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Diacylglycerol and Triacylglycerol as Responses in a Dual Response Surface-Optimized Process for Diacylglycerol Production by Lipase-Catalyzed Esterification in a Pilot Packed-Bed Enzyme Reactor

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Diacylglycerol (DAG) and triacylglycerol (TAG) as responses on optimization of DAG production using a dual response approach of response surface methodology were investigated. This approach takes the molecular equilibrium of DAG into account and allows for the optimization of reaction conditions to achieve maximum DAG and minimum TAG yields. The esterification reaction was optimized with four factors using a central composite rotatable design. The following optimized conditions yielded 48 wt % DAG and 14 wt % TAG: reaction temperature of 66.29 °C, enzyme dosage of 4 wt %, fatty acid/glycerol molar ratio of 2.14, and reaction time of 4.14 h. Similar results were achieved when the process was scaled up to a 10 kg production in a pilot packed-bed enzyme reactor. Lipozyme RM IM did not show any significant activity losses or changes in fatty acid selectivity on DAG synthesis during the 10 pilot productions. However, lipozyme RM IM displayed higher selectivity toward the production of oleic acid-enriched DAG. The purity of DAG oil after purification was 92 wt %.

KEYWORDS: Diacylglycerol; dual response; optimization; response surface methodology; packed-bed reactor; lipozyme RM IM; *Rhizomucor miehei* lipase; esterification; pilot plant

INTRODUCTION

Recent findings on the nutritional benefits of diacylglycerol (DAG) have attracted much attention on DAG research. DAG, particularly the 1,3-isoform, has been identified as having nutritionally beneficial effects such as the ability to reduce serum triacylglycerol (TAG) concentrations (*1*−*3*), body weight, and visceral fat (*4*−*6*). The energy value and absorption coefficient of DAG are similar to that of 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. Today, DAG is marketed as a functional cooking oil in Japan and the United States (*14*). The commercial 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 prepared either chemically or enzymatically. 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*). However, chemically prepared DAG often requires extensive purification steps to ensure desirable product quality. In the enzymatic approach, DAG can be prepared by esterification (*16*−*18*), glycerolysis (*19*, *20*), partial hydrolysis (*21*), interesterification (*22*), or a combination of partial hydrolysis and esterification reactions (*23*). Mostly, these investigations were carried out on a gram-scale laboratory setting and very few kilogram-scale pilot plant studies were reported. To date, Kristensen et al. (*21*) investigated glycerolysis of 15−20 kg of sunflower and rapeseed oils in a pilot stirred-tank batch reactor for DAG production.

In this work, an objective is to optimize the production of DAG by lipase-catalyzed esterification using response surface methodology (RSM) in a laboratory setting and to apply the optimized conditions in a scaled-up pilot packed-bed enzymatic reactor. RSM is a statistical technique for developing and optimizing processes; its response is influenced by several variables (*24*). RSM has the advantage 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 effect of individual variables and their combination of interac-

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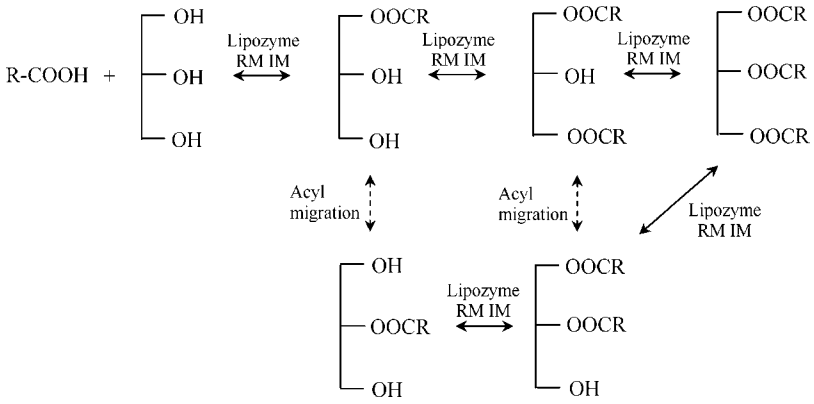
Scheme 1. Reaction Route of Lipozyme RM IM-Catalyzed Esterification of Fatty Acids and Glycerol



tions on the response. Unfortunately, there has been a certain inappropriate use of RSM for process optimization in the literature. Baş and Boyaci (*25*) reviewed the application of RSM in biochemical processes in the literature and observed some common mistakes made in some of the articles. One major mistake made was to fit the experimental data into a second-order polynomial equation. It can be argued that not all biochemical systems possess symmetrical curves that can be well-fitted into a second-order polynomial equation. As such, the use of a second-order polynomial may not be suitable to explain the effect of variables that are nonsymmetrical.

Another common inadequacy found in the literature on the use of RSM, specifically when the desired response is one of several products formed in a series of reversible biochemical reactions, is the use of only a single response for process optimization. In other words, the molecular equilibrium of the desired response is not taken into account during optimization. Although the use of a single response may be the easiest approach for optimization, the so-called optimized variables may not truly represent the actual optimization of the process for practical use since not all of the undesired responses are taken into consideration. For example, in the esterification of fatty acids with glycerol to produce acylglycerols such as DAG, the overall process involves a combination of three reactions (**Scheme 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 separated 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 byproduct of the esterification process and, thus, should be minimized as much as possible so as to achieve maximum process gain. As such, TAG should be regarded as an undesirable response and taken into consideration in the response surface optimization of DAG production. With these criteria taken into account, this work was 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.

In general, the response surface-optimized condition is only valid at the respective experimental setting at which the optimization was carried out. To date, there has been no literature report on the use of the laboratory-scale response surface-optimized conditions on a larger scale pilot plant setting. Therefore, it is also another objective of this work to investigate the possibility of up-scaling the response surface-optimized conditions.

MATERIALS AND METHODS

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 palmitic and oleic acids and glycerol with purities 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 high-performance liquid chromatography (HPLC) grade.

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 (S_r, 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, MN).

Esterification Reaction. Dioleopalmitin synthesis was carried out via esterification in a 50 mL conical flask. Palmitic and oleic acids (1:1 molar ratio) were added to glycerol at different substrate molar ratios (**Table 1**). The total substrate weight was fixed at 50 g. The substrate mixture was 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.

Acylglycerol Composition Analysis. Acylglycerol composition analysis was determined by reverse-phase 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 of volume was injected into the HPLC for analysis. The compositions of FFA, MAG, DAG, and TAG were calculated as wt % of the total acylglycerol content in the oil sample.

DAG Composition Analysis. Analysis of DAG components was performed using the method described by Lo et al. (*29*).

Production in Pilot Packed-Bed Enzymatic Reactor. A 16 L pilot packed-bed enzymatic reactor was used for this study. The pilot packed-bed enzymatic reactor was charged with 9 kg of FFAs 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 S_r = 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

Table 1. Experimental Runs for Five-Level–Four-Factor CCRD and the Comparison between Observed and Predicted Responses for DAG and TAG Yields[a]

					DAG (wt %)		TAG (wt %)	
no.	R_{temp}	Enz	S_r	R_{time}	actual	predicted	actual	predicted
1	55	4	2	3	45.92	46.80	23.68	23.57
2	75	4	2	3	57.31	55.88	30.70	30.59
3	55	10	2	3	50.68	48.67	27.07	26.96
4	75	10	2	3	38.31	40.87	31.55	31.44
5	55	4	3	3	41.88	40.45	36.46	36.57
6	75	4	3	3	38.42	39.30	41.84	41.95
7	55	10	3	3	47.65	50.21	28.88	28.99
8	75	10	3	3	34.19	32.18	36.77	36.88
9	55	4	2	8	51.4	51.49	38.61	38.50
10	75	4	2	8	54.67	54.04	34.13	34.02
11	55	10	2	8	45.93	46.97	45.33	45.22
12	75	10	2	8	41.06	40.56	50.50	50.39
13	55	4	3	8	44.13	43.50	36.84	36.95
14	75	4	3	8	35.72	35.81	34.23	34.34
15	55	10	3	8	31.85	31.35	49.48	49.59
16	75	10	3	8	13.67	14.71	82.43	82.54
17	45	7	2.5	5.5	29.72	29.71	14.30	14.32
18	85	7	2.5	5.5	36.91	36.89	41.83	41.82
19	65	1	2.5	5.5	43.42	44.51	21.75	21.70
20	65	13	2.5	5.5	30.16	29.07	24.35	24.34
21	65	7	1.5	5.5	36.15	36.14	28.47	28.89
22	65	7	3.5	5.5	21.73	21.75	46.08	45.66
23	65	7	2.5	0.5	25.74	25.76	29.75	29.73
24	65	7	2.5	10.5	45.71	45.71	22.89	22.86
25	65	7	2.5	5.5	43.21	41.70	18.68	20.71
26	65	7	2.5	5.5	41.15	41.70	20.90	20.71
27	65	7	2.5	5.5	41.52	41.70	20.97	20.71
28	65	7	2.5	5.5	42.55	41.70	19.20	20.71
29	65	7	2.5	5.5	39.64	41.70	23.54	20.71
30	65	7	2.5	5.5	42.13	41.70	20.99	20.71

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

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

enzyme bed at a flow rate of approximately 3.5 L/min. The enzyme bed had the following initial dimensions: bed diameter = 16.2 cm, and bed height = 5.5 cm.

Purification in a Pilot-Scale Short-Path Distillation. Crude DAG was purified using a 50 L falling film evaporator and short-path distillator. The distillation conditions are tabulated in **Table 2**. Other conditions were as follows: evaporator vacuum = 0.001 mbar, condenser temperature = 60 °C, and roller speed = 400 rpm.

Reusability of Lipozyme RM IM in Pilot Plant. Ten 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 %, was analyzed to determine enzyme reusability.

Statistical Analysis. The experimental data were analyzed by RSM using the software Design-Expert version 6.0.11 (Stat-ease Inc., Minneapolis, MN). 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.

Table 3. Regression Coefficients and Significance (*P*) Values for DAG Responses (Part A) and for TAG Responses (Part B) after Manual Elimination[a]

variables	coefficients	*P* value
	(A) DAG response	
intercept	−5281.07	<0.0001
R_{temp}	316.24	0.0002
Enz	7.04	<0.0001
S_r	199.07	<0.0001
R_{time}	20.47	0.0002
R_{temp}^2	−7.28	<0.0001
Enz^2	−0.14	0.0195
S_r^2	−63.02	<0.0001
R_{time}^2	−2.78	0.0073
$R_{temp} \times Enz$	−0.18	0.0002
$R_{temp} \times S_r$	−0.51	0.0009
$R_{temp} \times R_{time}$	−0.12	0.2558
$Enz \times S_r$	2.87	0.9515
$Enz \times R_{time}$	0.10	0.0010
$S_r \times R_{time}$	1.74	0.0016
R_{temp}^3	0.07	0.0007
S_r^3	6.70	0.0229
R_{time}^3	0.15	<0.0001
$R_{temp} \times Enz \times R_{time}$	0.01	0.0944
$Enz \times S_r \times R_{time}$	−0.52	0.0052
R_{temp}^4	-2.80×10^{-4}	<0.0001
	(B) TAG response	
intercept	20.71	<0.0001
R_{temp}	2.36	0.0057
Enz	6.07	<0.0001
S_r	4.19	<0.0001
R_{time}	10.12	<0.0001
R_{temp}^2	15.99	<0.0001
Enz^2	0.58	0.1255
S_r^2	4.14	<0.0001
R_{time}^2	1.40	0.0051
$R_{temp} \times Enz$	2.82	0.0004
$R_{temp} \times S_r$	1.96	0.0026
$R_{temp} \times R_{time}$	0.39	0.3624
$Enz \times S_r$	1.30	0.0167
$Enz \times R_{time}$	5.77	<0.0001
$S_r \times R_{time}$	0.22	0.6056
R_{temp}^3	1.13	0.0026
Enz^3	−1.35	0.0010
R_{time}^3	−2.96	<0.0001
$R_{temp} \times Enz \times S_r$	1.94	0.0028
$R_{temp} \times Enz \times R_{time}$	2.83	0.0004
$R_{temp} \times S_r \times R_{time}$	1.74	0.0046
$Enz \times S_r \times R_{time}$	3.42	0.0001
R_{temp}^4	−3.54	<0.0001
$R_{temp} \times Enz \times S_r \times R_{time}$	1.30	0.0167

[a] See **Table 1** for descriptions of abbreviations.

RESULTS AND DISCUSSION

Model Fitting for DAG. The experimental DAG data were best-fitted to a reduced quartic model by multiple regression after manual elimination. Factors and interactions that were not significant ($P > 0.1$) were eliminated from the model. The regression coefficients and *P* values for the DAG response are tabulated in **Table 3**, section a. 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 0.9842 and 0.9490, respectively, and the ANOVA showed no lack of fit of the model. The correlation between the predicted and the observed DAG responses was satisfactory (**Figure 1a**). 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

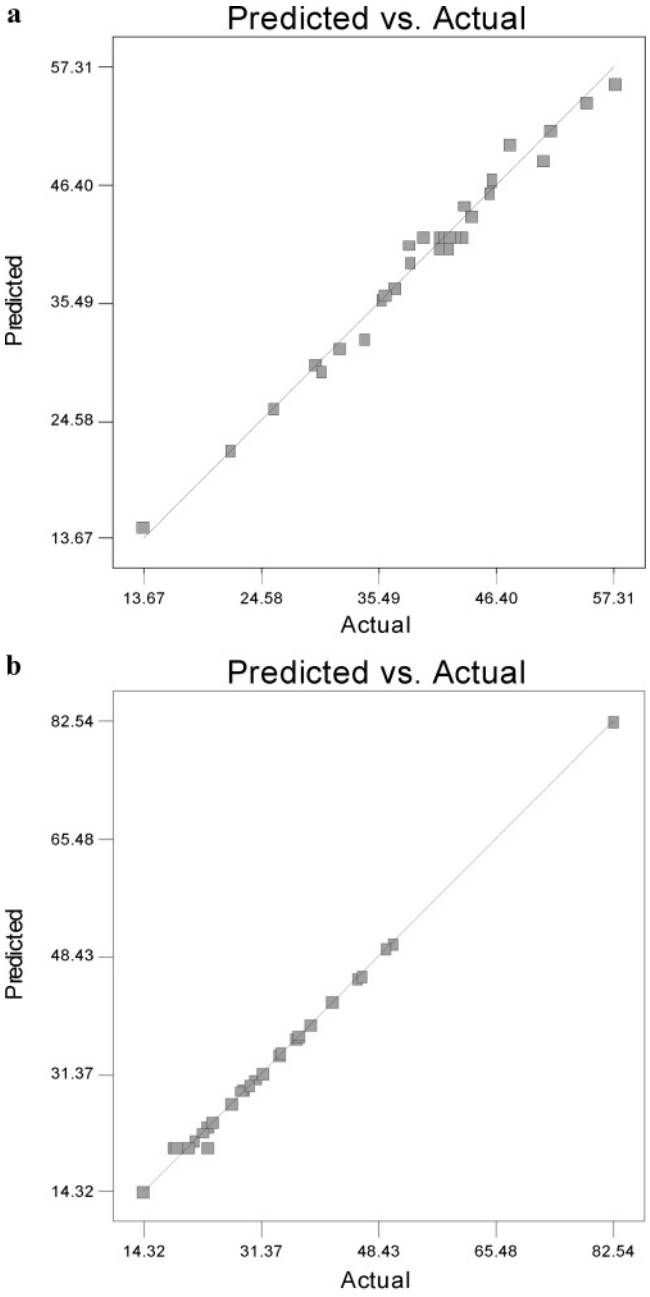


Figure 1. (**a**) Relationship between the observed and the predicted DAG responses. (**b**) Relationship between the observed and the predicted TAG responses.

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 Boyaci (*25*). The AAD value of the model was 2.24%. This means that the model for DAG response is acceptable for use in the optimization of DAG yield via esterification.

Model Fitting for TAG. The experimental TAG data were 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 3**, section b. 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 0.9972 and 0.9863, respectively, and the ANOVA showed no lack of fit of the model. The predicted and observed TAG responses were also sufficiently correlated (**Figure 1b**). The AAD value of the model was 1.38%. This means that the model for TAG response is suitable for use in the reverse optimization of TAG yield.

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 **Figure 2a**,**b**. All first-order coefficients of models for DAG response have positive effects. The second-order coefficient for S_r^2 has a negative effect, while most other coefficients have relatively small effects on DAG yield. R_{temp} has the most significant effect on DAG yield, followed by S_r. In the TAG response, first- and second-order coefficients have positive effects on yield, while third-order coefficients of Enz and R_{time} and fourth-order coefficient of R_{temp} showed significant negative effects. Similarly, R_{temp} is the most influential variable on TAG yield, followed by R_{time}.

Comparison of Response Contour Plots for DAG and TAG Yields. A clearer interpretation of the effects of variable interaction on DAG and TAG yields can be seen in the response contour plots of DAG and TAG (**Figure 3a**−**l**). From the contour plots (**Figure 3a**,**b**), at approximately the optimum operating temperature of lipozyme RM IM as suggested by the manufacturer ($R_{temp} = 65$ °C), DAG yield decreased by nearly 7 wt % while TAG yield slightly increased by about 3.3 wt % as Enz increased from 4 to 10 wt %. This effect suggests that increasing the dosage of lipozyme RM IM does not necessarily mean that a higher DAG yield can be attainable, especially when the system involves a reversible reaction with several products such as this. Higher lipase concentrations in the esterification system will increase the rate of the overall esterification reaction and may tend to sway the molecular equilibrium toward the extreme end of the esterification system, thereby gradually increasing the formation of TAG. Similar observations were also found in literature (*16*−*18*). In the corresponding TAG contour plot, a minimum point can be seen in the region of Enz = 4−5 wt %. This may suggest that lipozyme RM IM at 4−5 wt % concentrations may still give a reasonable DAG yield of approximately 47 wt %, while keeping TAG yield at the minimum level.

A similar observation was also noted in the interaction between S_r and R_{temp} on DAG and TAG yields (**Figure 3c**,**d**). At approximately 65 °C, DAG yield decreased by nearly 9 wt % while TAG yield gained by about the same figure as S_r increased from 2:1 to 3:1. This observation was expected as higher S_r stiochiometrically favors TAG formation. A stationary point was observed at $S_r = 2.15$ in the DAG plot, which corresponds to the lowest point of TAG yield, indicating that a fatty acid to glycerol molar ratio of 2.15 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 (**Figure 3e**,**f**) at approximately the suggested operating temperature of the lipase, the DAG yield was stationary at approximately 43 wt % from $R_{time} = 3.5$−4.5 h, before decreasing to 39.5 wt % as R_{time} approached 8 h. From the corresponding TAG contour plot, it can be observed that longer R_{time} resulted in higher TAG formation. On the basis of both the DAG and the TAG contour plots, it can be observed that an R_{time} of approximately 4 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*−*18*).

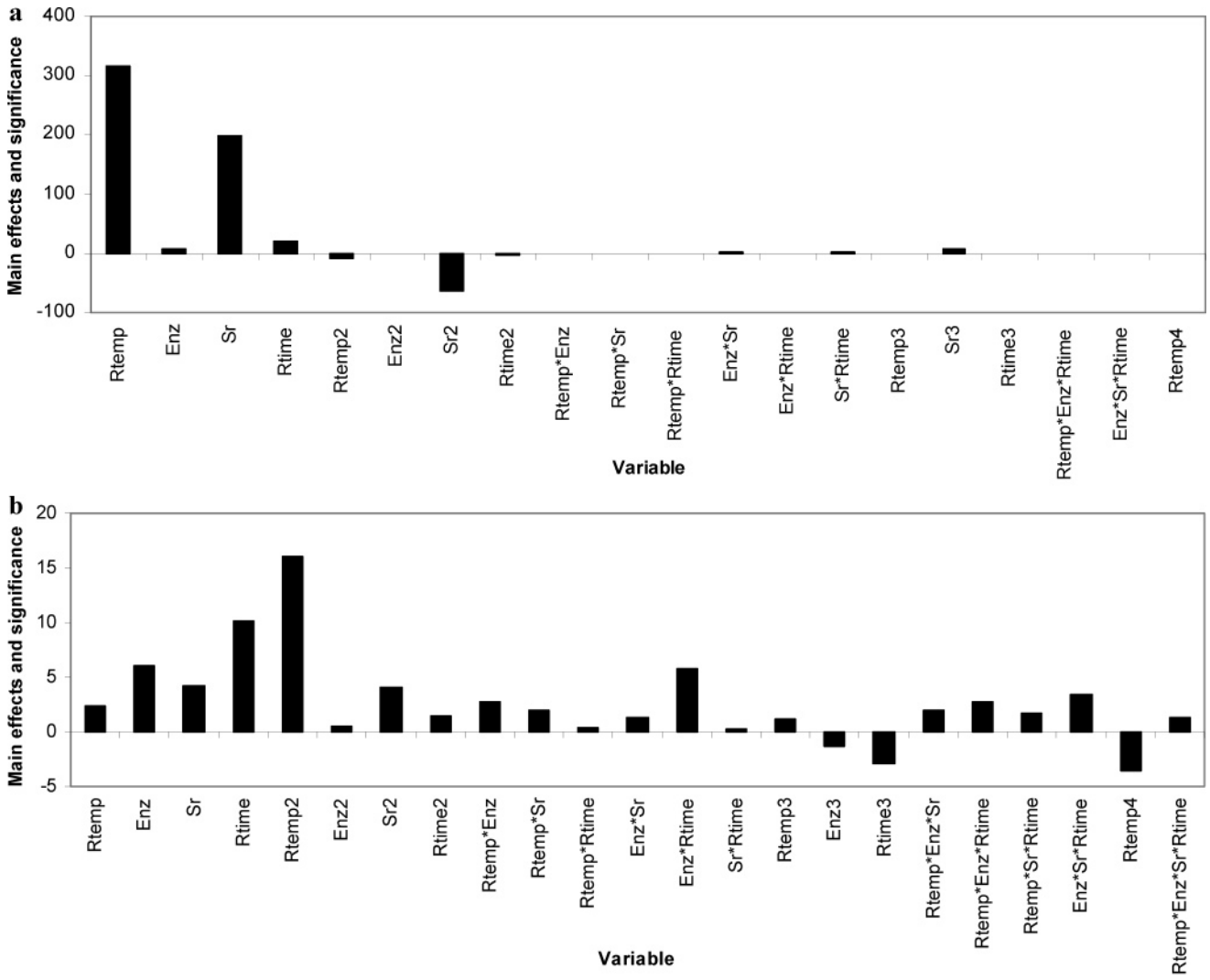


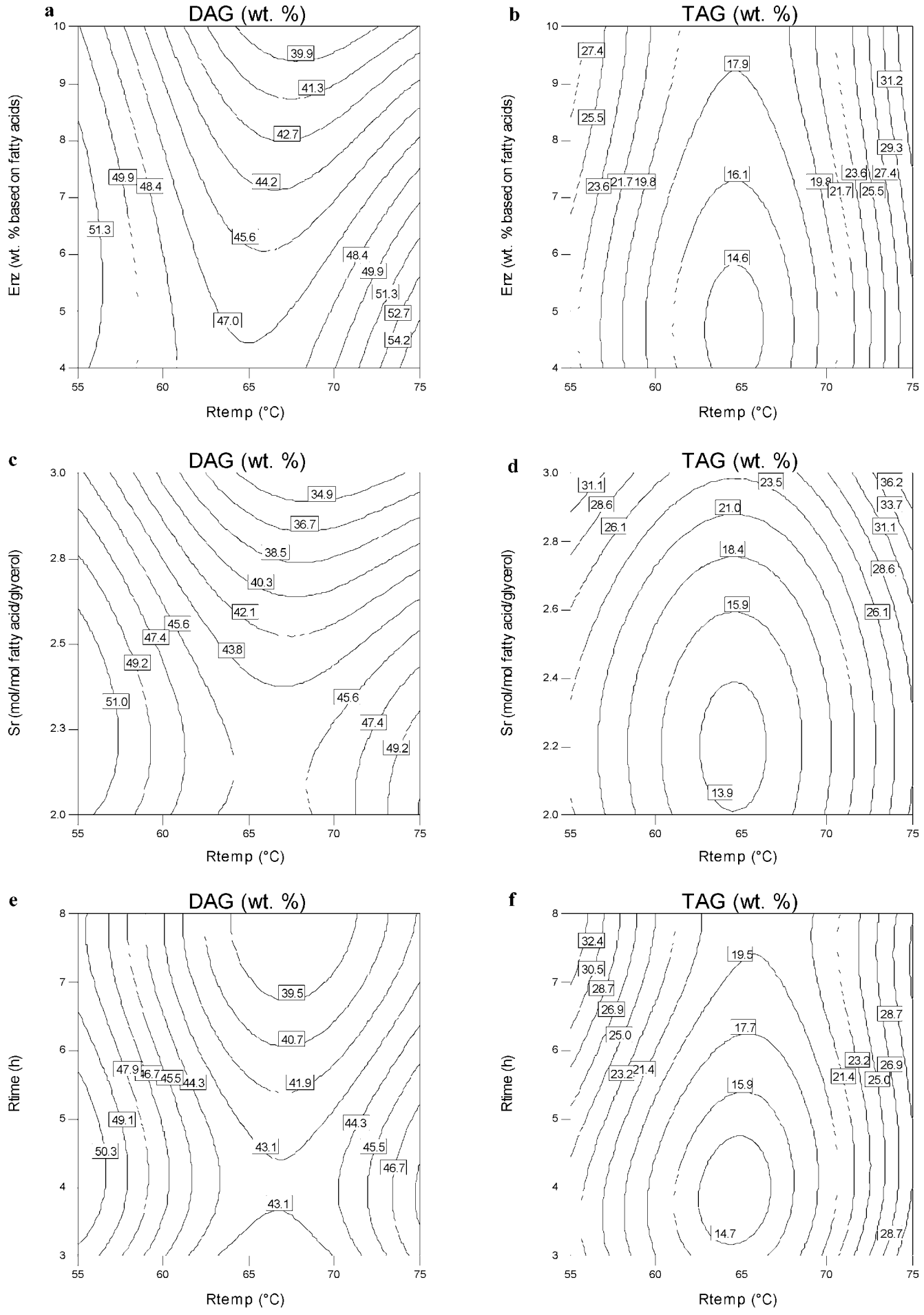
Figure 2. (**a**) Main effects and their significance of variables on lipozyme RM IM-catalyzed production of DAG. (**b**) Main effects and their significance of factors on lipozyme RM IM-catalyzed production of TAG.

On the basis of the contour plots (**Figure 3g**,**h**) showing the interactive effects of Sr and Enz, an increase in the effects of Sr and Enz, either individually or combined, will decrease DAG production and increase TAG yield. A positive peak at approximately Enz = 5.8 wt % and Sr = 2.2 was observed in the DAG contour plot. However, a negative peak was located within the vicinity (Enz = 4.5 wt % and S_r = 2.25) in the TAG contour plot. This indicates that the predicted conditions for optimum DAG production with minimal TAG formation fall within these points.

Similar patterns were also observed for the interactive effects of Enz and R_{time} (**Figure 3i**,**j**) and R_{time} and S_r (**Figure 3k**,**l**) on DAG and TAG yields. Positive peaks were observed at Enz = 6.2 wt %, R_{time} = 4.3 h and R_{time} = 4.1 h, S_r = 2.3 for DAG yields, while negative peaks at Enz = 4.9 wt %, R_{time} = 3.5 h and R_{time} = 3.5 h, S_r = 2.2 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 be 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. 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 toward 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.

Optimization of DAG Process. The most cost-efficient conditions for the production of DAG would use the lowest amount of lipase to achieve the highest DAG and lowest TAG yields in the shortest time. On the basis of 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 descending order of the 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 DAG yield (47.9 wt %) and minimal TAG (14.3 wt %) formation is as follows: R_{temp} = 66.29 °C, Enz = 4 wt %, S_r = 2.14, and R_{time} = 4.14 h.

Pilot Plant Production. The above optimized process conditions for DAG production were scaled up to a 16 L pilot packed-bed enzymatic reactor for model verification. The DAG and TAG yields from the 10 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


a
DAG (wt. %)
Enz (wt. % based on fatty acids)
Rtemp (°C)
39.9
41.3
42.7
44.2
45.6
47.0
48.4
49.9
51.3
52.7
54.2
49.9
48.4
51.3
b
TAG (wt. %)
Enz (wt. % based on fatty acids)
Rtemp (°C)
27.4
25.5
23.6
21.7
19.8
17.9
16.1
14.6
19.8
21.7
23.6
25.5
27.4
29.3
31.2
c
DAG (wt. %)
Sr (mol/mol fatty acid/glycerol)
Rtemp (°C)
34.9
36.7
38.5
40.3
42.1
43.8
45.6
47.4
49.2
51.0
45.6
47.4
49.2
d
TAG (wt. %)
Sr (mol/mol fatty acid/glycerol)
Rtemp (°C)
31.1
28.6
26.1
23.5
21.0
18.4
15.9
13.9
36.2
33.7
31.1
28.6
26.1
e
DAG (wt. %)
Rtime (h)
Rtemp (°C)
39.5
40.7
41.9
43.1
44.3
45.5
46.7
47.9
46.7
45.5
44.3
49.1
50.3
43.1
44.3
45.5
46.7
f
TAG (wt. %)
Rtime (h)
Rtemp (°C)
32.4
30.5
28.7
26.9
25.0
23.2
21.4
19.5
17.7
15.9
14.7
23.2
21.4
25.0
26.9
28.7
28.7

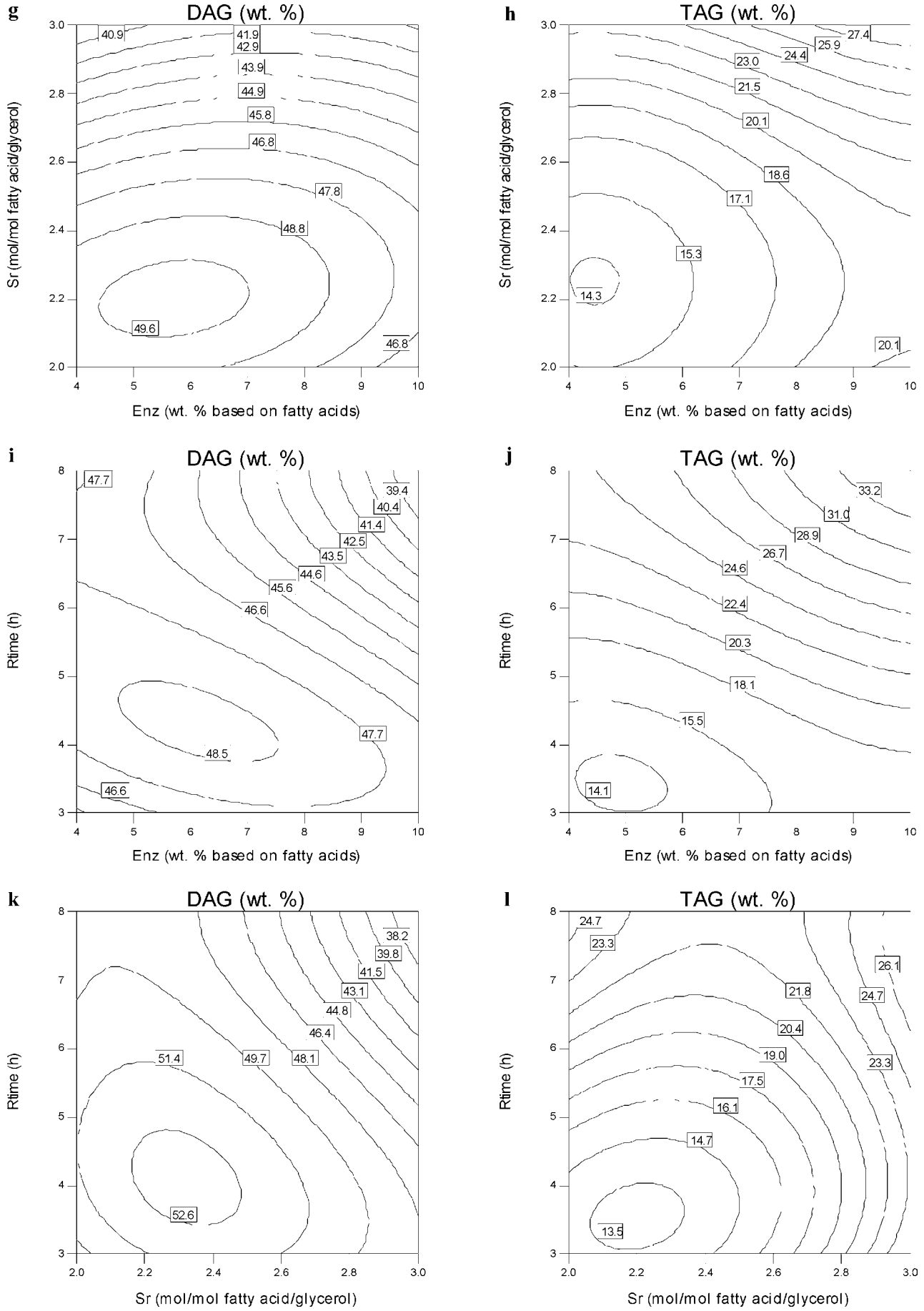


Figure 3. Contour plots of DAG and TAG responses showing interactions of factors: (**a**, **b**) Enz and R_{temp}, (**c**, **d**) S_r and R_{temp}, (**e**, **f**) R_{time} and R_{temp}, (**g**, **h**) S_r and Enz, (**i**, **j**) R_{time} and Enz, and (**k**, **l**) R_{time} and S_r.

Table 4. Optimized Esterification Reaction Conditions Based on Selected Criteria[a]

criteria	R_{temp}	Enz	S_r	R_{time}	DAG (wt %)	TAG (wt %)	desirability
minimum Enz	66.29	4.00	2.14	4.14	47.9	14.3	0.95
minimum Enz and R_{time}	65.96	4.00	2.11	3.00	45.6	14.7	0.94
minimum R_{temp}, Enz, and R_{time}	55.00	4.00	2.19	3.00	47.9	23.5	0.93
minimum R_{temp} and Enz	55.00	4.00	2.21	3.99	50.6	23.9	0.92
minimum R_{temp} and R_{time}	55.00	10.00	2.46	3.04	52.7	23.8	0.92
minimum R_{time}	62.05	5.52	2.20	3.00	45.6	14.3	0.92
neutral	66.29	4.01	2.14	4.15	47.9	14.3	0.91
minimum R_{temp}	55.00	10.00	2.46	3.07	52.7	23.8	0.91
minimum R_{temp} and R_{time} and maximum S_r	55.00	9.94	3.00	3.00	50.2	29.1	0.90
minimum R_{temp} and maximum S_r	55.00	10.00	2.99	3.00	50.3	28.9	0.88
maximum S_r and minimum R_{time}	55.22	9.97	3.00	3.00	49.9	28.8	0.88
minimum R_{temp}, Enz, and R_{time} and maximum S_r	55.00	4.00	2.81	3.09	43.6	31.4	0.86
minimum R_{temp} and Enz and maximum S_r	55.00	4.00	2.86	4.49	45.6	31.4	0.86
minimum Enz and R_{time} and maximum S_r	61.19	4.00	2.79	3.10	39.7	22.8	0.86
minimum Enz and maximum S_r	61.24	4.00	2.84	4.56	41.0	21.9	0.85
maximum S_r	55.15	10.00	3.00	3.00	50.0	28.8	0.85

[a] See **Table 1** for descriptions of abbreviations.

Table 5. DAG and TAG Yields and DAG Composition from Pilot Packed-Bed Enzyme Reactor Productions[a]

production no.	DAG yield (wt %)	error	TAG yield (wt %)	error	DAG composition (wt %)					
					1,3-OO	1,2-OO	1,3-PO	1,2-PO	1,3-PP	1,2-PP
1	43.8	4.1	19.2	4.9	4.8	11.4	12.7	5.8	0.2	0.3
2	47.7	0.2	16.9	2.6	5.2	12.5	14.1	6.3	0.2	0.3
3	46.8	1.1	14.9	0.6	5.1	12.3	13.9	6.0	0.2	0.3
4	47.2	0.7	12.8	1.5	5.1	12.4	13.9	6.2	0.2	0.3
5	47.2	0.7	13.3	1.0	5.1	12.2	13.7	6.2	0.2	0.4
6	46.3	1.6	11.0	3.3	5.0	12.1	13.6	6.1	0.1	0.3
7	45.2	2.7	12.3	2.0	5.0	11.8	13.4	5.8	0.2	0.3
8	46.4	1.5	11.7	2.6	5.0	12.2	13.7	6.1	0.2	0.3
9	46.9	1	13.5	0.8	5.1	12.2	13.6	6.2	0.2	0.4
10	47.3	0.6	12.4	1.9	5.1	12.4	13.9	6.2	0.2	0.3

[a] 1,3-OO = 1,3-diolein; 1,2-OO = 1,2-diolein; 1,3-PO = 1-palmitoyl-3-oleoyl-glycerol; 1,2-PO = 1-palmitoyl-2-oleoyl-glycerol; 1,3-PP = 1,3-dipalmitin; and 1,2-PP = 1,2-dipalmitin.

be attributed to the presence of some unimmobilized lipases in the enzyme preparation causing a tilt in the molecular equilibrium of DAG toward TAG synthesis as abovementioned. This goes to show that even after 10 consecutive pilot productions, lipozyme RM IM did not significantly lose enzyme activity at the optimized reaction conditions. In a related work (*20*), the author reported significant losses in enzyme activity and a drastic drop in DAG yield of 10 wt % after five consecutive runs in a pilot-scale batch stirred-tank enzyme reactor. On the basis of the current observation, lipozyme RM IM is best-suited to be used in a packed-bed configuration to ensure prolonged enzyme half-life and increased enzyme productivity.

DAG Composition. The crude DAG product from all 10 pilot productions were analyzed to check for any changes in DAG composition that may be caused by changes in fatty acid selectivity of lipozyme RM IM as a function of repeated usage. The DAG profiles of the 10 samples are shown in **Table 5**. There were no observed changes in fatty acid selectivity of the lipase as the pilot productions proceeded until the tenth batch. However, the DAG profiles in all of the pilot productions showed that the concentration of total diolein (16.2−17.7 wt %) was significantly higher than that of total dipalmitin (0.4−0.6 wt %). This observation revealed that, for DAG synthesis in the presence of equimolar concentrations of oleic and palmitic acids, lipozyme RM IM has a higher selectivity toward the synthesis of oleic acid-enriched DAG rather than palmitic acid-enriched DAG. A similar observation was also reported in literature (*30*).

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 DAG oil was approximately 92 wt %.

LITERATURE CITED

(1) Taguchi, H.; Watanabe, H.; Onizawa, K.; Nagao, T.; Gotoh, N.; Yasukawa, T.; Tsushima, R.; Shimazaki, H.; Itakura, H. Double-blinded controlled study on the effects of dietary diacylglycerol on postprandial serum and chylomicron triacylglycerol responses in healthy humans. *J. Am. Coll. Nutr.* **2000**, *19*, 789−796.

(2) Tada, N.; Watanabe, H.; Matsuo, N.; Tokimitsu, I.; Okazaki, M. Dynamics of postprandial remnant-like lipoprotein particles in serum after loading of diacylglycerols. *Clin. Chim. Acta* **2001**, *311*, 109−117.

(3) Yamamoto, K.; Asakawa, H.; Tokunaga, K.; Watanabe, H.; Matsuo, N.; Tokimitsu, I.; Yagi, N. Long-term ingestion of dietary diacylglycerol lowers serum triacylglycerol in Type II diabetic patients with hypertriglyceridemia. *J. Nutr.* **2001**, *131*, 3204−3207.

(4) Nagao, T.; Watanabe, H.; Goto, N.; Onizawa, K.; Taguchi, H.; Matsuo, N.; Yasukawa, T.; Tsushima, R.; Shimasaki, H.; Itakura, H. Dietary diacylglycerol suppresses accumulation of body fat compared to triacylglycerol in men in a double-blind controlled trial. *J. Nutr.* **2000**, *130*, 792−797.

(5) Maki, K. C.; Davidson, M. H.; Tsushima, R.; Matsuo, N.; Tokimitsu, I.; Umporowics, D. M.; Dicklin, M. R.; Foster, G. S.; Ingram, K. A.; Anderson, B. D.; Frost, S. D.; Bell, M. Consumption of diacylglycerol oil as part of a reduced-energy diet enhances loss of body weight and fat in comparison with consumption of a triacylglycerol control oil. *Am. J. Clin. Nutr.* **2002**, 1230−1236.

(6) Teramoto, T.; Watanabe, H.; Ito, K.; Omata, Y.; Furukawa, T.; Shimoda, K.; Hoshino, M.; Nagao, T.; Naito, S. Significant effects of diacylglycerol on body fat and lipid metabolism in patients on hemodialysis. *Clin. Nutr.* **2004**, *23*, 1122−1126.

(7) Taguchi, H.; Nagao, T.; Watanabe, H.; Onizawa, K.; Matsuo, N.; Tokimitsu, I.; Itakura, H. Energy value and digestibility of dietary oil containing mainly 1,3-diacylglycerol are similar to those of triacylglycerol. *Lipids* **2001**, *36*, 379−382.

(8) Watanabe, H.; Onizawa, K.; Naito, S.; Taguchi, H.; Goto, N.; Nagao, T.; Matsuo, N.; Tokimitsu, I.; Yasukawa, T.; Tsushima, R.; Shimasaki, H.; Itakura, H. Fat-soluble vitamin status is not affected by diacylglycerol consumption. *Ann. Nutr. Metab.* **2001**, *45*, 259−264.

(9) Yasunaga, K.; Glinsmann, W. H.; Seo, Y.; Katsuragi, Y.; Kobayashi, S.; Flickinger, B.; Kennepohl, E.; Yasukawa, T.; Borzelleca, J. F. Safety aspects regarding the consumption of high-dose dietary diacylglycerol oil in men and women in a double-blind controlled trial in comparison with consumption of a triacylglycerol control oil. *Food Chem. Toxicol.* **2004**, *42*, 1419−1429.

(10) Soni, M. G.; Kimura, H.; Burdock, G. A. Chronic study of diacylglycerol oil in rats. *Food Chem. Toxicol.* **2001**, *39*, 317−329.

(11) Sugano, M.; Akahoshi, A.; Nishida, E.; Shibata, A.; Ohkawa, Y. Dimethylbenz(a)anthracene-induced mammary tumorigenesis in Sprague-Dawley rats fed saturated and polyunsaturated triacylglycerols and diacylglycerols. *J. Oleo Sci.* **2002**, *9*, 583−588.

(12) Kasamatsu, T.; Ogura, R.; Ikeda, N.; Morita, O.; Saigo, K.; Watabe, H.; Saito, Y.; Suzuki, H. Genotoxicity studies on dietary diacylglycerol (DAG) oil. *Food Chem. Toxicol.* **2005**, *43*, 253−260.

(13) Meguro, S.; Osaki, N.; Onizawa, K.; Yajima, N.; Hase, T.; Matsuo, N.; Tokimitsu, I. Comparison of dietary triacylglycerol oil and diacylglycerol oil in protein kinase C activation. *Food Chem. Toxicol.* **2007**, doi: 10.1016/j.fct.2006.12.024.

(14) Yasukawa, T.; Katsuragi, Y. Diacylglycerols. In *Diacylglycerol Oil*; Katsuragi, Y., Yasukawa, T., Matsuo, N., Flickinger, B. D., Tokimitsu, I., Matlock, M. G., Eds.; AOCS Press: Champaign, Illinois, 2004; pp 1−15.

(15) Sonntag, N. O. V. Glycerolysis of fats and methyl esters—Status, review and critique. *J. Am. Oil Chem. Soc.* **1982**, *75*, 1359−1365.

(16) Lo, S. K.; Baharin, B. S.; Tan, C. P.; Lai, O. M. Lipase-catalysed production and chemical composition of diacylglycerols from soybean oil deodoriser distillate. *Eur. J. Lipid Sci. Technol.* **2004**, *106*, 218−224.

(17) Lo, S. K.; Baharin, B. S.; Tan, C. P.; Lai, O. M. Diacylglycerols from palm oil deodoriser distillate: Part 1. Synthesis by lipase-catalysed esterification. *Food Sci. Technol. Int.* **2004**, *10*, 149−156.

(18) Lo, S. K.; Baharin, B. S.; Tan, C. P.; Lai, O. M. Enzyme-catalyzed production and chemical composition of diacylglycerols from corn oil deodorizer distillate. *Food Biotechnol.* **2004**, *18*, 265−278.

(19) Yang, T.; Zhang, H.; Mu, H.; Sinclair, A. J.; Xu, X. Diacylglycerols from butterfat: Production by glycerolysis and short-path distillation and analysis of physical properties. *J. Am. Oil Chem. Soc.* **2004**, *81*, 979−987.

(20) Kristensen, J. B.; Xu, X.; Mu, H. Process optimisation using response surface design and pilot plant production of dietary diacylglycerols by lipase-catalysed glycerolysis. *J. Agric. Food Chem.* **2005**, *53*, 7059−7066.

(21) Plou, F. J.; Barandiarán, M.; Calvo, M. V.; Ballasteros, A.; Pastor, E. High-yield production of mono- and di-oleylglycerol by lipase-catalysed hydrolysis of triolein. *Enzyme Microb. Technol.* **1996**, *18*, 66−71.

(22) Weber, N.; Mukherjee, K. D. Solvent-free lipase-catalyzed preparation of diacylglycerols. *J. Agric Food Chem.* **2004**, *52*, 5347−5353.

(23) Yamada, Y.; Shimizu, M.; Sugiura, M.; Yamada, N. Process for producing diglycerides. KAO CORP (JP) Ed. Patent WO9909119, 1999.

(24) Myers, R. H.; Montgomery, D. C. *Response Surface Methodology: Process and Product Optimisation Using Designed Experiments*, 2nd ed.; Myers, R. H., Montgomery, D. C., Eds.; John Wiley and Sons: New York, 2002.

(25) Baş, D.; Boyacı, İ. H. Modeling and optimisation I: Usability of response surface methodology. *J. Food Eng.* **2007**, *78*, 836−845.

(26) Krishna, S. H.; Sattur, A. P.; Karanth, N. G. Lipase-catalyzed synthesis of isoamyl isobutyrate—Optimisation using a central composite rotatable design. *Process Biochem.* **2006**, *37*, 9−16.

(27) Shih, I.-L.; Hung, S.-H.; Chen, F.-Y.; Ju, H.-Y.; Shieh, C.-J. Optimized synthesis of lipase-catalysed L-menthyl butyrate by *Candida rugosa* lipase. *Food Chem.* **2007**, *100*, 1223−1228.

(28) Jeong, G.-T.; Park, D.-H. Response surface methodological approach for optimisation of enzymatic synthesis of sorbitan methacrylate. *Enzyme Microb. Technol.* **2006**, *39*, 381−386.

(29) Lo, S. K.; Baharin, B. S.; Tan, C. P.; Lai, O. M. Analysis of 1,2(2,3)- and 1,3-positional isomers of diacylglycerols from vegetable oils by reversed-phase high-performance liquid chromatography. *J. Chromatogr. Sci.* **2004**, *42*, 145−154.

(30) Lo, S. K.; Baharin, B. S.; Tan, C. P.; Lai, O. M. Diacylglycerols from palm oil deodoriser distillate: Part 2. Physical and chemical characterization. *Food Sci. Technol. Int.* **2004**, *10*, 157−161.

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