LiCl) for 2 days at room temperature. In general, the purified PFAD dissolved in the *t*-BuOMe. (0.38 g : 1 ml). The absorbed glycerol was added to make the molar ratio between fatty acid distillate and glycerol of 0.75 to 2.25 mol. The molecular sieves at various amount (25, 30, and 35 wt%) were then added and the reaction was started by adding lipase (2.0, 2.5, and 3.0 wt%). The mixture was incubated at 45°C in a shaker at 110 rpm. The reaction products were sampled at 24 h and stored at -20°C until they were analyzed.

2.6 DAG purification

The free fatty acid that remained in the reaction mixture was removed by neutralization with NaOH. The reaction mixture was dissolved with 100 ml petroleum ether and titrated with 1N NaOH until reaching the phenolphthalein end-point. Water:ethanol (1:1 v/v) 50 ml was added to the titrate mixture and then shaken and transferred in a separate funnel. The aqueous layer at the bottom was removed and the organic layer was collected. The organic solvent was removed by vacuum evaporation (Lo *et al*., 2004b).

2.7 Analysis of the reaction products

HPTLC plates were pre-developed to full length in hexane:diethyl ether (1:1v/v) before drying under stream hot air. A 10 µl of each lipid sample was spotted to the plates. These plates were then developed to a distance of 4.5 cm using methyl acetate:isopropanol:chloroform:methanol:0.25% (w/v) KCl (25:25:25:10:9 by volume) as the developing solvent. The solvents were evaporated under stream hot air, the plates were then dried in a desiccator over dry NaOH for 30 minutes. After that the plates were developed again in hexane:diethyl ether:glacial acetic acid (80:20:2 by volume) to 9.5 cm. The plates were dipped with 3% (w/v) cupric acetate in 8% (v/v) phosphoric acid solution, followed by charring at 160°C for 20 min. The characterization of DAG was performed on a TLC scanner at 546 nm (Olsen and Henderson, 1989).

3. Results and Discussion

3.1 Selection of factor levels

Figure 1 shows the effect of molecular sieve, enzyme concentration and substrate molar ratio on DAG conversion. By increasing the amount of the molecular sieves up to 30 wt%, DAG significantly increased from 21 to 31% ($p \leq 0.05$). But above 30 wt% molecular sieves, the yield of DAG significantly decreased ($p \leq 0.05$) (Figure 1A). Similar results were reported in previous studies (Lo *et al*., 2004a, 2004b). Therefore, the design points for optimization of DAG production were 25, 30 and 35 wt% molecular sieves. DAG synthesis using 1.0 wt% enzyme was low, but increased with increasing enzyme up to 2.5 wt% (~ 33%). However, with further increasing amounts of enzyme the yield of DAG significantly decreased ($p \leq 0.05$) (Figure 1B). These results are also consistent with other reports (Kristensen *et al*., 2005b; Kim and Lee, 2006; Radazi *et al*., 2011). Thus, the enzyme concentration levels of 2.0, 2.5 and 3.0 were chosen as the lower, middle and upper points, respectively. The substrate molar ratio is another important factor that affects the reaction products. The results for effect of substrate molar ratio (fatty acid distillate:glycerol) showed that when the molar ratio increased from 1:1 to 4:1, the percentage of diacylglycerols significantly decreased ($p \leq 0.05$) (Figure 1C). The three design points selected for substrate molar ratio were 0.75, 1.5 and 2.25.

3.2 Response surface analysis and model evaluation

The data for the response variable Y (% DAG conversion) obtained from the 17 experimental points (Table 2) was used for a statistical analysis to optimize the process variables; substrate molar ratio, enzyme concentration and amount of molecular sieve. The yields of DAG ranged from 32.7 to 46.9% with a mean value of 39.4%. The best-fitting model was determined by multiple regression with backward elimination. The un-significant factors and interactions were also removed from the model. The estimated regression coefficient of the response surface models for the DAG conversion along with the corresponding coefficient of determination values (R^2) and lack of fit test are shown in Table 3. The linear coefficient indicates that the DAG yield is positively correlated with enzyme concentration and the amount of molecular sieve. On the other hand, the quadratic coefficient of all independent variables shows a negative correlation with conversed DAG yield. The R^2 and adjusted R^2 were 0.87 and 0.84 respectively. These results imply that the response surface model can explain more than 84 percent of the variation in the response variables studied. Therefore, the R^2 values of the response models are sufficiently high, to indicate that the response surface model is workable and can be used for estimation of the mean response and the subsequent optimization stages. The lack of fit, which measures the fitness of the model, was found to be non-significant ($p>0.05$), suggesting that the number of experiments were sufficient for determining the effect of independent variables on percentage DAG yield (Montgomery; 2001). As shown in Table 3, the DAG yield was significantly ($p<0.05$) influenced by the main effects of enzyme concentration and molecular sieve and the quadratic effects of all independent variables, as well as the interaction effect. The following response surface model (Eq. (2)) was fit to the three independent vari-

Table 2. Uncoded and coded levels of independent variables in the experimental design and DAG yield (response)

Treatment runs	Substrate molar ratio (X_1, mol)		Enzyme concentration (X_2, wt%)		Molecular sieve (X_3, wt%)		DAG Yield (%)[1]
	Uncoded	Coded	Uncoded	Coded	Uncoded	Coded	
1	0.75	-1	2.0	-1	25	-1	38.0±0.3^{d}
2	2.25	1	2.0	-1	25	-1	35.2±0.3^{b}
3	0.75	-1	3.0	1	25	-1	37.1±1.1^{cd}
4	2.25	1	3.0	1	25	-1	41.6±0.1^{f}
5	0.75	-1	2.0	-1	35	1	45.0±0.3^{i}
6	2.25	1	2.0	-1	35	1	35.6±1.1^{b}
7	0.75	-1	3.0	1	35	1	32.7±2.2^{a}
8	2.25	1	3.0	1	35	1	36.4±0.4^{bc}
9	1.50	0	2.5	0	21.6	-1.68	42.2±0.4^{fg}
10	1.50	0	2.5	0	38.4	+1.68	40.2±0.4^{e}
11	1.50	0	1.66	-1.68	30	0	38.4±0.6^{d}
12	1.50	0	3.34	+1.68	30	0	39.8±0.4^{e}
13	2.76	+1.68	2.5	0	30	0	35.1±0.5^{b}
14	0.24	-1.68	2.5	0	30	0	40.3±0.8^{e}
15	1.50	0	2.5	0	30	0	43.1±1.0^{gh}
16	1.50	0	2.5	0	30	0	43.8±0.9^{hi}
17	1.50	0	2.5	0	30	0	46.9±0.5^{j}

[1] Means ± standard deviation having the same letters are not significantly different at the 5% level

Table 3. Regression coefficients and p-value of the response surface models and statistical analysis

Regression Term	DAG yield (Y, %)	
	Coefficient	Probability (p-value)[1]
Intercept (b_0)	-122.76	0.000
Enzyme concentration (b_2)	58.015	0.000
Molecular sieve (b_3)	5.846	0.000
Substrate molar ratio^2 (b_{11})	-4.693	0.000
Enzyme concentration^2 (b_{22})	-8.798	0.000
Molecular sieve^2 (b_{33})	-0.059	0.000
Substrate molar ratio x Enzyme concentration (b_{12})	7.338	0.000
Substrate molar ratio x Molecular sieve (b_{13})	-0.185	0.000
Enzyme concentration x Molecular sieve (b_{23})	-0.852	0.000
R^2	0.87	-
R^2 (adj)	0.84	-
Regression	-	0.000
Lack-of-Fit	-	0.942

[1] The p-value more than 0.05 is not significantly different at the 5% level

ables (X_1, X_2 and X_3) of the response variables (Y):

$$Y = -112.76 + 58.015X_2 + 5.846X_3 - 4.693X_1^2 - 8.798X_2^2 - 0.059X_3^2 + 7.338X_1X_2 - 0.185X_1X_3 - 0.852X_2X_3 \quad (2)$$

3.3 Effect of the independent variables on percentage DAG

The response surface plot of DAG yield from various combinations of substrate molar ratio, enzyme concentration and the amount of molecular sieve is shown in Figure 2. The

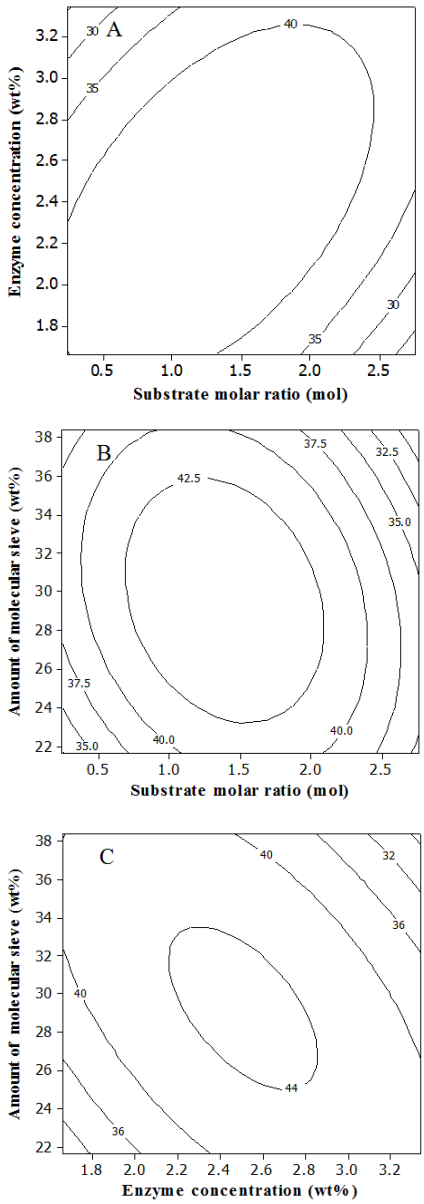

Figure 2. Contour plots of DAG responses showing interactions of the independent factors: enzyme concentration and substrate molar ratio (A), molecular sieves and substrate molar ratio (B), and molecular sieves and enzyme concentration (C)

interaction between substrate molar ratio and enzyme concentration significantly affected the DAG yield. These results indicate that the effect of substrate molar ratio on DAG yield changed when enzyme concentrations were changing and the effect of enzyme concentration also changed when substrate molar ratio was changing (Figure 2A). At low levels of substrate molar ratio (fatty acid distillate / glycerol < 0.75 mol), increasing enzyme concentration did not affect DAG yield until enzyme concentrations increased to more than 2.5 wt%, when DAG yield then decreased.At high levels of substrate molar ratio (> 2.25 mol), at the first period, the increased enzyme concentrations increased the maximum yield of DAG. When enzyme concentrations were increased to more than 3.0 wt%, DAG yield also decreased. Increasing the enzyme concentration did not increase yield but might increase the reaction rate (Kristensen *et al*., 2005b; Fregolente *et al*., 2008). Large amounts of enzyme may have caused the hydrolysis of glycerides and a poorer mixing of the reaction resulting of increased free fatty acid and decreased yield (Kristensen *et al*., 2005b). Moreover, none of their active sites are exposed to the substrates and resulted in the molecules of the enzyme tending to aggregate together. However, small amounts of enzyme may have been insufficient for complete substrates conversion within the specified reaction period (Radazi *et al*., 2011).

The interaction between substrate molar ratio and the amount of molecular sieve also significantly affected the DAG yield (Figure 2B). At low levels of the substrate molar ratio (fatty acid distillate / glycerol < 0.75 mol), increasing the amount of molecular sieve causes increased DAG yield up to the maximum. After that the increased molecular sieve (>30 wt%), DAG yield was decreased. Similar results were found at high substrate molar ratios (> 2.25 mol), and these results showed that the yield of DAG slightly increased with increased the amount of the molecular sieve. When molecular sieve concentration was continuously increased, the yield of DAG decreased. For increased or decreased molar ratios, the percentage of DAG decrease may be due to the amount of fatty acids in the reaction mixture exceeding the amount required for optimal DAG production (Lo *et al*., 2004a, 2004b). Similar results were reported for the lipase-catalyzed production of mono- or dipalmitoylglycerol in hexane (Kwon *et al*., 1995). The optimum pH for the enzyme was 7. Under acidic conditions, *Rhizomucor miehei* lipase activity was lower than that under alkaline conditions (Wu *et al*., 1996). The pH of the reaction mixture gradually decreased with increased molar ratio. This may further affect the enzyme activity.

Furthermore, the interaction between the amount of molecular sieve and enzyme concentration also significantly affected the DAG yield (Figure 2C). At low levels of molecular sieve (< 25 wt%), the yield of DAG increased with increasing enzyme concentrations. When the enzyme concentration increased to more than 2.5wt%, it did not affect the DAG yield, whereas at high levels of the amount of the molecular sieve (>35 wt%), the increase in enzyme concentrations did not affect the DAG yield until the enzyme concentrations were increased to more than 3.0 wt%, leading to a decrease in DAG yield. Lipase-catalyzed reactions are reversible and controlled by the water content of the reaction mixture. In general, adding the proper amount of molecular sieves, water removal agents, is able to improve the degree of esterification and provide a higher synthesis rate. However, a small amount of water is needed to maintain enzyme activity (Li and ward, 1993; Gulati *et al*., 2003; Lo *et al*., 2004a, 2004b; Nagesha *et al*., 2004; Chaiyoso *et al*., 2006; Chong *et al*., 2007; Fregolente *et al*., 2008). At higher water contents this

can cause the hydrolysis of glycerides, therefore the degree of synthesis is decreased. The hydrolysis increased the amount of FFA. The addition of molecular sieves shifted the equilibrium towards ester synthesis and prevented hydrolysis by absorbing excess water in the system. Moreover, there are also other methods for the removal of water formed during the reaction. The water can be removed by reducing pressure (Weber and Mukherjee, 2004; Lo *et al.*, 2008; Tangkam *et al.*, 2008).

3.4 Model validation and optimization

The adequacy of the response surface equations was demonstrated by a comparison between the experimental values and the predicted values based on a response regression (Mirhosseinia *et al.*, 2008). Model verification was carried out using combinations of variables at different levels within the experimental range. Under various conditions, the mean experimental value for DAG yield was not significantly different ($p>0.05$) from the predicted value (Table 4). The high correlation coefficients (≈0.903, Figure 3) also confirmed that a close agreement between experimental data and the predicted values calculated using the models had been obtained. The closer the experimental and predicted results, the better they explain the adequacy of the regression equation (Rossa *et al.*, 2011). Response optimizations were performed to measure the optimum levels of independent variables, substrate molar ratio, amount of molecular sieve and amount of enzyme, required to achieve the desired DAG yield. The process providing the maximal DAG yield would involve samples with low substrate molar ratio and with middle enzyme concentration and molecular sieve (Figure 2). To determine the exact optimum points for all the independent variables necessary to achieve the optimized condition, a numerical optimization was utilized. The results showed that the esterification using the ratio between free fatty acid distillate and glycerol of 1.23 mol containing 31.1 wt% molecular sieve and 2.32 wt% of enzyme provided the maximal DAG yield. Under these optimum conditions, the predicted DAG yield was 44.9%.

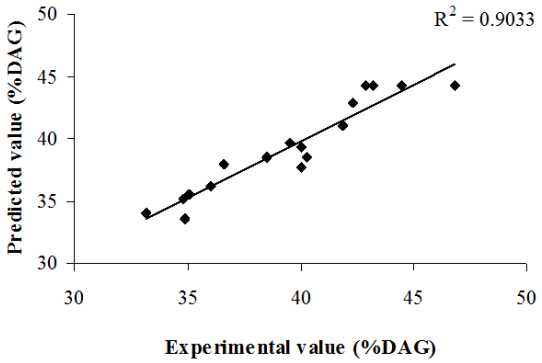

Figure 3. Relationship between the experimental and the predicted DAG responses

3.5 Product purification

The DAG from palm oil distillate was further partially purified by removal of FFA. After alkaline neutralization, the DAG yield could be increased up to 70% and FFA composition in the final product was reduced to less than 13%. The partially purified DAG had a pale yellowish color and was semisolid at room temperature. The major fatty acids in DAG product were palmitic acid (71.5%), oleic acid (23.3%) and stearic acid (4.2%), which were not significantly different from initial PFAD ($p>0.05$).

4. Conclusions

The palm fatty acid distillate is a typical co-product of the palm oil industry, which can be used successfully as a substrate for production of DAG. RSM was successfully applied to model and optimize lipase-catalyzed esterification to produce the DAG. A molar ratio between fatty acid distillate and glycerol of 1.23, molecular sieve of 31.1 wt% and enzyme concentration of 2.32 wt% provided the maximum predicted DAG yield of 44.9%. After removal of FFA, the DAG produced in this study has a DAG and TAG composition of approximately 70% and 15%, respectively.

Table 4. DAG yield from combination of variables at different levels within the experimental range for model verification

Independent variables			DAG Yield (%)	
Substrate molar ratio (X_1, mol)	Enzyme concentration (X_2, wt%)	Molecular sieve (X_3, wt%)	Experimental value*	Predicted value*
2.00	2.7	33	40.50±1.66	41.1
2.25	2.3	28	38.76±2.88	39.3
2.25	2.9	34	38.43±2.59	38.0

* not significantly different at the 5% level

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