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Response surface modeling of 1-stearoyl-3(2)-oleoyl glycerol production in a pilot packed-bed immobilized *Rhizomucor miehei* lipase reactor

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ABSTRACT

A dual response approach using diacylglycerol (DAG) and triacylglycerol (TAG) as responses for optimization of 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG synthesis using response surface methodology (RSM) was investigated. Four variables from a lipase-catalyzed esterification reaction were optimized using a central composite rotatable design. The following optimized conditions yielded 51 wt.% DAG and 22 wt.% TAG: reaction temperature of 55 °C, enzyme dosage of 9.5 wt.%, fatty acid/glycerol molar ratio of 2.1 and reaction time of 3 h. Results were repeatable at 10 kg production scale in a pilot packed-bed enzyme reactor. No significant losses in enzyme activity or changes in fatty acid selectivity on DAG synthesis were observed during the five pilot productions. Lipozyme RM IM showed selectivity towards the production of stearic acid enriched DAG. The purity of DAG oil after purification was 90 wt.%.

1. Introduction

Latest reports on the nutritional benefits of diacylglycerol (DAG) have renewed interest on DAG research. DAG, particularly the 1,3-isoform, has been confirmed of having certain nutritional benefits such as the ability to reduce serum triacylglycerol (TAG) concentration [1–3], body weight and visceral fat [4–6]. There is no significant difference in energy value and absorption coefficient between DAG and TAG oil [7]. Numerous studies on the safety aspects of DAG on humans [3–5,8,9] and animals [10–13] demonstrated no adverse effects. Currently, DAG is marketed as a functional cooking oil in Japan and the U.S. [14]. The commercially available DAG oil contains approximately 80% DAG, of which approximately 56% exists as *sn*1,3-DAG and the remainder as *sn*1,2(2,3)-DAG, and about 20% TAG [14].

DAG can be synthesized either by a chemically or enzymatically catalyzed reaction. In the chemical approach, the process is conducted at high temperatures (220–260 °C) using an inorganic catalyst such as sodium, potassium or calcium hydroxides [15]. Generally, chemically synthesized DAG requires extensive purification steps to ensure desirable product quality. In the enzymatic approach, DAG can be prepared by esterification [16–19], glycerolysis [20,21], partial hydrolysis [22], interesterification [23] or a combination of partial hydrolysis and esterification reactions [24]. Most of these studies were carried out on a gram-scale laboratory setting and very few kilogram-scale pilot plant studies were reported.

In this work, an objective is to optimize the production of 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG by lipase-catalyzed esterification using response surface methodology (RSM) in a laboratory setting and to apply the optimized conditions to a scaled-up pilot packed-bed enzymatic reactor. RSM is a statistical tool for developing and optimizing processes with one or more responses that are influenced by several variables [25]. The advantage of RSM is that it allows the user to gather large amounts of information from a small number of experiments. The use of RSM is also possible to observe the effects of individual variables and their combinations of interactions on the response. If the desired response is one of several products formed in a series of reversible biochemical reactions, it is appropriate to use more than one response for process optimization. In a reversible reaction process, the use of multiple responses will take into account the effect of molecular equilibrium of the desired response during optimization. Although the use of a single response may be the simplest approach, the so-called

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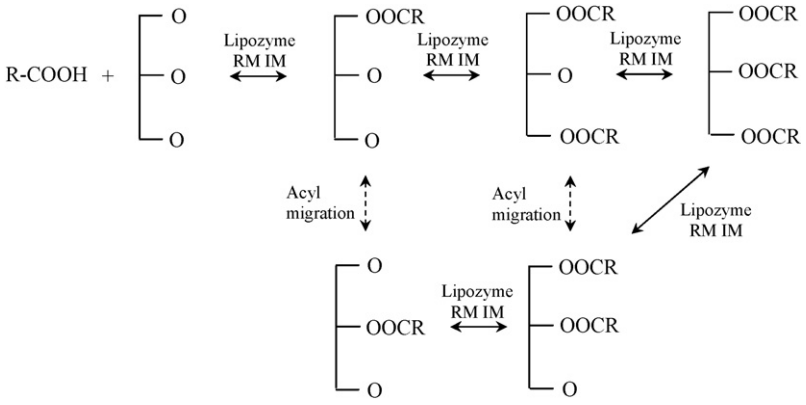

Fig. 1. Reaction route of Lipozyme RM IM-catalyzed esterification of fatty acids and glycerol.

optimized variables may not truly represent the actual optimization of the process for practical application since not all of the undesired responses are taken into account. For example, in the esterification of fatty acids with glycerol to produce acylglycerols such as DAG, the overall process involves a combination of 3 reactions (Fig. 1) producing monoacylglycerol (MAG), DAG and TAG as esterification products while some unreacted free fatty acids (FFA) and glycerol remain as residues. In a practical point-of-view, MAG, FFA and glycerol will be removed from DAG and TAG by distillation and reused as substrates for the subsequent runs of DAG production. Therefore, in a response surface optimization point-of-view, MAG, FFA and glycerol can be regarded as neutral responses as they do not pose a significant irrecoverable process loss. On the other hand, the formed TAG is an economically undesirable by-product of the esterification process, and thus, should be minimized so as to achieve maximum process gain. As such, TAG should be regarded as an undesirable response and factored in during optimization of DAG production. With these criteria noted, this work is carried out to reinvestigate the use of RSM on DAG yield optimization by lipase-catalyzed esterification. A five-level-four-factor RSM is employed in this study.

From a statistical point-of-view, the response surface optimized conditions are only valid using the respective experimental setting at which the optimization was carried out. Currently, there has been very few literature reporting on the use of the laboratory-

Table 1
Experimental runs for five-level-four-factor central composite rotatable design and the comparison between observed and predicted responses for DAG and TAG yields

No.	R_{temp}	Enz	Sr	R_{time}	DAG (wt.%)		TAG (wt.%)	
					Actual	Predicted	Actual	Predicted
1	55	4	2	3	21.31	20.96	33.61	33.23
2	75	4	2	3	38.96	38.61	53.41	53.79
3	55	10	2	3	52.73	52.38	29.38	29.76
4	75	10	2	3	33.33	32.98	58.58	58.20
5	55	4	3	3	30.73	30.38	39.2	39.58
6	75	4	3	3	31.45	31.10	63.72	63.34
7	55	10	3	3	24.41	24.06	57.83	57.45
8	75	10	3	3	23.74	23.39	63	63.38
9	55	4	2	8	32.48	32.83	53.56	53.94
10	75	4	2	8	54.67	55.02	34.13	33.75
11	55	10	2	8	19.3	19.65	59.9	59.52
12	75	10	2	8	34.52	34.87	58.06	58.44
13	55	4	3	8	26.01	26.36	59.25	58.87
14	75	4	3	8	11.68	12.03	83.25	83.63
15	55	10	3	8	36.77	37.12	46.52	46.90
16	75	10	3	8	19.14	19.49	65.46	65.08
17	45	7	2.5	5.5	13.16	13.56	59.51	59.43
18	85	7	2.5	5.5	26.64	26.71	48.39	48.57
19	65	1	2.5	5.5	34.02	34.69	35.11	35.05
20	65	13	2.5	5.5	16.98	16.79	81.58	81.52
21	65	7	1.5	5.5	19.95	19.25	68.69	68.73
22	65	7	3.5	5.5	22.31	22.84	59.45	59.46
23	65	7	2.5	0.5	24.3	25.72	41.20	41.36
24	65	7	2.5	10.5	23.03	21.61	74.75	74.80
25	65	7	2.5	5.5	42.21	42.75	26.25	25.50
26	65	7	2.5	5.5	43.25	42.75	25.63	25.50
27	65	7	2.5	5.5	42.21	42.75	25.25	25.50
28	65	7	2.5	5.5	44.04	42.75	25.09	25.50
29	65	7	2.5	5.5	43.42	42.75	25.95	25.50
30	65	7	2.5	5.5	41.35	42.75	24.81	25.50

R_{temp} = reaction temperature (°C); Enz = enzyme concentration (wt.% based on amount of fatty acids); Sr = substrate molar ratio (mol/mol fatty acids/glycerol); R_{time} = reaction time (h).

scale response surface optimized conditions on a larger scale pilot plant setting. Therefore, it is also another objective of this work to investigate on the possibility of up-scaling of the response surface optimized conditions.

2. Materials and methods

2.1. Materials

Lipozyme RM IM (*Rhizomucor miehei* lipase immobilized on a macroporous anion exchange resin) purchased from Novozymes A/S (Bagsvaerd, Denmark) was used for DAG synthesis. Commercial stearic and oleic acids and glycerol with a purity of 99.0%, 77.5% and 99.8%, respectively, were purchased from Cognis Oleochemicals (M) Sdn. Bhd. (Telok Panglima Garang, Malaysia). Acetone and acetonitrile were of HPLC grade.

2.2. Experimental design

A five-level-four-factor central composite rotatable design (CCRD) with a total of 30 experiments (Table 1) was applied in this study [26–28]. The variables and their levels were reaction time (R_{time}, 3–8 h), enzyme concentration (Enz, 4–10 wt.% of fatty acid mass), reaction temperature (R_{temp}, 55–75 °C), and substrate molar ratio (Sr, 2–3 mol/mol fatty acid/glycerol). The responses were DAG (wt.%) and TAG (wt.%) yields. This design was generated by the use of the software Design-Expert version 6.0.11 (Stat-ease Inc., Minneapolis, USA).

2.3. Esterification reaction

1-Stearoyl-3(2)-oleoyl glycerol-enriched DAG synthesis was carried out via esterification in a 50 ml conical flask. Stearic and oleic acids (1:1 molar ratio) were added to glycerol at different substrate molar ratios (Table 1). The total substrate weight is fixed at 50 g. The substrate mixture is mixed with Lipozyme RM IM at various concentrations and incubated at different temperatures and reaction times (Table 1) under magnetic stirring at approximately 500 rpm and reduced pressure of 133 mbar to remove excess water in the reaction system.

2.4. Acylglycerol composition analysis

Acylglycerol composition analysis was determined by reverse-phase high-performance liquid chromatography (RP-HPLC). Elution of total FFA and MAG, total DAG and total TAG was done using a LiChrospher® 100 RP-18e 5 μm (250 mm × 4 mm) column from Merck KgaA (Darmstadt, Germany). An isocratic elution using acetone and acetonitrile mixture at 1:1 (v/v) ratio was used. Reaction samples were dissolved in chloroform at approximately 50 mg/ml and 10 μl volume were injected into the HPLC for analysis. The compositions of FFA, MAG, DAG, and TAG are calculated as wt.% of the total acylglycerol content in the oil sample.

2.5. Diacylglycerol composition analysis

Analysis of DAG composition in the 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG was performed using the method described [29]. A 5% (w/v) sample was prepared in chloroform. Ten microlitres of the sample was injected into a LiChrospher 100RP-18e 5 μm (250 mm × 4 mm) reversed-phase HPLC column using a Shimadzu LC-10AD HPLC system (Kyoto, Japan) equipped with a Shimadzu SPD-10AV ultraviolet (UV) detector set at 205 nm. The mobile phase comprised of 100% (v/v) acetonitrile. Flow rate of the mobile phase was isocratically set at 1.1 ml/min, while the oven temperature was at 40 °C.

2.6. Production in pilot packed-bed enzymatic reactor

A 16 l pilot packed-bed enzymatic reactor was used in this study. The pilot packed-bed enzymatic reactor was charged with 9 kg of free fatty acids and 1.44 kg of glycerol before carrying out the esterification reaction at the following optimized settings; R_{time} = 4.14 h, Enz = 4 wt.% of fatty acid mass, R_{temp} = 66.29 °C, and Sr = 2.14. Reactions were conducted under vacuum at 133 mbar to remove water from the system. The fatty acids and glycerol were thoroughly mixed using two vertically aligned three-bladed impellers in the feed tank at a speed of approximately 500 rpm and equilibrated at 65 °C before contacting the substrate with the enzyme bed at a flow rate of approximately 3.5 l/min. The enzyme bed has the following initial dimensions; bed diameter = 16.2 cm, and bed height = 5.5 cm.

2.7. Purification in a pilot-scale short-path distillation

Crude DAG was purified using a 50 l falling film evaporator and short-path distillation unit. The distillation conditions are tabulated in Table 2. Other conditions were; evaporator vacuum = 0.001 mbar, condenser temperature = 60 °C, roller speed = 400 rpm.

2.8. Reusability of Lipozyme RM IM in pilot plant

Five consecutive pilot productions of DAG were conducted at the optimized settings to test the reusability of the commercial immobilized lipase. DAG yield, given as wt.%, were analyzed to determine enzyme reusability.

2.9. Statistical analysis

The experimental data were analyzed by RSM using the software Design-Expert version 6.0.11 (Stat-ease Inc., Minneapolis, USA). Model fitting to equations of up to the fourth-order polynomial was performed to determine the goodness-of-fit. The responses were fitted to the variables by multiple regression. The quality of fit of the model was evaluated by the coefficients of determination (R^2 and adjusted R^2), the analysis of variance (ANOVA) and the absolute average deviation (AAD) analysis. The model was refined after insignificant coefficients were examined and manually eliminated.

3. Results and discussion

3.1. Model fitting for DAG

The experimental DAG data was best-fitted to a reduced quartic model by multiple regression after manual elimination. Factors and interactions that are not significant ($P > 0.1$) are eliminated from

Table 2
Short-path distillation conditions used for DAG purification

Distillation step	Feed	Residue	Distillate	Feed rate (l/h)	Evaporator temperature (°C)
Step 1	Crude DAG	DAG and TAG	FFA and MAG	10	180
Step 2	Residue from step 1	TAG	DAG	10	250

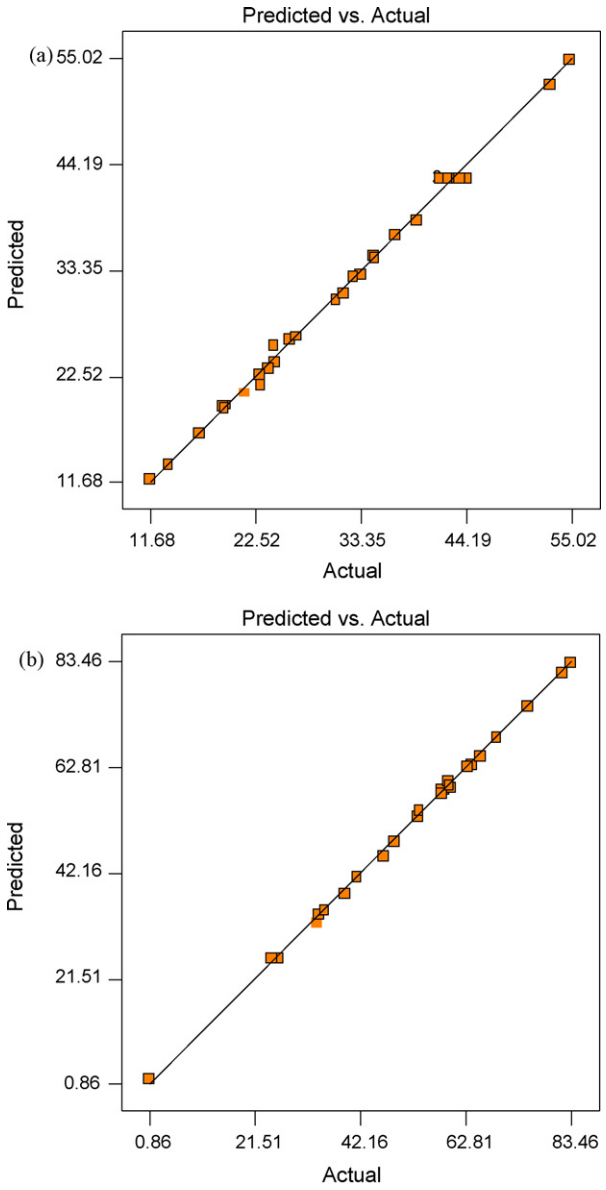

Fig. 2. (a) Relationship between the observed and predicted DAG responses. (b) Relationship between the observed and predicted TAG responses.

the model. The regression coefficients and *P* values for the DAG response are tabulated in Table 3a. Most of the variables have *P* values below 0.01, except for the terms required to retain hierarchy of the model. The coefficient of determination (R^2) and the adjusted R^2 of the model were 0.9970 and 0.9857, respectively, and the analysis of variance (ANOVA) showed no lack of fit of the model. The correlation between the predicted and observed DAG responses was satisfactory (Fig. 2a). To further verify the accuracy of the selected model, AAD analysis was performed on the data. AAD analysis provides information on the degree of deviation of the predicted and observed data. Therefore, for an accurate model, it is desirable that the AAD value be as small as possible. Acceptable values of R^2 and AAD mean that the equation of the model represents the true behavior of the system and it can be used for interpolation in the experimental setting. The AAD value is calculated based on the equation by Baş and Boyacı [30]. The AAD value of the model was 1.9%. This means that the model for DAG response is acceptable for use in the optimization of DAG yield via esterification.

Table 3a
Regression coefficients and significance (*P*) values for DAG response after manual elimination

Variables	Coefficients	*P* value
Intercept	−5784.77	<0.0001
R_{temp}	307.91	0.1519
Enz	109.50	0.0441
Sr	593.43	<0.0001
R_{time}	49.97	0.0098
R_{temp}^2	−6.95	0.0004
Enz^2	0.63	<0.0001
Sr^2	−143.86	<0.0001
R_{time}^2	−0.76	<0.0001
$R_{temp} \times Enz$	−1.37	0.0001
$R_{temp} \times Sr$	−2.09	<0.0001
$R_{temp} \times R_{time}$	−0.19	0.2337
$Enz \times Sr$	−37.60	0.0728
$Enz \times R_{time}$	−14.22	0.0025
$Sr \times R_{time}$	−12.36	0.0799
R_{temp}^3	0.07	0.0020
Enz^3	−0.05	0.0003
Sr^3	16.29	<0.0001
$R_{temp} \times Enz \times Sr$	0.46	0.0003
$R_{temp} \times Enz \times R_{time}$	0.16	0.0020
$R_{temp} \times Sr \times R_{time}$	0.02	<0.0001
$Enz \times Sr \times R_{time}$	4.99	<0.0001
R_{temp}^4	−2.7E−04	<0.0001
$R_{temp} \times Enz \times Sr \times R_{time}$	−0.05	0.0010

See Table 1 for description of abbreviations.

3.2. Model fitting for TAG

The experimental TAG data was also best-fitted to a reduced quartic model by multiple regression after manual elimination. Insignificant ($P > 0.1$) factors and interactions were eliminated from the model. The regression coefficients and *P* values for the TAG response are tabulated in Table 3b. Most of the variables have *P* values below 0.01, except for the terms required to retain hierarchy of the model. The coefficient of determination (R^2) and the adjusted R^2 of the model were 0.9959 and 0.9908, respectively, and the analysis of variance (ANOVA) showed no lack of fit of the model. The predicted and observed TAG responses were also sufficiently cor-

Table 3b
Regression coefficients and significance (*P*) values for TAG response after manual elimination

Variables	Coefficients	*P* value
Intercept	4272.50	<0.0001
R_{temp}	−216.44	<0.0001
Enz	−35.39	0.0002
Sr	−617.82	<0.0001
R_{time}	59.43	0.0002
R_{temp}^2	4.76	0.2532
Enz^2	−1.80	<0.0001
Sr^2	206.95	<0.0001
R_{time}^2	−0.29	<0.0001
$R_{temp} \times Enz$	0.44	0.4494
$R_{temp} \times Sr$	−0.24	<0.0001
$R_{temp} \times R_{time}$	−1.32	<0.0001
$Enz \times Sr$	19.22	<0.0001
$Enz \times R_{time}$	1.86	0.0010
$Sr \times R_{time}$	−18.07	0.9856
R_{temp}^3	−0.05	<0.0001
Enz^3	0.13	<0.0001
Sr^3	−22.45	<0.0001
R_{time}^3	0.10	<0.0001
$R_{temp} \times Enz \times Sr$	−0.21	<0.0001
$R_{temp} \times Enz \times R_{time}$	0.02	0.0004
$R_{temp} \times Sr \times R_{time}$	0.42	<0.0001
$Enz \times Sr \times R_{time}$	−1.30	<0.0001
R_{temp}^4	1.65E−04	<0.0001

See Table 1 for description of abbreviations.

related (Fig. 2b). The AAD value of the model was 0.8%. This means that the model for TAG response is suitable for use in the reverse optimization of TAG yield.

3.3. Comparison of main effects of variables on DAG and TAG yields

The main effects of variables on DAG and TAG yields and their significance are shown in Fig. 3a and b. All first-order coefficients of models for DAG response have positive effects. Second-order coefficient for Sr has a negative effect while most other coefficients have relatively small effects on DAG yield. In general, Sr has the most significant effect on DAG yield, followed by R_{temp}. In the TAG response, most of the first- and second-order coefficients had negative effects on yield, except for R_{temp} and Sr^2. The effects of other higher order coefficients were minor. Similarly, Sr is the most influential variable on TAG yield, followed by R_{temp}.

3.4. Comparison of response contour plots for DAG and TAG yields

A better understanding of the interactive effects of variables on DAG and TAG yields can be seen in the response contour plots of DAG and TAG (Fig. 4a–l). From the contour plots (Fig. 4a and b), at approximately the recommended optimum operating temperature of Lipozyme RM IM (R_{temp} = 65 °C), DAG yield shifted slightly by 3 wt.% while TAG yield changed by 5 wt.%, as Enz increased from 4 to 10 wt.%. However, the maximum DAG and minimum TAG yields were not observed to be at 65 °C, but instead in the region of 56 °C. This effect suggests that the recommended optimum temperature of a commercial lipase may not necessarily mean the best operating temperature for a reaction. Another important point to note is that a higher dosage of Lipozyme RM IM does not necessarily mean a higher DAG yield, especially when the system involves a reversible reaction with several products such as this. An excess of lipase in the esterification system will increase the rate of the overall esterification reaction and may tend to sway the molecular equilibrium towards the extreme end of the esterification system, thereby gradually increasing the formation of TAG. Similar findings were reported in literature [16–19]. In the corresponding TAG contour plot, a minimum point was observed at the region of Enz = 7–8 wt.%. This suggests that Lipozyme RM IM at 7–8 wt.% concentrations may provide a satisfactory DAG yield of approximately 44 wt.%, while keeping TAG yield at the lowest level.

Similar findings were also observed in the interaction between Sr and R_{temp} on DAG and TAG yields (Fig. 4c and d). At 56 °C, DAG yield decreased by approximately 8 wt.% while TAG yield gained by about 12 wt.% as Sr increased from 2:1 to 3:1. This observation was reasonable as higher Sr stoichiometrically favors TAG formation. A

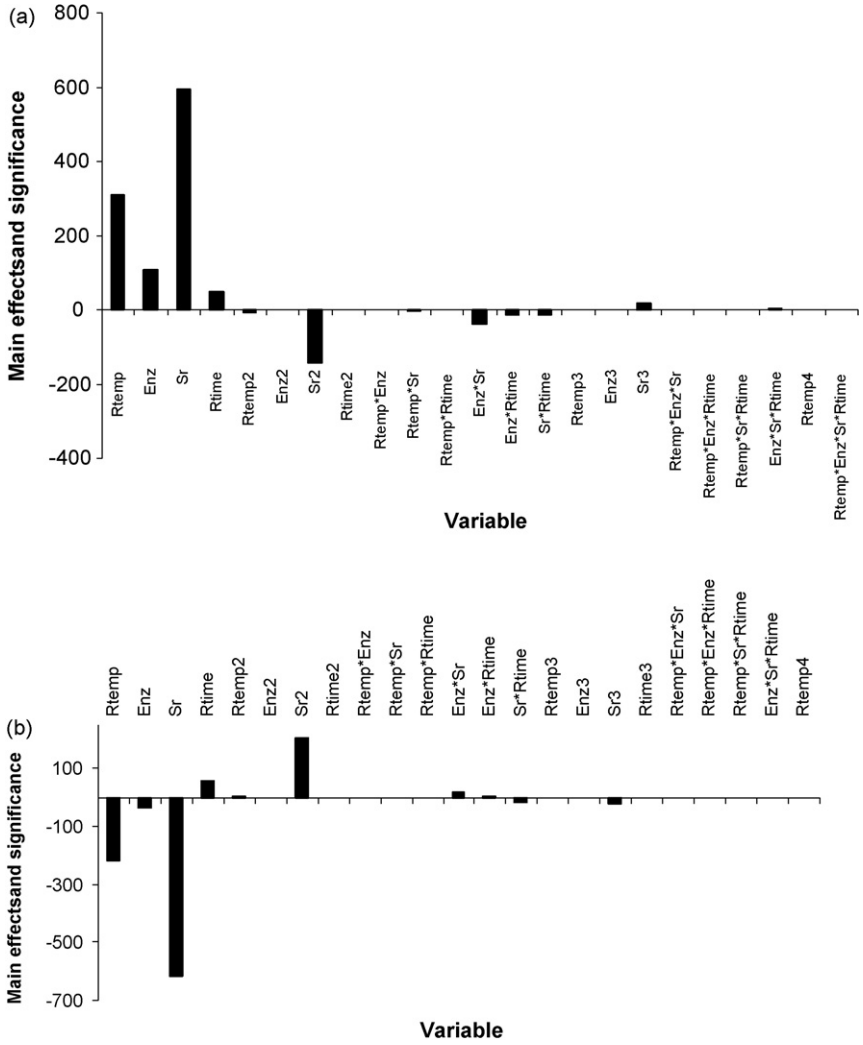

Fig. 3. (a) Main effects and their significance of variables on Lipozyme RM IM-catalyzed production of DAG. (b) Main effects and their significance of factors on Lipozyme RM IM-catalyzed production of TAG.

peak was observed at Sr = 2.35 in the DAG plot, which corresponds to the lowest point of TAG yield, indicating that a fatty acid to glycerol molar ratio of 2.35 may be the optimum condition for highest DAG yield and lowest TAG yield.

In the interaction between R_{time} and R_{temp} on DAG and TAG yields (Fig. 4e and f) at 56 °C, the DAG yield peaked at approximately 45 wt.% at 5–6 h of reaction, while the corresponding TAG yield was minimal at 19 wt.% at the 4–5 h period. Based on both the DAG and TAG contour plots, it can be observed that an R_{time} of approximately 5 h is ideal for maximizing DAG formation and minimizing TAG formation. This observation is in accordance with previous reports showing that a further increase in reaction time did not significantly increase DAG yield but significantly increased TAG yield [16–19].

Based on the contour plots (Fig. 4g and h) showing the interactive effects of Sr and Enz, an increase in the effects of Sr and Enz, either individually or combined, will generally decrease DAG production and increase TAG yield. A positive peak at approxi-

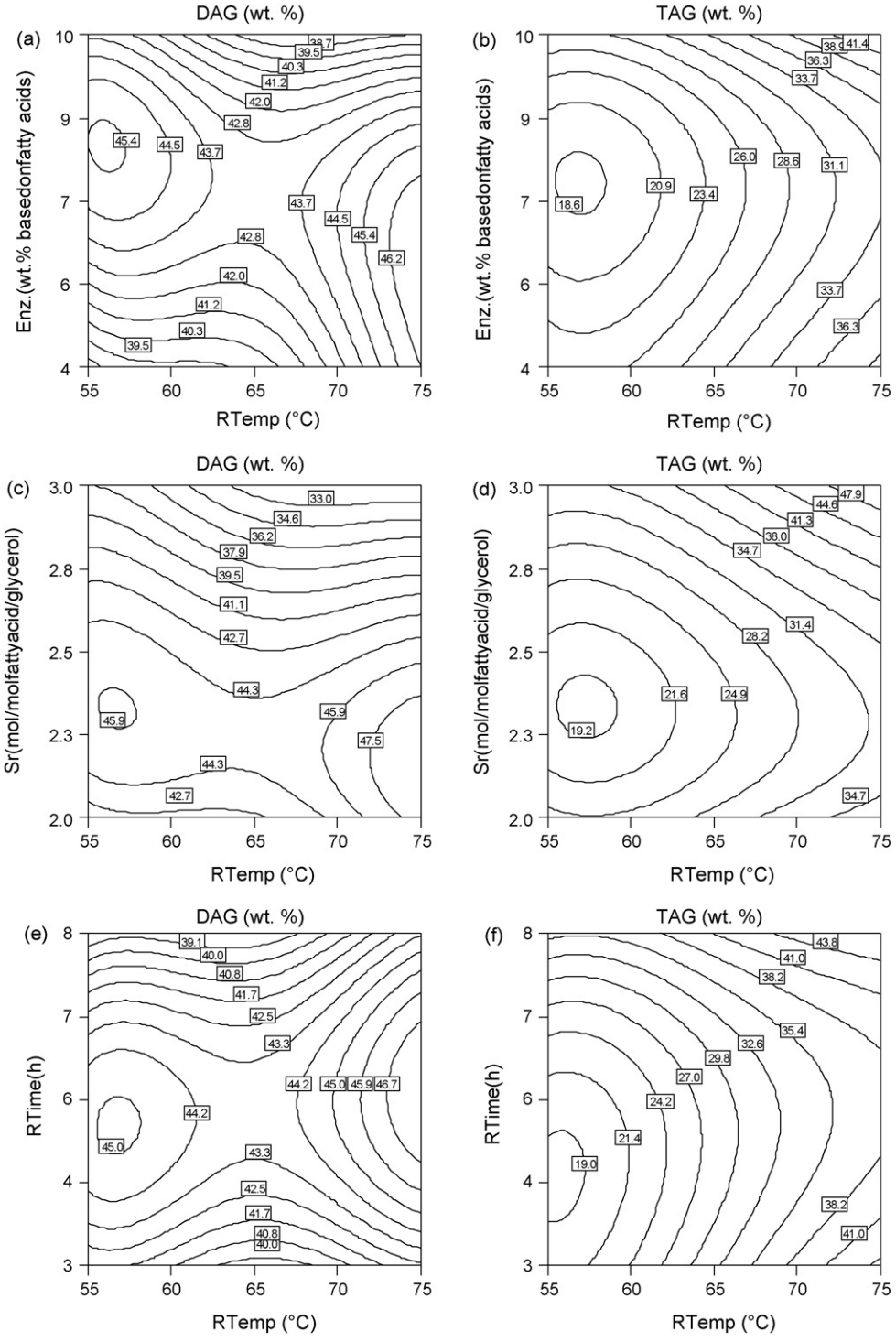

Fig. 4. Contour plots of DAG and TAG responses showing interactions of factors: (a, b) Enz and R_{temp}, (c, d) Sr and R_{temp}, (e, f) R_{time} and R_{temp}, (g, h) Sr and Enz, (i, j) R_{time} and Enz, and (k, l) R_{time} and Sr.

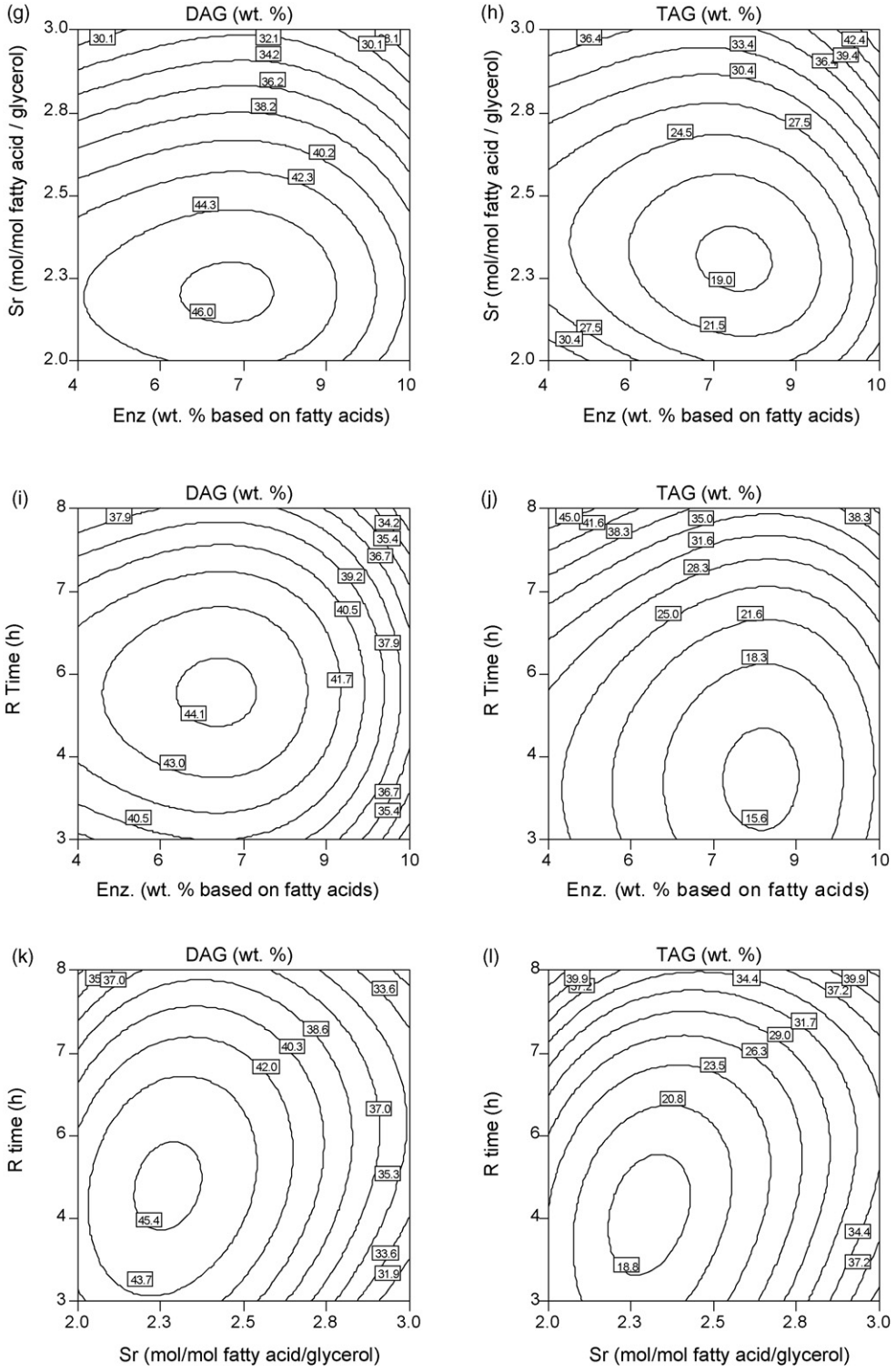

Fig. 4. (*Continued*).

mately Enz = 6.8 wt.% and Sr = 2.3 was observed in the DAG contour plot. However, a negative peak was located within the vicinity (Enz = 7.5 wt.% and Sr = 2.4) in the TAG contour plot. This indicates that the predicted conditions for optimum DAG production with minimal TAG formation may fall within these regions.

Similar patterns were also observed for the interactive effects of Enz and R_{time} (Fig. 4i and j), and R_{time} and Sr (Fig. 4k and l) on DAG and TAG yields. Positive peaks were observed at Enz = 6.6 wt.%, R_{time} = 5.5 h; and R_{time} = 5.3 h, Sr = 2.3 for DAG yields, while negative peaks at Enz = 8.1 wt.%, R_{time} = 3.7 h; and R_{time} = 3.7 h, Sr = 2.3 for TAG yields were noted. An increase in the effects of Enz and R_{time}, either individually or combined, will decrease DAG production and increase TAG yield. Again, this observation can explained by the fact that this system involves a series of reversible reactions, whereby the desired component is formed as a product in one reaction and serves as a substrate for another reaction in the system.

Table 4
Optimized esterification reaction conditions based on selected criteria

Criteria	R_{temp}	Enz	Sr	R_{time}	DAG	TAG	Desirability
Minimum R_{temp} and R_{time}	55.00	9.46	2.09	3.00	51.39	22.25	0.98
Minimum R_{time}	55.00	9.46	2.09	3.00	51.05	22.11	0.97
Minimum R_{temp}	55.02	9.48	2.10	3.04	51.24	23.48	0.97
Neutral	55.00	9.46	2.10	3.00	51.29	24.39	0.96
Minimum Enz	75.00	4.00	2.02	7.01	54.67	31.84	0.95
Minimum R_{temp} and Enz	55.00	4.80	2.40	5.25	40.01	24.73	0.86
Minimum R_{temp} and maximum Sr	55.00	7.68	2.76	5.43	42.68	26.69	0.85
Minimum R_{temp}, Enz and R_{time}	55.00	5.36	2.43	3.10	36.89	22.83	0.84
Minimum R_{temp}, R_{time} and maximum Sr	55.00	7.82	2.59	3.13	41.87	25.18	0.83
Minimum Enz and R_{time}	75.00	4.00	2.17	4.56	48.48	38.30	0.82
Maximum Sr	55.84	7.68	2.75	5.39	42.75	25.99	0.82
Minimum R_{temp}, Enz and maximum Sr	55.00	4.68	2.78	4.99	37.29	31.17	0.81
Maximum Sr and minimum R_{time}	55.91	7.75	2.60	3.16	41.72	24.94	0.80
Minimum R_{temp}, Enz, R_{time} and maximum Sr	55.00	4.00	2.85	3.37	32.17	34.39	0.80
Minimum Enz, R_{time} and maximum Sr	56.27	4.00	2.83	3.33	32.45	33.79	0.78
Minimum Enz and maximum Sr	56.11	4.65	2.76	4.96	37.50	30.46	0.77

See Table 1 for description of abbreviations.

In this case, after a certain R_{time} whereby the substrates for DAG synthesis start to deplete causing the molecular equilibrium of the overall system to tilt towards the extreme end of the system, the DAG formed from the esterification of MAG and FFA will serve as a substrate for the synthesis of TAG, thereby gradually decreasing DAG yield and increasing TAG yield.

3.5. Optimization of DAG process

The most cost-efficient conditions for the production of DAG via esterification would be to use the lowest amounts of lipase and glycerol to achieve the highest DAG and lowest TAG yields in the shortest time. Based on the contour plots, a set of approximate reaction conditions based on the required criteria for optimum DAG and minimum TAG yields can be predicted (Table 4). The optimized reaction conditions are sorted by order of descending desirability. Desirability is an objective function that reflects the desirable ranges for each factor and is defined as the geometric means of all transformed factors. The most desirable reaction condition for optimum 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG yield (51 wt.%) with minimal TAG (22 wt.%) formation is as follow: R_{temp} = 55 °C, Enz = 9.5 wt.%, Sr = 2.1 and R_{time} = 3 h.

3.6. Pilot plant production

The optimized process variables for 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG production were applied to a 16 l pilot packed-bed enzymatic reactor for model verification. The DAG and TAG yields from the five pilot productions are tabulated in Table 5. Except for the first production, all other pilot productions produced DAG and TAG yields similar or close to their predicted yields. The lower than predicted DAG yield and higher than predicted TAG yield observed in the first production may be explained by the presence of some loosely bound lipases in the enzyme preparation causing a shift in the molecular equilibrium of DAG towards TAG synthesis as mentioned earlier. This observation reveals that even after five consecutive pilot productions, Lipozyme RM IM did not significantly lose its activity to catalyze esterification at the optimized reaction conditions. In a related work [21], the authors reported significant losses in enzyme activity and a large decrease in DAG yield of 10 wt.% after five consecutive runs in a pilot-scale batch stirred-tank enzyme reactor. The current results indicate that Lipozyme RM IM is best suited to be used in a packed-bed configuration to ensure prolonged enzyme half-life and increased enzyme productivity.

3.7. Diacylglycerol composition

DAG compositions from the crude 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG product of all five pilot productions were analyzed to check for changes in DAG composition that may be caused by fatty acid selectivity of Lipozyme RM IM as a function of repeated usage. DAG profiles of the five samples are shown in Table 5. There were no observed changes in fatty acid selectivity of the lipase as the pilot productions progressed to the fifth batch. DAG profiles in all the pilot productions showed that the concentration of total diolein (24.3–25.9 wt.%) were similar to that of total distearin (24.5–26.1 wt.%). The results revealed that the synthesis of 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG using equimolar concentrations of oleic and stearic acids catalyzed by Lipozyme RM IM showed high preference towards the synthesis of stearic acid-enriched DAG. Interestingly, this observation was on the contrary to Lo et al. [19] who reported on a high selectivity of Lipozyme RM IM towards the synthesis of 1,3(2)-diolein and 1-palmitoyl-3(2)-oleoyl glycerol at equimolar concentrations of oleic and palmitic acids. Another interesting observation to note is that although distearin has the highest concentration among DAG moieties produced, the production is largely skewed towards the synthesis of

Table 5
DAG and TAG yields and DAG composition from pilot packed-bed enzyme reactor productions

Production no.	DAG yield (wt.%)	Error	TAG yield (wt.%)	Error	DAG composition (wt.%)					
					1,3-OO	1,2-OO	1,3-SO	1,2-SO	1,3-SS	1,2-SS
1	41.6	2.5	29.1	2.8	1.6	0.5	11.2	3.3	25.0	0.5
2	48.2	0.6	23.5	1.4	2.1	0.5	12.8	3.8	28.3	0.5
3	49.3	1.6	21.2	0.9	1.7	0.4	13.5	3.7	30.7	0.4
4	51.1	1.3	19.0	2.6	2.0	0.6	13.7	4.0	29.6	0.4
5	49.8	0.5	22.3	2.3	1.5	0.6	12.5	4.0	28.3	0.5

1,3-OO = 1,3-diolein; 1,2-OO = 1,2-diolein; 1,3-SO = 1-stearoyl-3-oleoyl-glycerol; 1,2-SO = 1-stearoyl-2-oleoyl-glycerol; 1,3-SS = 1,3-distearin; 1,2-SS = 1,2-distearin.

its 1,3-isoform. Based on the current and previous findings, it can be postulated that the selectivity of Lipozyme RM IM towards the production of DAG from long-chain fatty acids, such as palmitic, oleic, and stearic acids, could be based on structural configuration rather than the degree of unsaturation of the fatty acids.

3.8. Pilot plant purification

Purification of DAG was performed to separate unreacted glycerol, FFA, MAG and TAG from DAG. After purification by SPD, the purity of the 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG oil was approximately 90 wt.%.

4. Conclusions

Dual response surface optimization using DAG and TAG as responses is useful in predicting the optimum conditions for high DAG and low TAG yields in a multiple step esterification reaction. The optimum conditions yielding 51 wt.% of 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG and 22 wt.% TAG are as follow: R_{temp} = 55 °C, Enz = 9.5 wt.%, Sr = 2.1 and R_{time} = 3 h. The optimized process variables were reproducible at 10 kg production scale in a pilot packed-bed enzyme reactor. No significant enzyme activity losses or changes in fatty acid selectivity on 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG synthesis were noted during the five pilot productions. In addition, Lipozyme RM IM showed selectivity towards the synthesis of stearic acid enriched DAG. A purity of approximately 90 wt.% of 1-stearoyl-3(2)-oleoyl glycerol-enriched DAG oil was obtained.

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