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Production of a diacylglycerol-enriched palm olein using lipase-catalyzed partial hydrolysis: Optimization using response surface methodology

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Abstract

Partial hydrolysis using Lipozyme RMIM lipase in a solvent-free system was used to produce a diacylglycerol (DAG)-enriched palm olein. Response surface methodology (RSM) was applied to model and optimize the reaction conditions namely water content (30–70 wt% of enzyme mass), enzyme load (5–15 wt% of oil mass), reaction temperature (45–85 °C) and reaction time (6–16 h). Well fitting models were successfully established for both DAG yield ($R^2 = 0.8788$) and unhydrolysed triacylglycerol (TAG) ($R^2 = 0.8653$) through multiple linear regressions with backward elimination. Chi-square test indicated that there were no significant ($P > 0.05$) differences between the observed and predicted values for both models. All reaction conditions had positive effects on DAG yield and negative effects on unhydrolysed TAG. Optimal reaction conditions were: 50 wt% water content, 10 wt% enzyme load, 65°C of reaction temperature and 12 h of reaction time. The process was further up-scaled to a 9 kg production in a continuous packed bed bioreactor. Results indicated that upscaling was possible with a similar DAG yield (32 wt%) as in lab scale. Purification of the DAG oil using short path distillation yielded a DAG-enriched palm olein with 60 wt% DAG and 40 wt% TAG which is suitable for margarine, spread or shortening applications.

Keywords: Partial hydrolysis; Diacyglycerol; *Rhizomucor meihei*; Optimization; Response surface methodology; Palm olein; Central composite rotatable design (CCRD)

1. Introduction

Diets high in fats and oils are risk factors for obesity and heart diseases (Katsuragi et al., 2004). Professional and public health organizations have recommended reduced consumption of high fats and oils diets to decrease the prevalence of obesity and heart diseases. However, fats and oils are also important sources of energy, essential fatty acids and fat-soluble vitamins (Yang, Zhang, Mu, Sinclair, & Xu, 2004). Thus, recommendations and guidelines on the intake of good fat and a balancing of types of fats have recently been issued (Katsuragi et al., 2004).

Diacylglycerols (DAGs) are esters of glycerol in which two of the hydroxyl group are esterified with fatty acids (FAs). They present in two different isomeric forms which are 1,2(2,3) DAG and 1,3 DAG. It occurs as a natural

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component of acylglycerols in various fats and oils at levels up to 10% (w/w) (Katsuragi et al., 2004). DAG is also commonly used as emulsifiers and stabilizers in food, cosmetics and pharmaceutical industries. The latest application of DAG is being marketed as a functional cooking oil in Japan and in United States (Flickinger & Matsuo, 2003).

Studies on both animals and humans have shown the beneficial health effects of DAG. Although DAG has similar digestibility and energy value as triacylglycerol (TAG), it has the ability to decrease postprandial lipids level (Taguchi et al., 2001; Taguchi et al., 2000; Yamamoto et al., 2001). Consumption of DAG is also shown to reduce body weight and accumulation of visceral abdominal fat (Maki et al., 2002; Nagao et al., 2000). It is suggested that the beneficial health effects of DAG is due to differences in digestion and absorption of TAG and DAG (Kondo, Hase, Murase, & Tokimitsu, 2003).

DAG can be produced enzymatically through esterification (Berger, Laumen, & Schneider, 1992; Lo, Baharin, Tan, & Lai, 2004; Lo, Baharin, Tan, & Lai, 2004; Watanabe et al., 2003) and glycerolysis (Kristensen, Xu, & Mu, 2005a, 2005b; Yamane, Kang, Kawahara, & Koizumi, 1994) processes. In lipase-catalyzed esterification, DAG is synthesized through esterification of FA and glycerol with simultaneous removal of water. Glycerolysis, on the other hand, involved removal of an acyl moiety from the TAG molecule and by acylation of monoacylglycerol (MAG) formed during reaction. Selection of enzymes, particularly their carrier materials, is crucial to avoid clumping of enzyme particles during glycerolysis reactions. Besides esterification and glycerolysis, DAG can also be produced through partial hydrolysis (Plou, Barandiaran, Calvo, Mallesteros, & Pastor, 1996). Purification is an essential process to increase the DAG yield in any product. In the production of DAG, it is advantageous to have higher amounts of MAG while the TAG is simultaneously reduced, since it is easier to separate MAG and DAG than DAG and TAG. Besides, the high MAG formed in the reaction can be separated and sold as food emulsifiers or reused again in the production of DAG (Kristensen et al., 2005a, Kristensen, Xu, & Mu, 2005b).

Palm oil ranks first in the worldwide production of edible oils (Malaysian Palm Oil Board, 2006). Because of its high content of saturated FAs, palm oil and its fractions such as palm olein, has received negative consumer perceptions. The potential health benefits of a DAG-enriched palm olein will improve the uses of palm olein in various food applications. Furthermore, following lipase-catalyzed partial hydrolysis of palm olein, the remaining oil obtained after MAG removal will have a high DAG and low TAG composition. The final product, which is the healthier DAG-enriched palm olein can then be incorporated in the formulations of margarine, shortening and spreads without the necessary removal of TAG. So far, to the best of our knowledge, the use of partial hydrolysis using Lipozyme RMIM lipase to produce a DAG-enriched palm olein has received little attention.

The aim of this study was to model and optimize partial hydrolysis of palm olein using Lipozyme RMIM lipase to produce a DAG-enriched oil by considering both DAG yield and unhydrolysed TAG. Optimization is done through response surface methodology (RSM). RSM has several advantages as compared to the classical optimization method as it enables observations of interaction effects of multiple parameters on the response. It also offers a large amount of information from a small number of experiments (Myers & Montgomery, 2002). In this study, four reaction conditions namely reaction temperature, reaction time, water content and enzyme load were optimized to produce a DAG-enriched palm olein. Subsequently, the optimized reaction conditions were applied in a 9 kg production using a continuous packed bed bioreactor. The final reaction products were purified using short path distillation (SPD).

2. Materials and methods

2.1. Materials

Palm olein (iodine value, 56 g of I_2/100 g of oil) was provided by Jomalina Sdn. Bhd. (Tanjung Panglima Garang, Selangor, Malaysia). Commercial immobilised lipase from *Rhizomucor miehei* (Lipozyme RMIM) was obtained from Novozymes A/S (Bagsvaerd, Denmark). All chemicals and solvents used were either of analytical or high-performance liquid chromatography (HPLC) grade, respectively.

2.2. Experimental design

A central composite rotatable design (CCRD) consisting of four variables was used in this study. The four variables and their levels were water content (Wa, 30–70 wt% of enzyme mass), enzyme load (En, 5–15 wt% of oil mass), reaction temperature (Te, 45–85 °C) and reaction time (Ti, 6–16 h). This design generated a total of 30 experiments with different settings by using Design Expert release 6.0.6 software (Minneapolis, Minnesota, USA) (Table 1). All experiments were carried out in a randomized order to minimize the effect of unexplained variability in the observed response due to extraneous factor.

2.3. Partial hydrolysis of palm olein

Partial hydrolysis of palm olein at different reaction conditions were performed in 250 ml screw-capped round bottomed flask. Firstly, oil bath was heated to the desired temperature. The flask containing immobilized lipase was then heated and maintained at the desired temperature by using the oil bath. Desired water content was then added to the flask and gently stirred by using magnetic stirrer. Finally, a total of 50 g of palm olein was added to the reaction mixture. The reaction was stopped by filtering the lipase by using a two layer cheese cloth. Two milliliters of the reaction mixture were then withdrawn for determination of acylglycerol composition.

Table 1
Central composite design and responses for the lipase-catalyzed partial hydrolysis to produce a DAG-enriched palm olein

Exp. no.	Factors[a]				Responses (wt%)	
	Wa (%)	En (%)	Te (°C)	Ti (hour)	$DAG_{(y)}$	$_{(un)}TAG$
1	60.00	7.50	55.00	8.50	29.46	31.92
2	40.00	7.50	55.00	8.50	26.95	48.35
3	60.00	7.50	75.00	13.50	30.31	33.55
4	60.00	7.50	75.00	8.50	29.33	34.18
5	60.00	12.50	55.00	8.50	31.99	33.72
6	50.00	10.00	85.00	11.00	29.37	34.94
7	40.00	12.50	75.00	13.50	30.88	27.22
8	60.00	12.50	55.00	13.50	30.41	27.41
9	40.00	12.50	55.00	8.50	30.2	31.32
10	50.00	10.00	65.00	6.00	24.45[b]	55.89[b]
11	40.00	12.50	75.00	8.50	29.93	26.07
12	40.00	7.50	75.00	8.50	29.69	35.33
13	70.00	10.00	65.00	11.00	30.2	28.9
14	60.00	7.50	55.00	13.50	28.64	31.59
15	50.00	10.00	65.00	11.00	33.02	25.46
16	50.00	10.00	45.00	11.00	28.31	42.51
17	50.00	10.00	65.00	11.00	32.50	27.18
18	60.00	12.50	75.00	8.50	29.32	32.67
19	50.00	10.00	65.00	11.00	32.01	29.44
20	50.00	10.00	65.00	11.00	30.82	26.91
21	40.00	12.50	55.00	13.50	29.63	35.16
22	50.00	15.00	65.00	11.00	32.00	24.92
23	50.00	5.00	65.00	11.00	27.16	47.04
24	50.00	10.00	65.00	11.00	31.74	28.45
25	40.00	7.50	55.00	13.50	28.37	42.24
26	30.00	10.00	65.00	11.00	28.16	35.24
27	50.00	10.00	65.00	16.00	32.44	20.87
28	50.00	10.00	65.00	11.00	32.26	29.37
29	60.00	12.50	75.00	13.50	31.17	22.63
30	40.00	7.50	75.00	13.50	31.91	29.97

[a] Wa = Water content (wt% of enzyme mass); En = Enzyme load; (wt% of oil mass); Te = Reaction temperature (°C); Ti = Reaction time (h), and $DAG_{(y)}$ = Diacyglycerol yield (wt%); $_{(un)}TAG$ = Unhydrolysed TAG yield (wt%).
[b] Outlier excluded from modeling and optimization.

2.4. Pilot plant production

Ten continuous productions were conducted in a 16 L packed bed bioreactor. The packed bed bioreactor consisted of a 10 L reaction vessel and a 6 L filtration vessel. Optimal amount of immobilized enzyme was packed in the filtration vessel. Optimal water content was then added to the enzyme. A total of 9 kg of palm olein was added to the reactor vessel and heated up to the optimal temperature. Once the oil was heated, it was stirred by using impeller stirrer and introduced into the top of the packed bed filtration vessel by using a centrifugal pump at a flow rate of 850 ml/min. Two millimeters of reaction mixture were withdrawn at determined intervals for analysis of acylglycerol composition. At the end of the reaction time, products were removed from the bottom of the packed bed vessel.

2.5. Purification of DAG

The final product from the partial hydrolysis process consisted of FFA, MAG, DAG and TAG. FFA and MAG can be easily separated from the final product by SPD, but the separation of TAG from DAG is difficult due to the small vapor pressure difference between DAG and TAG (Watanabe et al., 2003). Therefore, TAG is not removed and remained in the final product. To remove FFA and MAG from the reaction mixture, the non varying conditions for the distillation process were: evaporator and degasser vacuum, 0.01 mbar; roller speed, 350 rpm; and condenser temperature, 60 °C. The varying distillation conditions were: evaporator temperature, 175 °C; feed rate, 2.5 l/h and heat exchanger temperature, 80 °C. DAG purity is defined as

$$\text{DAG purity } (\%) = \frac{\text{DAG } (\%)}{[\text{DAG } (\%) + \text{TAG } (\%)]} \times 100$$

2.6. Analysis of acylglycerol composition

The acylglycerol composition was determined using reverse-phase high performance liquid chromatography (RP-HPLC). The RP-HPLC samples were prepared by dissolving 10% of reactant into appropriate amount of acetone. The samples were then filtered through a 0.45 μm nylon membrane filter to remove impurities. Ten microliters of the sample were then injected into a Merck KGaA

(Darmstadt, Germany) LiChrospher® 100 RP-18e 5 μm (250 mm × 4 mm) column using a Shimadzu SCL-10A VP HPLC (Kyoto, Japan) equipped with a Shimadzu Sil-10AD VP auto injector (Kyoto, Japan) and a Shimadzu RID-10A refractive index detector (Kyoto, Japan) under isocratic conditions. The mobile phase comprised of a mixture of acetone and acetonitrile (50:50, v/v) and was set at a flow rate of 1 ml/min. The oven temperature was maintained at 40 °C. Reference standards of commonly found fatty acids and acylglycerols moieties in palm olein (palmitoyl, oleoyl, linoleoyl and stearoyl) were used to determine the range of retention times at which these compounds were eluted. Due to the limitations of the available HPLC instrument, a distinct separation of FFA and MAG elution regions could not be obtained. Thus, these compounds were quantified as a group. The retention time of the sum of FFA and MAG ranged from 2.6 to 5.8 min, while those of DAG and TAG ranged from 5.8 to 9.9 min and 17.5 to 45 min, respectively. The reaction products were analyzed based on their sum of total FFA and MAG, total DAG and total TAG contents. The contents of FFA and MAG, DAG and TAG were expressed as wt% of the total weight of the sample.

3. Results and discussion

3.1. Model fitting

RSM was applied to model the DAG yield [$DAG_{(y)}$] and unhydrolysed TAG [$_{(un)}$TAG], respectively, with four reaction parameters: water content (Wa), enzyme load (En), reaction temperature (Te) and reaction time (Ti). Table 1 shows the levels of $DAG_{(y)}$ and $_{(un)}$TAG at each of the 30 experimental sets generated. Outlier (experiment 10) was excluded in modeling $DAG_{(y)}$ and $_{(un)}$TAG to enhance the fit of the models. The best fitting models were determined through multiple linear regressions with backward elimination whereby insignificant factors and interactions were removed from the models. The accuracy of the models was evaluated by coefficient of determination (R^2 and adjusted R^2 values) and absolute average deviation (AAD) (Bas & Boyaci, 2007). The AAD was calculated using the following equation

$$\mathrm{AAD} = \left\{ \left(\sum_{i=1}^{p} \left(|y_{i,\mathrm{exp}} - y_{i,\mathrm{cal}}| / y_{i,\mathrm{exp}} \right) \right) \Big/ p \right\} \times 100$$

Values of R^2, adjusted R^2 and AAD for $DAG_{(y)}$ and $_{(un)}$TAG were 0.8788, 0.8114, 1.44% and 0.8653, 0.8014, 6.54%, respectively. ANOVA (Table 2) also showed that both regression models for $DAG_{(y)}$ and $_{(un)}$TAG were statistically good with a significance level of $P < 0.0001$ and the models had no significant ($P > 0.05$) lack of fit. Thus, well-fitting models for $DAG_{(y)}$ and $_{(un)}$TAG were successfully established.

3.2. Main effects and interaction between parameters

There are many contributing factors that affect the degree of hydrolysis, such as enzyme concentrations, reaction temperature, reaction time and water content of the reaction mixture. Table 3 shows that $DAG_{(y)}$ was positively affected by all four reaction parameters. Among all four reaction parameters, En had the greatest effect on $DAG_{(y)}$, followed by Te, Wa and Ti. The effect of En on $DAG_{(y)}$ increased from low to medium level and thereafter, further increase in En did not significantly increase the $DAG_{(y)}$ (Fig. 1a). Further increase in En only speeded up the reaction, but did not increase the $DAG_{(y)}$. There are two possible reasons for this. Firstly, DAG which contains a hydroxyl group is more hydrophilic in nature; thus, are more susceptible to hydrolysis as compared to TAG (Katsuragi et al., 2004). As the wt% of DAG in palm olein increased from low to medium level of En, lipase started

Table 3
Regression coefficients and *P*-values for $DAG_{(y)}$ in wt% after backward elimination

Variables[a]	Regression coefficients	*P*-values
Intercept	32.03	<0.0001
Wa	0.30	0.0562
En	0.77	<0.0001
Te	0.38	0.0192
Ti	0.26	0.1218
Wa^2	−0.72	<0.0001
En^2	−0.62	0.0003
Te^2	−0.80	<0.0001
WaTe	−0.48	0.0157
EnTe	−0.55	0.0068
TeTi	0.47	0.0167

[a] Wa = Water content (wt% of enzyme mass); En = Enzyme load; (wt% of oil mass); Te = Reaction temperature (°C); Ti = Reaction time (h), and $DAG_{(y)}$ = Diacyglycerol yield (wt%); $_{(un)}$TAG = Unhydrolysed TAG yield (wt%).

Table 2
ANOVA table for $DAG_{(y)}$ and $_{(un)}$TAG

	Df		SS		MS		*F*-values		*P*-values	
	$DAG_{(y)}$	$_{(un)}$TAG	$DAG_{(y)}$	$_{(un)}$TAG	$DAG_{(y)}$	$_{(un)}$TAG	$DAG_{(y)}$	$_{(un)}$TAG	$DAG_{(y)}$	$_{(un)}$TAG
Model	10	9	66.71	1066.39	6.67	118.49	13.05	13.56	< 0.0001	<0.0001
Residual	18	19	9.20	166.05	0.51	8.74				
Lack of fit	13	14	6.41	153.82	0.49	10.99	0.88	4.49	0.6099	0.0533
Pure error	15	15	2.80	12.23	0.56	2.45				

Df = degree of freedom; SS = sum of squares; MS = mean square.

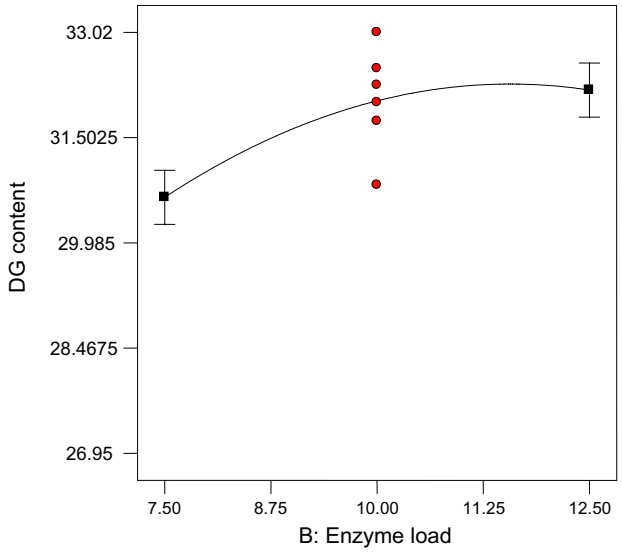

Fig. 1a. Main effect plot showing the effect of En on $DAG_{(y)}$.

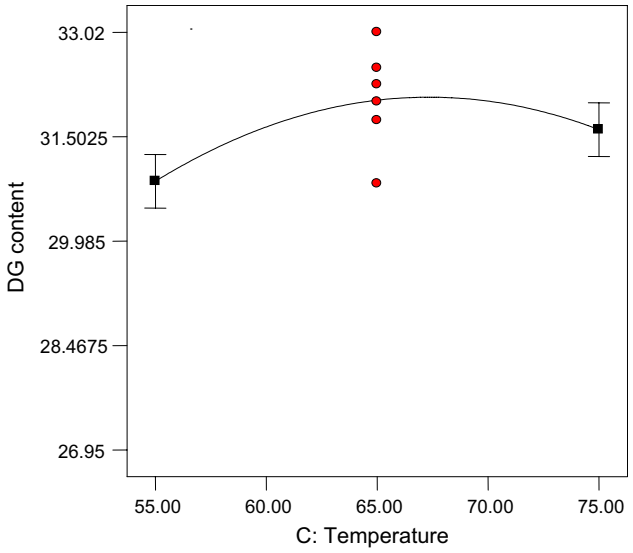

Fig. 1b. Main effect plot showing the effect of Te on $DAG_{(y)}$.

to hydrolyze DAG as well. Thus, $DAG_{(y)}$ did not increase significantly with further increment of En beyond medium level (10 wt%). $DAG_{(y)}$ remained constant until the rate of DAG hydrolysis equilibrated the rate of TAG hydrolysis at approximately 12 wt% of En. After 12 wt% of En, $DAG_{(y)}$ started to decrease as the rate of DAG hydrolysis became greater than rate of TAG hydrolysis. Secondly, the addition of higher amounts of En in a viscous oil system (solvent-free systems) could cause improper mixing of reaction mixtures and thereby promoting limitations to mass transfer (Kristensen et al., 2005a, 2005b). Therefore, for cost reduction and food application purposes, it would be desirable to select a low enzyme load as the cost of enzymes is the most important factor in reducing the production costs.

Reaction temperature plays an important role in enzyme activation/deactivation and thus, also strongly affects the DAG yield. A higher reaction temperature allows a higher rate of DAG formation. Although an increase in temperature enhances the hydrolytic rate, the lipases become more susceptible to thermal deactivation. The optimum temperature recommended by the manufacturer for maximum Lipozyme RMIM lipase activity is between 30 and 70 °C. Fig. 1b shows that the highest DAG yield was obtained at 65 °C which falls within the manufacturer's recommended optimal temperature range.

With P values of more than 0.05, Wa and Ti had a insignificant effect on $DAG_{(y)}$. This is in contrary to other studies on hydrolysis reactions that focus on producing FFA and glycerol but not intermediates MAG and DAG, as in this study. In such cases, where higher hydrolysis is preferred, Wa and Ti are of high significance (Chu, Quek, & Baharin, 2003; Shankar, Agrawal, Sarkar, & Singh, 1997; Wanasundara & Shahidi, 1998). The effect of Wa on $DAG_{(y)}$ increased slightly from low to center level and then decreased slightly to high level of Wa (Fig. 1c). Fig. 1d shows that reaction time had a positive linear effect on

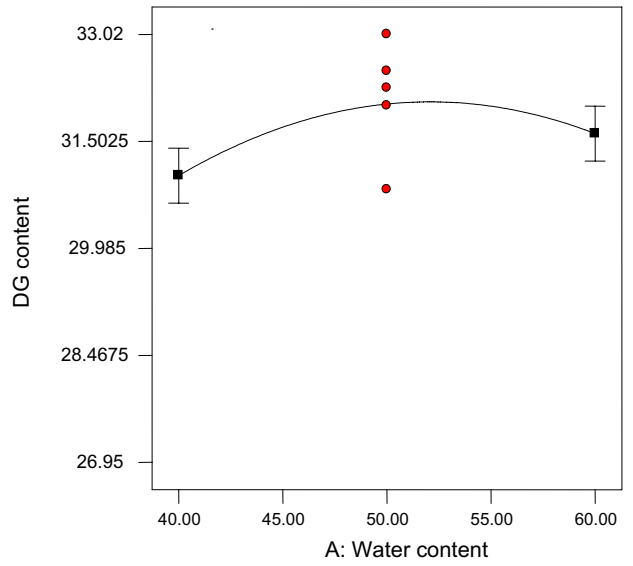

Fig. 1c. Main effect plot showing the effect of Wa on $DAG_{(y)}$.

$DAG_{(y)}$ with a slight increase from low to high level of Ti. Although both Wa and Ti had insignificant effects on $DAG_{(y)}$, they were not removed by backward elimination to maintain the hierarchy of the model. The quadratic terms for Wa, En and Te had negative effects on $DAG_{(y)}$. All four reaction parameters had significant ($P < 0.05$) negative effects on $_{(un)}$TAG, with En having the greatest effect followed by Te, Wa and Ti (Table 4). The quadratic terms for Wa, En and Te had positive effects on $_{(un)}$TAG.

Among the three interaction parameters which had significant effects on $DAG_{(y)}$, interaction between En and Te had the greatest effect on $DAG_{(y)}$ (Table 3). To evaluate the interaction between En and Te, response surface plot was constructed for medium level of Wa and high level of Ti as these levels of parameters had the most significant ($P < 0.05$) effect on $DAG_{(y)}$ (Fig. 2). Based on the response

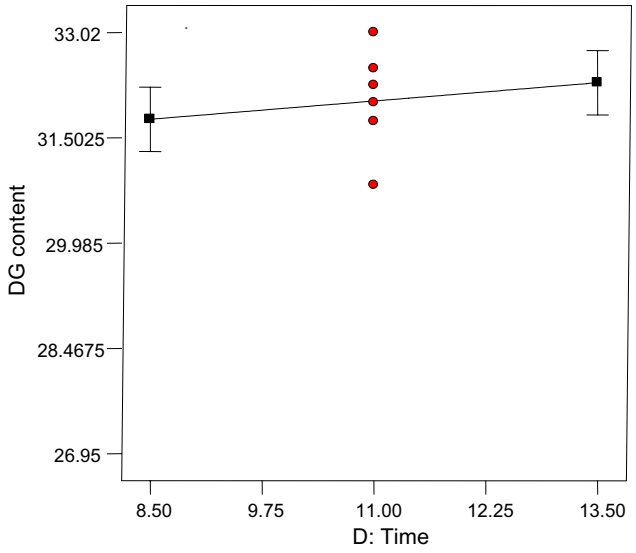

Fig. 1d. Main effect plot showing the effect of Ti on $DAG_{(y)}$.

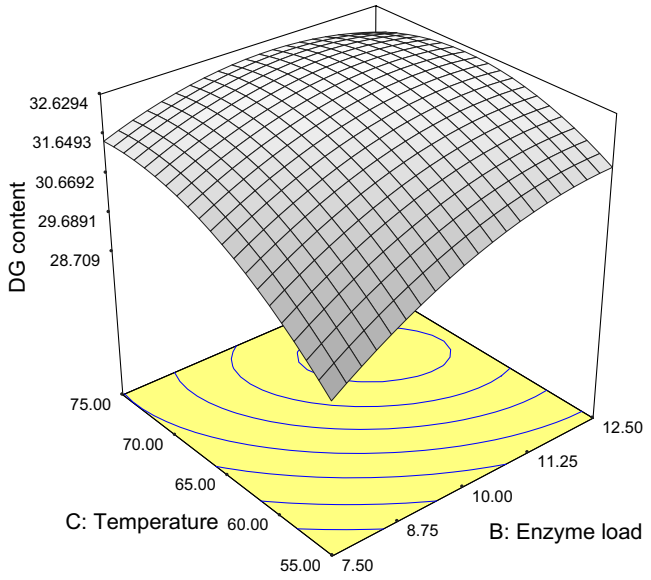

Fig. 2. Response surface plot of interaction between En and Te on $DAG_{(y)}$ at center level of Wa and high level of Ti.

Table 4
Regression coefficients and *P*-values for $_{(un)}$TAG after backward elimination

Variables[a]	Regression coefficients	*P*-values
Intercept	27.15	<0.0001
Wa	−1.69	0.0112
En	−3.97	<0.0001
Te	−2.30	0.0012
Ti	−1.82	0.0138
Wa^2	1.07	0.0760
En^2	2.05	0.0020
Te^2	2.74	0.0001
WaEn	1.33	0.0057
WaTe	2.30	0.0074

[a] Wa = Water content (wt% of enzyme mass); En = Enzyme load; (wt% of oil mass); Te = Reaction temperature (°C); Ti = Reaction time (h), and $DAG_{(y)}$ = Diacyglycerol yield (wt%); $_{(un)}$TAG = Unhydrolysed TAG yield (wt%).

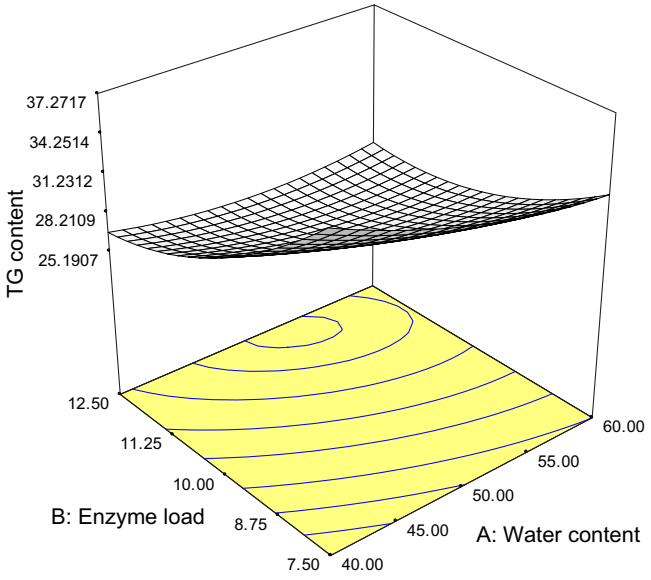

Fig. 3. Response surface plot of interaction Wa and En on $_{(un)}$TAG at center levels of Te and Ti.

surface plot, it is observed that $DAG_{(y)}$ increased at higher En and Te. However, after approximately 12 wt% of En, further increased of En and Te started to decrease $DAG_{(y)}$. As noted previously, DAG has greater hydrolyzability as compared to TAG. Increment of En and Te beyond a certain level decreases the $DAG_{(y)}$. This is due to the fact that rate of DAG hydrolysis has become greater than the rate of TAG hydrolysis.

The most significant interaction parameter for $_{(un)}$TAG was interaction between Wa and En (Table 4). Based on the response surface plot which was constructed for center level of Te and Ti, it is observed that $_{(un)}$TAG decreased at higher Wa and En (Fig. 3). It would therefore appear that the lower the ratio of oil to water, the higher the extent of hydrolysis. Nevertheless, after reaching approximately 53 wt% of Wa, increase in Wa and En did not further decrease $_{(un)}$TAG. This observation is in accordance with Chu et al. (2003) and Shankar et al. (1997) findings. The possible explanation for this is that the absorption of excess water on the hydrophilic macroporous anion resin had completely submerged the lipase resulting in the inability of the substrates to reach the surface bound lipase. Lipase is immobilized on hydrophilic support such as anionic resin. It requires a small hydration layer to increase its catalytic activity as lipase catalyzes reactions at oil–water interface (Paez, Medina, Rubio, Moreno, & Grima, 2003). Nevertheless, instead of forming a thin mono-hydration layer, excess of water content may submerge the lipase totally. Therefore, substrates are unable to reach the lipase molecule and the lipase active sites are unable to access the interface.

3.3. Optimization of partial hydrolysis and model verification

In order to produce a DAG-enriched palm olein, partial hydrolysis was optimized to produce high $DAG_{(y)}$ and low $_{(un)}$TAG. By using the optimizer function of Design Expert, a total of six optimized reaction conditions were generated (Table 5). As expected, En was kept at center level which was approximately 10% and Ti was kept at high level which was approximately 12 h to obtain high $DAG_{(y)}$ and low $_{(un)}$TAG composition. Our observation showed that after approximately 10 wt% of En, $DAG_{(y)}$ did not increase with increase in En. Thus, from an industrial point of view, to produce a high purity DAG oil, En was optimized at the lowest possible level. Our finding also showed that $_{(un)}$TAG decreased with Ti. Thus, Ti was optimized at high level at 12 h in order to increase the purity of DAG oil produced. Both Wa and Te were kept at several levels ranging from low to center level. It was observed that reaction conditions with lower Wa required higher Te to produce a high purity DAG-enriched oil. At lower Wa, higher Te is needed to drive the reaction condition towards hydrolyzing more TAG. Therefore, optimal Wa and Te should be carefully selected. Center level of Wa and Te at approximately 50 wt% and 65°C, respectively, seemed desirable as overall cost of production and purification at these reaction conditions would be lowered.

The model predicted a yield of approximately 32 wt% of $DAG_{(y)}$ and 23% of $_{(un)}$TAG for all six optimal reaction conditions generated. A chi-square test was performed to verify the adequacies of the models established. Chi-square test showed there were no significant ($P > 0.05$) differences between the observed and predicted values for both model $DAG_{(y)}$ and $_{(un)}$TAG (Table 5). The chi-square values for both $DAG_{(y)}$ (0.22) and $_{(un)}$TAG (1.07) were much smaller than the cut of points (11.07) at $\alpha = 0.05$ and $df = 5$. Therefore, both models were adequate to predict $DAG_{(y)}$ and $_{(un)}$TAG in partial hydrolysis with high accuracy.

3.4. Scaling up of partial hydrolysis

Ten continuous batches of 9 kg production were conducted in a 16 L packed bed bioreactor to examine the possibility of direct up scaling. At the optimal levels of En (10 wt% of oil mass), Wa (50 wt% of enzyme mass), Te (65°C) and Ti (12 h), the models predicted $DAG_{(y)}$ and $_{(un)}$TAG to be 32.13 wt% and 26.43 wt%, respectively. Table 6 shows the level of $DAG_{(y)}$ and $_{(un)}$TAG for the ten consecutive batches. Chi-square test showed that there were no significant differences ($P > 0.05$) between the predicted and observed values for both $DAG_{(y)}$ and $_{(un)}$TAG. The chi-square values for both $DAG_{(y)}$ (9.43) and $_{(un)}$TAG (12.77) were smaller than the cut off points (16.9) at $\alpha = 0.05$ and $df = 9$. Therefore, optimal levels of En, Wa, Te and Ti can be up-scaled to produce a DAG-enriched palm olein achieving similar $DAG_{(y)}$ as in the lab scale.

Table 6
Percentage of $DAG_{(y)}$ and $_{(un)}$TAG in ten continuous productions

Batch no.	$DAG_{(y)}$ (%)	$_{(un)}$TAG (%)
1	28.45 ± 0.36	20.30 ± 1.13
2	27.98 ± 0.07	18.22 ± 0.28
3	27.21 ± 0.11	23.44 ± 0.30
4	26.51 ± 0.05	21.22 ± 0.50
5	26.97 ± 0.45	19.89 ± 0.17
6	26.45 ± 0.08	19.90 ± 1.92
7	26.48 ± 0.38	21.99 ± 1.76
8	25.5 ± 0.11	22.83 ± 0.37
9	25.95 ± 0.19	20.02 ± 0.18
10	25.52 ± 0.05	20.33 ± 0.48
	$\chi^2 = 9.43$	$\chi^2 = 12.77$

$DAG_{(y)}$ = diacyglycerol yield (wt%); $_{(un)}$TAG = unhydrolysed TAG yield (wt%).

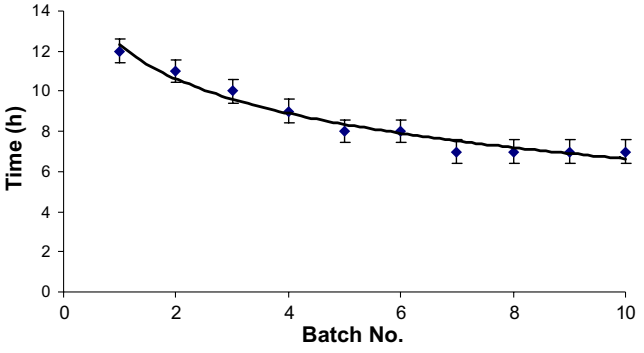

Fig. 4. Ti required to achieve process equilibrium for the ten consecutive batches of continuous productions.

Table 5
Optimization of partial hydrolysis and model verification by Chi-square test

Factors				$DAG_{(y)}$ (%)		$_{(un)}$TAG (%)	
Wa (%)	En (%)	Te (°C)	Ti (h)	Observed	Predicted	Observed	Predicted
50.80	11.69	68.22	13.50	32.40	32.61	24.33	23.13
50.95	11.62	68.09	13.50	31.88	32.62	25.89	23.17
51.21	11.91	67.22	13.50	31.68	32.59	25.71	23.12
51.26	11.54	69.54	13.50	32.23	32.60	24.31	23.23
48.44	12.50	68.82	13.50	31.74	32.41	21.12	22.88
54.46	12.18	64.07	13.50	30.22	32.46	21.32	23.55
				$\chi^2 = 0.22$		$\chi^2 = 1.07$	

Wa = Water content (wt% of enzyme mass); En = Enzyme load; (wt% of oil mass); Te = Reaction temperature (°C); Ti = Reaction time (h), and $DAG_{(y)}$ = Diacyglycerol yield (wt%); $_{(un)}$TAG = Unhydrolysed TAG yield (wt%).

The reusability of the enzymes is important in any protocol designed for commercial application. As noted earlier, Chi-square test showed there were no significant differences between the predicted and observed values for both $DAG_{(y)}$ and $_{(un)}TAG$ ($P > 0.05$) for all ten consecutive production runs. Thus, it is evident that the immobilized lipase can be used at least 10 times (120 h) to produce similar DAG(y).

A study on the acylglycerol composition at each hour interval up to 12 h for the ten consecutive production runs revealed that at the optimized conditions of En, Wa and Te, the Ti required to achieve process equilibrium decreased from 12 h from the first batch to 7 h at the tenth batch (Fig. 4). The possible reason for this is Lipozyme RMIM lipase contains Wa in the enzyme particles due to enzyme binding and carrier absorption, thus, initial addition of Wa may submerge lipase totally and limit the accessibility of the lipase active site access to the oil–water interface. Hence, longer Ti is required to achieve process equilibrium. During each batch, Lipozyme RMIM lipase released the bound water into substrate stream. Thus, at subsequent batches, addition of Wa is sufficient only to form a mono-hydration layer without submerging the lipase. Lipase is able to catalyze hydrolysis at oil–water interface effectively; hence, shorten the Ti required to achieve process equilibrium. The observation is not seen during the laboratory scale runs where longer Ti is required to achieve the maximum yield. Possible reason for this is that during laboratory scale run, mechanical stirring crushed the immobilized lipase particles while in packed bed continuous reactor, this phenomenon did not occur.

3.5. Product purification

The DAG-enriched palm olein was further purified using short path distillation. After removal of FFA and MAG, the DAG and TAG composition in the final product was, 60% and 40%, respectively. The purified DAG-enriched palm olein had a pale yellowish color, was semi-solid at room temperature and may find suitable applications as a potential healthy hard stock in margarine, spread or shortening formulations.

4. Conclusions

RSM was successfully applied to model and optimize partial hydrolysis to produce a DAG-enriched palm olein by considering both $DAG_{(y)}$ and $_{(un)}TAG$. Through the models, main effects and interactions between the four reaction parameters (Wa, En, Te and Ti) were successfully elucidated. Optimal reaction conditions were: 50 wt% water content, 10 wt% enzyme load, 65°C of reaction temperature and 12 h of reaction time. The optimized conditions can be scaled-up to a continuous pilot production in a 16 L packed bed bioreactor. The DAG-enriched palm olein produced in this study has a DAG and TAG composition of 60% and 40%, respectively after removal of FFA and MAG. It is also expected to have characteristics that are suitable for several food applications such as margarine, spreads, mayonnaise and shortenings.

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