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Enzymatic Production of Monoacylglycerols Containing Polyunsaturated Fatty Acids through an Efficient Glycerolysis System

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The aim of the study was to develop an efficient glycerolysis system for the enzymatic production of monoacylglycerols (MAGs) containing polyunsaturated fatty acids. Glycerolysis has been widely applied in industry for the chemical production of food MAGs under high temperature. The enzymatic glycerolysis system at 40−70 °C is unfortunately a multiphase system, which leads to the lower reaction efficiency. A *tert*-butyl alcohol system was developed after careful evaluation and more than 20-fold of the reaction efficiency from this system was obtained compared to the solvent-free system. Novozym 435 was employed as a catalyst in the glycerolysis from the screening. In the batch reaction system with *tert*-butyl alcohol, temperature higher than 40 °C was favored. The glycerol/oil ratio was best in the study with 4.5 while the solvent weight ratio from 1 to 3 had little effect. In general, 60−70% yield can be obtained at 2 h in the stirred tank reactor. The continuous glycerolysis was conducted in a packed bed reactor. MAG yield up to 70% was reached at 30−40 min residence time. The continuous glycerolysis was more sensitive to the amount of *tert*-butyl alcohol, and in the weight ratio to oil more than 2 was favored. The continuous process was optimized with the assistance of response surface methodology. Optimal conditions for the packed bed reactor after all considerations were recommended as glycerol/oil 4:1 (mol/mol), temperature 40 °C, and residence time 45 min. The operation stability study showed that there was no slight reduction of reaction performance at more than 30 days, implying a high feasibility in practical applications.

KEYWORDS: Bioreactor; *Candida antarctica* lipase; emulsifier; glycerolysis; monoacylglycerols; Novozym 435; optimization; sunflower oil

INTRODUCTION

Commercial food monoacylglycerols (MAGs) are manufactured by chemical glycerolysis of fats and oils in a batch or a continuous process. High temperature (220−250 °C) and inorganic alkaline catalysts are used to accelerate the reaction. Continuous operation produces better quality of MAGs than the batch method because there is less damage to the fat with shorter heating and reaction time. Limitations of the chemical processes include formation of undesirable dark color and burnt taste, maximum MAG yield of 40−60%, and adoption of expensive molecular distillation. In particular, chemical processes are not quite suitable to those heat-sensitive oils and fats concerning nutritional or biological properties (*1*−*3*).

Lipase-catalyzed glycerolysis of oils and fats at atmospheric pressure and lower temperature have attracted interest in both academia and industry, which is believed to be a practical alternative method for the chemical methods in the production of commercial MAGs. Several glycerolysis systems have been investigated with or without organic solvents, with immobilized or nonimmobilized enzymes, and in microemulsion or other media. In general, the MAG yields of enzymatic glycerolysis were usually low (*2*−*7*). In a typical reaction strategy called solid-phase reaction, glycerolysis was carried out below the critical temperature (T_c), in which MAGs were instantaneously crystallized so as to break the reaction equilibrium, leading to the high yield of MAGs (*8*−*9*). However, the reaction was very long (up to a week), and it was not very practical from an industrial point of view. The reuse of enzymes could also be difficult. In other solvent-free systems, it was difficult to attain a high yield of MAGs in the enzymatic glycerolysis (*5*, *10*−*11*). Solvents such as *n*-hexane, dioxane, acetonitrile, acetone, and *tert*-butyl alcohol or their mixtures were used in the lipase-catalyzed interesterification (*5*, *11*). However, there have been no careful evaluations on potential and practical applications with a view of industrial processing.

Glycerolysis system with an immobilized lipase as catalyst is a three-phase system: a hydrophobic oil phase, a hydrophilic glycerol phase, and a solid enzyme phase. Since the more

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hydrophilic characteristics of the enzyme, glycerol often binds to the enzyme particles so that the excess of the oil molecules to the enzyme is difficult. The mass transfer of glycerol is also limited. Because of this reason, the reaction efficiency is usually low even though the efficiency can be improved through optimization in a low range. It is reported that glycerol can be immobilized on silica gel so as to overcome the problem (*2*−*3*). The improvement is only minor, not to say the difficulty in practical operations. Therefore, a solvent medium is actually an important solution to improve the homogeneity of the system.

The single solvent that could hold oil and glycerol in a homogeneous system is actually very difficult to find, especially considering the safety issue of the solvent for food applications. The hydrocarbon solvents are generally impossible for this purpose. After ruling out those toxic or uncommon solvents from the list, very few are left, especially considering the effect on enzyme activity. Among those left, a few alcohols more than five carbons can be considered since they contain a polar −OH group and a nonpolar carbon chain. This gives a possibility to hold oil and glycerol in one system.

Alcohols are naturally reaction competitors to glycerol, especially those primary alcohols. The use of tertiary alcohols is therefore the primary consideration since the tertiary structure will have strong steric hindrance for the enzyme activity. This assumption is actually confirmed by previous studies with *tert*-butyl alcohol (*5*, *10*, *12*−*13*). Jansen et al. (*14*) also found that the use of *tert*-butyl alcohol favored the synthesis of monoesters. Higher yield of MAGs was achieved in the glycerolysis with *tert*-butyl alcohol (*11*).

Batch-stirred tank reactors are commonly available in a factory. It has been also widely used in many studies (*2*−*3*). On the other hand, packed bed reactors are the most frequently used reactors for immobilized lipases. They are best used continuously on a commercial scale so as to minimize labor and overhead costs. Packed bed reactors need relatively low power input and have the lowest reactor volume, because of the high enzyme/substrate ratio maintained. The highest enzyme-to-substrate ratio will result in the highest reaction rate and the least reaction time needed to reach a certain extent of conversion (*10*, *15*−*16*).

In this study, we intended to build an efficient system for the enzymatic production of monoacylglycerols from polyunsaturated oils. The process should be simple and easy-handling. The reaction time should be short but the yield should be reasonably high, at least comparable to the chemical process. For this intention, we selected *tert*-butyl alcohol as the solvent and Novozym 435 as catalyst after screening for the reaction system. Both reactors were studied concerning parameters and optimal conditions. The process stability has also been evaluated.

MATERIALS AND METHODS

Materials. Sunflower oil, *tert*-butyl alcohol, and glycerol were supplied by Danisco A/S, Brabrand, Denmark. The quality characteristics of the sunflower oil were water content of 0.06%, free fatty acid content 0.05%, and peroxide value 0.3 meq/kg. The glycerol was analytical grade with 0.2% water. The properties of *tert*-butyl alcohol are boiling point 83 °C, melting point 25 °C, relative density (water = 1) 0.8, vapor pressure (20 °C) 4.1 kPa, relative vapor density (air = 1) 2.6, flash point 11 °C, autoignition temperature 470 °C, explosive limit 2.4−8.0 vol % in air, octanol/water partition coefficient (log $P_{o/w}$) 0.4, and appearance colorless liquid or crystalline powder. Lipozyme TL IM, a silica granulated *Thermomyces lanuginosa* lipase, Lipozyme RM IM, an immobilized lipase from *Rhizomucor miehei*, and Novozym 435, a lipase from the *Candida antarctica* submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and adsorbed on a macroporous resin, were supplied by Novozymes A/S, Bagsvaerd, Denmark. Linoleic acid, monolinolein, dilinolein, and trilinolein standards were purchased from Sigma (St. Louis, MO). All other reagents and solvents were of analytical grades.

Enzymatic Glycerolysis in a Batch Reactor. The mixture of 20 g of sunflower oil, required amount of glycerol, and required amount of *tert*-butyl alcohol were incubated in a capped 50-mL flask at the designed conditions under 700 rpm magnetic stirring in a water bath. The reaction was initiated by the addition of lipase. At selected intervals, 0.5 mL of the reaction mixture was withdrawn and mixed with 1 mL of chloroform. The mixture was filtered through a microfilter (0.3−0.5 μm) to remove the lipase. All samples were stored at −20 °C prior to analysis. Experimental repeatability for batch reactions was conducted through three experiments under the following conditions: temperature 40 °C, glycerol/oil molar ratio 4.5:1, *tert*-butyl alcohol/oil 2.2:1 (wt/wt), 15 wt % Novozym 435 (based on oil and glycerol), and no additional water. The standard deviations were calculated for MAG 1,2-DAG, 1,3-DAG, TAG, and FFA as less than ±2.6%, 1.2%, 1.5%, 1.6%, and 0.3%, respectively, for all the data under 1-, 2-, and 3-h reaction times.

Enzymatic Glycerolysis in Continuous Reactor. Oil, *tert*-butyl alcohol, and glycerol were well-mixed in a feeding flask and maintained at 35 °C. The mixture was pumped through a continuous reactor (packed bed column with Novozym 435) at the designed conditions. The detail process setup, porosity determination, and operation method are described elsewhere (*17*−*18*). Under each setting conditions, the samples or products were collected from the outlet after running in two residence times to ensure a representative sample. A 5-mL reaction mixture was collected and 1 mL of the sample was added to 1 mL chloroform. The prepared samples were stored at −20 °C until analysis.

Analysis of Lipid Profiles by High-Performance Thin-Layer Chromatography (HPTLC). The lipid profiles were analyzed by a HPTLC system (DESAGA GmbH, Wiesloch, Germany) consisting of an AS 30 TLC Applicator, Densitometer CD 60 Scanner, and ProQuant Control and Evaluation Software. TLC plates (20 × 10 cm, Silica gel 60, Merck, Darmstadt, Germany) were developed (washed) by chloroform in an HPTLC horizontal developing chamber (20 × 10 cm, Muttene, Switzerland), dried at 120 °C for 30 min, and stored in a desiccator for later uses. The sample from glycerolysis was applied to the activated TLC plate by HPTLC Application AS 30. On each plate, three standard solutions (linoleic acid, monolinolein, dilinolein, and trilinolein in chloroform) were also applied and used for calibration. The plate was developed in a developing chamber with chloroform/acetone (90:10, v/v) (the chamber was allowed to saturate for 10 min before development). After development, the plate was dried with a fan for 10 s and in air 30 min, sprayed with a charring agent (a solution of 15.6 g cupric sulfate pentahydrate, 9.4 mL phosphoric acid (85%), and 100 mL distillate water) by an automatic sprayer, and dried at 180 °C for 15 min in an oven. After cooling to ambient temperature, the plate was scanned lane by lane at 510 nm using Densitometer CD 60. The data from the scanning were processed by the ProQuane software. Results were calculated on the basis of standards and expressed as weight percentages. Content values are reported as the mean of three runs (standard deviation less than ±2.4%, ±0.9%, ±1.2%, ±1.9%, and ±0.4% for MAG, 1,2-DAG, 1,3-DAG, TAG, and FFA, respectively).

Experimental Design and Statistical Analysis. Response surface methodology (RSM) enables evaluation of effects of multiple parameters, alone or in combination, on response variables. Software Modde 6.0 (Umetri, Umeå, Sweden) was used to assist the design, statistical analysis, and reaction optimization. A 3 factor fractional factorial design with five central points was adopted to optimize the continuous glycerolysis of sunflower oil. Three factors chosen for the optimization were reaction temperature (Te), molar ratio of glycerol/oil (G/O), and residence time (*t*). The variables and their levels were selected from single factors studies and are presented in **Table 1**. The quadratic response surface model was fitted as the following equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum {}^{2}_{i=1} \sum_{j=i+1}^{3} \beta_{ij} X_i X_j \qquad (1)$$

Table 1. Set Factor Levels and Observed Responses in Response Surface Methodology Experiments for Enzymatic Glycerolysis in the Packed Bed Reactor

exp. no.[a]	Te (°C)	G/O (mol:mol)	residence time (min)	MAG (wt %)	1,2-DAG (wt %)	1,3-DAG (wt %)
1	40	2	30	50.3	17.3	22.9
2	60	2	30	57.1	15.3	24.2
3	40	5	30	69.8	11.7	18.5
4	60	5	30	68.0	12.2	19.8
5	40	2	50	60.7	15.3	24.0
6	60	2	50	65.7	12.8	21.5
7	40	5	50	70.8	10.2	19.0
8	60	5	50	70.9	11.8	17.2
9	35	3.5	40	70.8	11.3	17.9
10	65	3.5	40	69.7	11.4	18.8
11	50	1.25	40	53.4	13.8	22.5
12	50	5.75	40	72.2	10.8	17.0
13	50	3.5	25	71.5	10.9	17.6
14	50	3.5	55	69.2	11.2	19.6
15	50	3.5	40	67.2	13.1	19.7
16	50	3.5	40	68.9	11.5	19.7
17	50	3.5	40	67.2	12.4	20.4
18	50	3.5	40	68.3	11.5	20.2
19	50	3.5	40	68.1	12.1	19.8

[a] Abbreviations: exp. no. (experimental number), Te (temperature), G/O (glycerol/oil), MAG (monoacylglycerol), DAG (diacylglycerol).

where Y is the response (the yield of MAGs, wt %), β_0 = intercept, β_i = first-order model coefficients, β_{ii} = quadratic coefficients for the ith variable, β_{ij} = interaction coefficients for the interaction of variables i and j, and X_i = independent variables. Second-order coefficients were generated by regression analysis with backward elimination. Responses were first fitted for the factors by partial least-squares regressions. The fit of the model was evaluated by the coefficients of determination (R^2) and analysis of variance. The insignificant factors were eliminated from evaluation and the model was finally refined.

RESULTS AND DISCUSSION

Lipase Screening. There are three commercially available immobilized lipases which are popular to the public: Novozym 435 (nonspecific), Lipozyme RM IM (1,3-specific), and Lipozyme TL IM (1,3-specific). They are widely used in the preparation of structured lipids (*15*). Under the *tert*-butyl alcohol medium (2/1 wt/wt to oil), the glycerolysis of sunflower oil with the three lipases was carried out at 40 °C, 15 wt % lipase load (based on total substrates), 4.5:1 (mol/mol) glycerol/oil, and reaction time 8 h. Novozym 435 showed highest activity with 71% yield of MAGs, while Lipozyme RM IM gave the lowest yield with only 36%. Lipozyme TL IM showed slight lower activity (67% yield) than Novozym 435, but the mixture became a semisolid fluid, leading to difficulty for enzyme recovery. This is due to the silica characteristics of the lipase carrier since the particle structure cannot be maintained in such a hydrophilic system (*19*). The lower activity of Lipozyme RM IM is due to its water sensitivity since the alcohol medium will deprive the water from the enzymes whereas Novozym 435 has no problem in such polar systems as previously studied (*20*). As a general conclusion, Novozym 435 was selected for further process studies. Since its low water requirement, the process can have a big benefit, that is, a lower free fatty acid content in the products. This has been confirmed by experiments (data not shown). This is a very important issue for industrial applications since higher free fatty acid (FFA) content will lead to the loss of oils as well as the difficulty of the process.

Novozym 435-Catalyzed Glycerolysis in Batch Reactor. A typical time course of sunflower oil glycerolysis in the batch reactor is given in **Figure 1**. Under the set conditions, the equilibrium was reached in about 2 h with no triacylglycerols (TAGs) left and MAG yield up to 70%. When 3 wt % additional water was added to the system, there was no significant difference of the time course except that FFA content was increased from 4 to 18% (data not shown). Therefore, no additional water is needed for Novozym 435 in the glycerolysis system with *tert*-butyl alcohol as the medium. The MAG yield after equilibrium from the time course is the same as the theoretical value under the substrate molar ratio 4.5:1. The theoretical molar percentages of MAGs, diacylglycerols (DAGs), and TAGs under this ratio are 80.75, 17.92, and 1.33%, respectively, when glycerol is removed from the calculation (*21*). If the molar percentages are changed into weight percentages with the molecular weight of sunflower oil fatty acids, the corresponding weight percentages are approximately 70, 27, and 3%, respectively. This indicates that the reaction reached maximum chemical equilibrium.

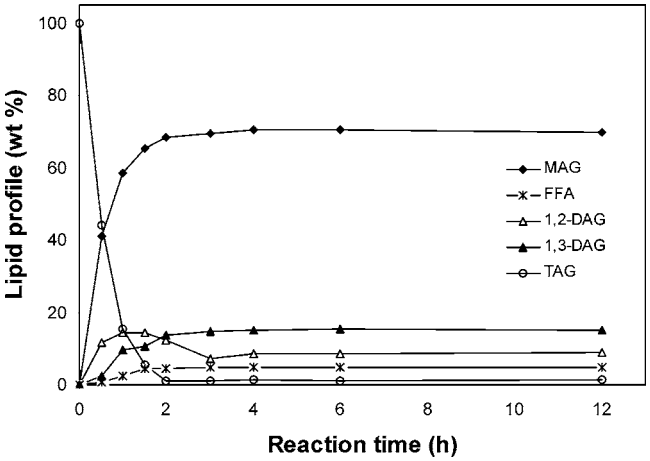

Figure 1. Glycerolysis time courses of sunflower oil in *tert*-butyl alcohol. Reaction conditions: temperature 40 °C, glycerol/oil molar ratio 4.5:1, *tert*-butyl alcohol/oil 2.2:1 (wt/wt), 15 wt % Novozym 435 (based on oil and glycerol), and no additional water. Abbreviations: MAG (monoacylglycerol), DAG (diacylglycerol), TAG (triacylglycerol).

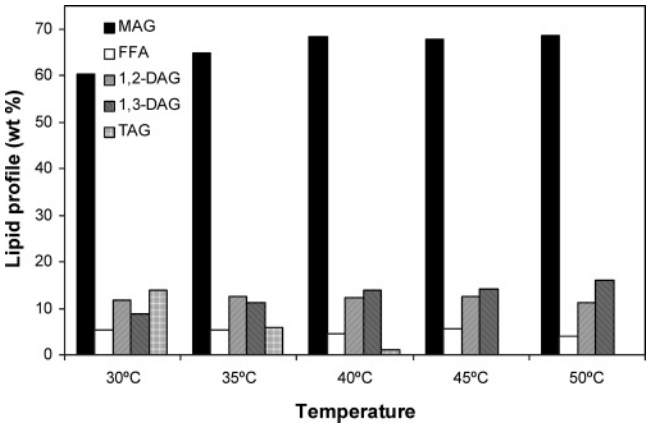

Figure 2. Effects of temperature on glycerolysis of sunflower oil in *tert*-butyl alcohol. Reaction conditions: glycerol/oil 4.5:1 (mol/mol), *tert*-butyl alcohol/oil 2.2:1 (wt/wt), reaction time 2 h, 15 wt % Novozym 435 (based on oil and glycerol), no additional water, and 700 rpm stirring. See **Figure 1** for abbreviations.

Temperature effect on the glycerolysis of sunflower oil was carried out at 30, 35, 40, 45, and 50 °C. The result is shown in **Figure 2**. As the reaction temperature was increased, there was a trend in higher formation of MAGs. However, the MAG yield was almost constant after 40 °C and all TAGs were converted.

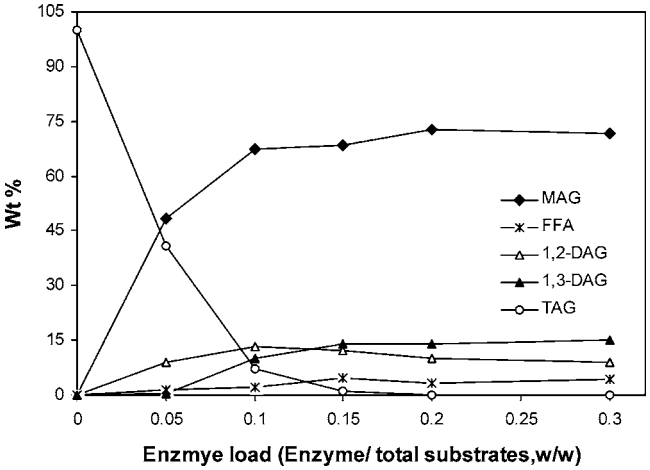

Figure 3. Effects of enzyme load on glycerolysis of sunflower oil in *tert*-butyl alcohol. Reaction conditions: temperature 40 °C, glycerol/oil 4.5:1 (mol/mol), *tert*-butyl alcohol/oil 2.2:1 (wt/wt), reaction time 2 h, no additional water, and 700 rpm stirring. **See Figure 1** for abbreviations.

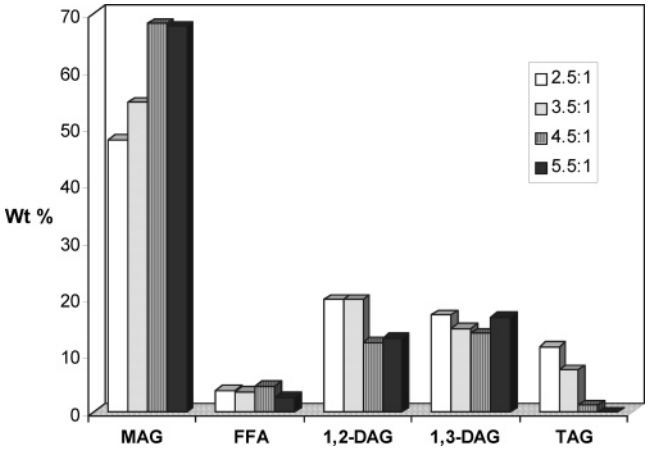

Figure 4. Effects of glycerol/oil molar ratio on glycerolysis of sunflower oil in *tert*-butyl alcohol. Reaction conditions: temperature 40 °C, *tert*-butanol/oil 2.2:1 (wt/wt), reaction time 2 h, 15 wt % Novozym 435 (based on oil and glycerol), and no additional water. See **Figure 1** for abbreviations.

It might indicate that temperature is not crucial for such a system, but this could be also because the reaction equilibrium has reached a 70% yield. In general, 40 °C was used in the following studies since lower temperature was recommended with respect to the product quality.

In the enzyme load study, glycerolysis of sunflower oil was conducted at the Novozym 435 load of 5, 10, 15, 20, and 30-wt % based on glycerol and oil. The results are shown in **Figure 3**. Enzyme load more than 10% resulted in little increase of MAG yield. The same situation to the temperature could exist since 70% yield had been already achieved after 10% enzyme load. In the used conditions, 10−15% enzyme load is enough for the maximum reaction performance.

The effect of substrate ratio can be in two ways. The increase of glycerol amount will increase the theoretical equilibration value as previously discussed. On the other hand, the glycerol amount will definitely affect the system polarity so as to influence the system stability and homogeneity. The experiments were conducted in the following molar substrate ratios between glycerol and oil: 2.5:1, 3.5:1, 4.5:1, and 5.5:1. The results are indicated in **Figure 4**. Less glycerol certainly produced less MAG since the maximum yield was also reduced. However, more glycerol did not produce more MAG as well since 4.5:1 and 5.5:1 (mol/mol) glycerol/oil had little difference in MAG yield. This is definitely not due to the equilibration problem. An optimal ratio must exist considering both effects of glycerol on the reaction equilibrium and the system homogeneity. In this study, 4.5:1 (mol/mol) glycerol/oil was taken for the batch reaction system.

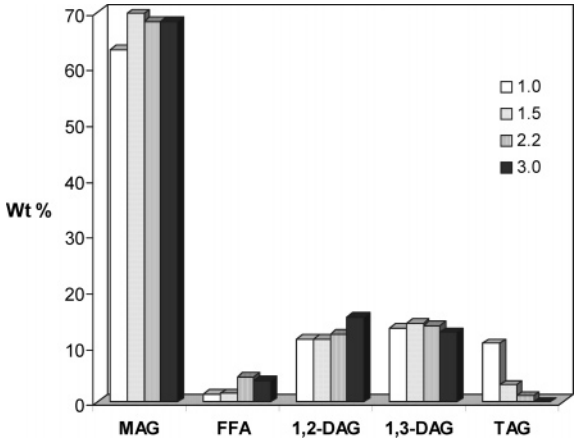

Figure 5. Effects of *tert*-butyl alcohol/oil ratio (wt/wt) on glycerolysis of sunflower oil. Reaction conditions: temperature 40 °C, glycerol/oil molar ratio 4.5:1, 15 wt % Novozym 435 (based on oil and glycerol), and no additional water. See **Figure 1** for abbreviations.

The solvent medium in one way can help improve the system homogeneity and stability as well as reduce the viscosity and mass transfer limitations. On the other hand, the medium will reduce the concentration of substrates so as to reduce the reaction rates as indicated by the Michaelis−Menten equation. The amount of *tert*-butyl alcohol has therefore double effects. The effect was evaluated in the weight ratio of *tert*-butyl alcohol to oil of 1:1, 1.5:1, 2.2:1, and 3:1. The results are given in **Figure 5**. In general, the change from 1:1 to 3:1 had little difference in MAG yields, especially after 1.5:1. FFA was increased, however, when high amount of solvent was used, probably because of the water brought in by the solvent. This implies that an optimal amount does exist considering different aspects. The ratio 1.5:1 is selected for the batch reaction system from this study.

Novozym 435-Catalyzed Glycerolysis in Continuous Packed Bed Reactor. In a packed bed reactor, enzyme amount is fixed. The feeding fluids that contain the substrates pass through the enzyme bed so that the reaction happens during the contact with the enzyme. The time where a molecule goes through the enzyme bed under the ideal plug flow is defined as residence time. The residence time can be calculated as $t = V\epsilon/F$, where t is the residence time (min), V is the volume of the enzyme bed (mL), F is the volume flow rate of the feeding (mL/min), and ϵ is the porosity of the enzyme (0.47). Because of the highest enzyme/substrate ratio in packed bed reactors, reaction rates are high and, consequently, mass transfer will have strong impact on the reaction efficiency. Viscosity of the fluids is thus an essential factor for the reaction efficiency. In this aspect, solvent plays an important role in the reduction of the viscosity.

A residence time course study, which will give the basic information of the reaction in the system, can be conducted through pumping through the enzyme bed with different flow rates. Glycerolysis of sunflower oil was carried out at the flow rates as 0.5, 1.0, 1.5, 2.0, and 2.8 mL/min in the packed enzyme bed reactor (the equivalent residence time: 187, 95, 63, 47, and 34 min, respectively). The contents of MAG, FFA, 1,2-

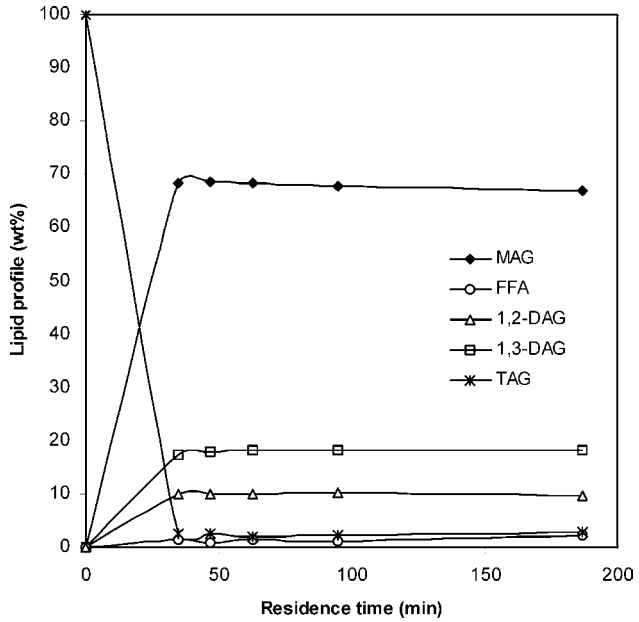

Figure 6. Effects of residence time on continuous glycerolysis catalyzed by Novozym 435. Conditions: glycerol/oil molar ratio 4.5:1, temperature 40 °C, no additional water, and *tert*-butyl alcohol/oil 2.2:1 (w/w). The continuous reactor (glass column, L 38 cm, o.d. 5 cm, i.d. 2.6 cm) was packed with 65 g Novozym 435. See **Figure 1** for abbreviations.

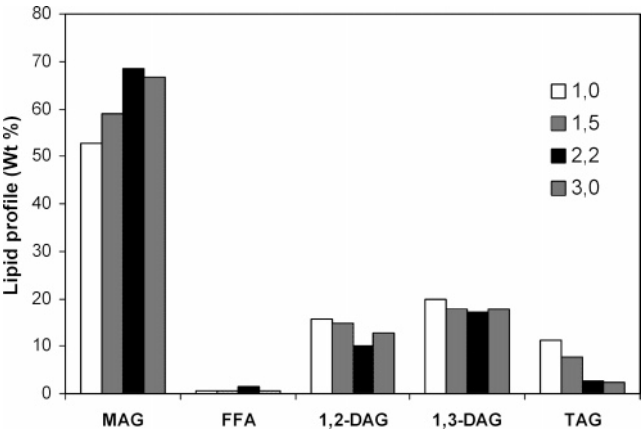

Figure 7. Effects of ratio of *tert*-butyl alcohol with oil on continuous glycerolysis catalyzed by Novozym 435. Conditions: glycerol/oil molar 4.5:1, flow rate 2.8 mL/min and temperature 40 °C. Other conditions see **Figure 6**. See **Figure 1** for abbreviations.

DAG, 1,3-DAG, and TAG in the products at the course of residence time are indicated in **Figure 6**. The glycerolysis in the packed bed reactor is much faster than in the batch reactor as shown in **Figure 1**. MAG contents reached 70% in the continuous glycerolysis at 30−40 min residence time. FFA content was lower in the continuous reactor than in the batch reactor in general.

In batch reactors, the solvent amount was not crucial as shown in **Figure 5**. As discussed above, it can have stronger influence in the packed bed reactor because of the consideration of mass transfer. The continuous glycerolysis were carried out at the weight ratio of *tert*-butyl alcohol to oil 1:1, 1.5:1, 2.2:1, and 3:1. The reaction results are indicated in **Figure 7**. The influence in the figure was more significant than in **Figure 5**. The ratio 2.2:1 was the best in the experiment. This indicates that the continuous glycerolysis in the packed bed reactor was more sensitive to the amount of *tert*-butyl alcohol than in the batch reactor. The *tert*-butyl alcohol/oil 2.2:1 was selected for the following optimization.

Table 2. Model Coefficients and Probability (*P*) of the Polynomials after Backward Elimination

	MAG yield (wt %)	
variables	coefficient	P
constant	68.910	2.63e-018
G/O[a]	4.882	4.53e-007
t	1.959	2.12e-003
G/O*G/O	−1.805	3.47e-003
*t***t*	−1.259	2.22e-002
G/O**t*	−1.412	1.22e-002

[a] Abbreviations: reaction temperature (Te), molar ratio of glycerol/oil (G/O), and residence time (*t*).

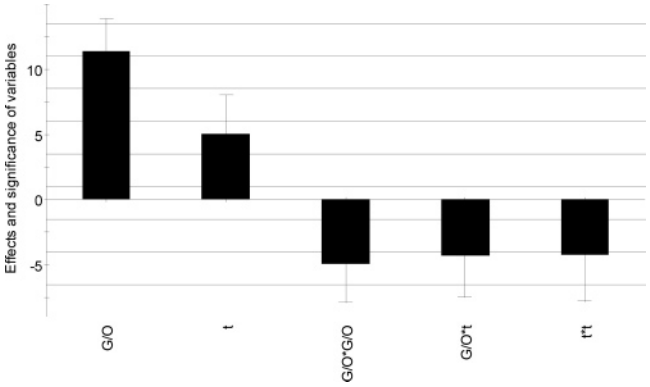

Figure 8. Effect and significance plot of parameters on MAG yield generated by the RSM optimization model. G/O = glycerol/oil ratio, Te = temperature (°C), and *t* = reaction time (min).

The two other important parameters, enzyme bed temperature and glycerol/oil ratio, have been studied in the batch system. The change may not very crucial when moving the system to the packed bed system. However, they probably have stronger interactions with residence time in the packed bed reactor. To optimize the system with such three parameters in the packed bed reactor, a statistical experimental design was used with the assistance of response surface methodology. This will allow evaluating the individual parameters as well as their interactions. The practical experimental setting is given in **Table 1** including responses from the experiments. Partial least-squares regression was used to fit the responses. One outlier was detected and eliminated. The insignificant variables were refined also in steps by backward elimination. The coefficient of determination (R^2) was 0.93 for MAG yield. The observed results and the predicted results were also well correlated (data not shown). The model for the MAG yield was generally satisfactory for the evaluation of such a system. The fit for DAG yields was not acceptable, probably because of the migration reactions between the 1,3-DAGs and 1,2(2,3)-DAGs. The model coefficients and *P*-values for the yield of MAG are given in **Table 2**.

Mathematical models (yield of MAG as response) were established and used for interpretation, predictions, and optimization.

The model can be used for the evaluation of parameters. The effect and significance of each parameter can be seen from the plot of main effects (**Figure 8**). The molar ratio of glycerol to oil (G/O) was the most significant factor for MAG yield. A close look of the effect is given in **Figure 9**. The MAG yield increases to about 73% at the ratio of 5. Further increase of glycerol/oil ratio, however, will not lead to more MAG

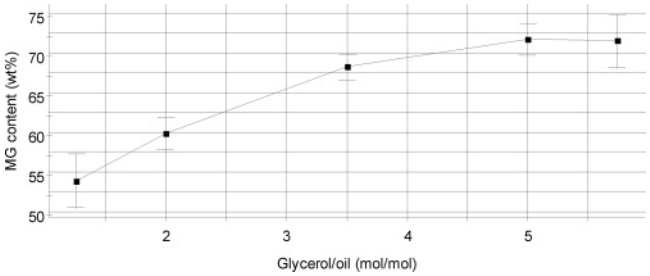

Figure 9. Effect of glycerol/oil ratio on the MAG yield generated by the RSM optimization model. Other conditions: residence time 40 min, temperature 50 °C, *tert*-butanol/oil 2.2:1 (w/w), and no additional water.

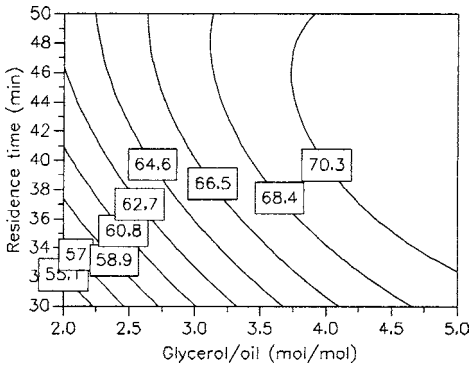

Figure 10. Contour plots between molar glycerol/oil and residence time (min) for MAG yields in continuous glycerolysis catalyzed by Novozym 435. Other conditions: temperature 50 °C, *tert*-butyl alcohol/oil 2.2:1 (w/w), and no additional water. See **Figure 1** for abbreviations.

formation; instead, a slight reduction will occur even though the theoretical yield is increased. This in general agrees with **Figure 4** in the batch system. Residence time had considerably low effect from **Figure 8**, which also agrees well to the single factor study (**Figure 6**). Temperature had little effect on MAG content in this design and therefore was eliminated. This was consistent with the results in the batch reactor (**Figure 2**).

Figure 10 is the contour plot generated from the model for the MAG yield between glycerol/oil ratio and residence time, the two left factors in the model. There were interactions between glycerol/oil and residence time since the second-order effect was significant (**Table 2**). Using the contour plot, optimal parameters could be obtained under different considerations. A higher ratio of glycerol/oil favored more formation of MAG from the contour plot as well as from **Figure 8**. However, downstream processing and enzyme stability should be considered also in the choice of parameters. Glycerol/oil 4:1 (mol/mol), temperature 40 °C, and residence time 45 min are recommended as optimal conditions for the enzymatic production of MAGs after various considerations.

Operational Stability. The lipase-catalyzed glycerolysis, in fact, is yet used in the commercial production although it possesses many advantages over chemical methods. The major issue for a large-scale production is the lipase cost. Long life span or high lipase stability in glycerolysis would make it more attractive for use at commercial plants. In the continuous operation in this study, the activity of the lipase was considerably stable during the 31 day running (**Figure 11**). No FFA was detected in the reaction after 15 days. There were no signs of decreasing in the lipase activity on the last running day. The experiment was terminated simply because of the high consumption of materials. Anyway, a life span up to a few months can be expected from the study.

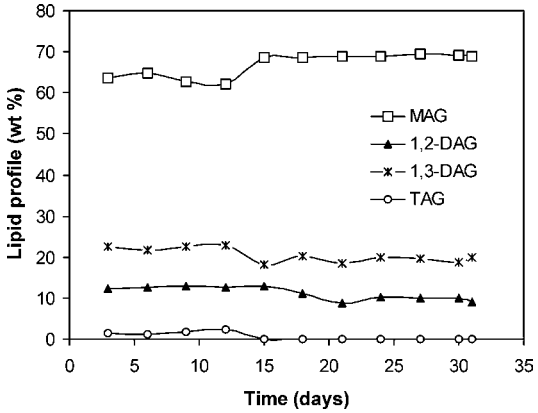

Figure 11. Operation stability during the continuous Novozym 435-catalyzed glycerolysis of sunflower oil. Reaction conditions: glycerol/oil 3.5:1 (mol/mol), temperature 40 °C, residence time 40 min, and *tert*-butyl alcohol/oil 2:1 (w/w). Other conditions see **Figure 6**. See **Figure 1** for abbreviations.

Product Purification. The product collected from the stability study was subject to the removal of *tert*-butyl alcohol by distillation and short-path distillation. Under 160 °C evaporation temperature of the short-path distillation, the composition of the distillate was glycerol 0.30%, free fatty acids 1.40%, MAGs 96.75%, DAGs 1.55%, and TAGs 0.00%. The composition of the residue was 0.05%, 0.30%, 10.7%, 83.38%, and 5.58%, respectively. It can be seen that the distillate had a very high purity of monoacylglycerols. The fatty acid composition of the MAG fraction (distillate) is (mol %) 16:0 1.1, 18:1n-9 14.6%, 18:2n-6 83.8, and others 0.5. The residue can be added to the process for further reaction or can be treated as another product for cooking oils after further refining and deodorization since diacylglycerols have been also used as healthy oil in the market.

ABBREVIATIONS USED

DAG, diacylglycerol; FFA, free fatty acid; MAG, monoacylglycerol;

RSM, response surface methodology; TAG, triacylglycerol.

SAFETY

The product and process have no particular safety problems. The solvent, *tert*-butyl alcohol, needs particular care. The basic properties of the solvent are given in the Materials part. The solvent can mix with air, which is explosive when the content is above the limit. The inhalation of the solvent can lead to a consciousness problem. Contact with eyes should be avoided. The solvent is allowed to be used in the food industry. Detail safety issues can be found from the web sites of the producers.

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