Production of Heat-Sensitive Monoacylglycerols by Enzymatic Glycerolysis in *tert*-Pentanol: Process Optimization by Response Surface Methodology

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ABSTRACT: The aim of this study was to optimize production of MAG by lipase-catalyzed glycerolysis in a tert-pentanol system. Twenty-nine batch reactions consisting of glycerol, sunflower oil, tert-pentanol, and commercially available lipase (Novozym®435) were carried out, with four process parameters being varied: Enzyme load, reaction time, substrate ratio of glycerol to oil, and solvent amount. Response surface methodology was applied to optimize the reaction system based on the experimental data achieved. MAG, DAG, and TAG contents, measured after a selected reaction time, were used as model responses. Well-fitting quadratic models were obtained for MAG, DAG, and TAG contents as a function of the process parameters with determination coefficients (R^2) of 0.89, 0.88, and 0.92, respectively. Of the main effects examined, only enzyme load and reaction time significantly influenced MAG, DAG, and TAG contents. Both enzyme amount and reaction time showed a surprisingly nonlinear relationship between factors (process parameters) and responses, indicating a local maximum. The substrate ratio of glycerol to oil did not significantly affect the MAG and TAG contents; however, it had a significant influence on DAG content. Contour plots were used to evaluate the optimal conditions for the complex interactions between the reaction parameters and responses. The optimal conditions established for MAG yield were: enzyme load, 18% (w/w of oil); glycerol/oil ratio, 7:1 (mol/mol); solvent amount, 500% (vol/wt of oil); and reaction time, 115 min. Under these conditions, a MAG content of 76% (w/w of lipid phase) was predicted. Verification experiments under optimized reaction conditions were conducted, and the results agreed well with the range of predictions.

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KEY WORDS: *Candida antarctica* lipase, glycerolysis, monoacylglycerols, response surface methodology (RSM), sunflower oil, *tert*-pentanol.

MAG are amphiphilic molecules with both a hydrophilic and a hydrophobic part, giving excellent emulsifying properties. They are approved by the European Union (EU) as food-grade additives, have GRAS (Generally Recognized As Safe) status by the U.S. Food and Drug Administration, and can be used *quantis satis* (no maximum level permitted is specified) according to the European Parliament and Council Directive (1–3). Accordingly, MAG contribute to a large worldwide market and are found in varied applications, for example, food, cosmetics, pharmaceuticals, and plastic products (1,2).

Today, commercial MAG are manufactured by chemical glycerolysis of fats/oils and glycerol at high temperatures (220—250°C), using inorganic alkaline catalysts in a nitrogen gas atmosphere. The use of high temperature has some drawbacks, such as a dark color, burnt taste, and high energy consumption (1,2,4–7).

Commercial chemical glycerolysis usually provides 35–60% MAG, 35–50% DAG, 1-20% TAG, 1–10% FFA, and the alkali metal salts (1,2,4). According to the World Health Organization and the EU directive, MAG and DAG of FA are required to contain at least 70 wt% MAG + DAG, at least 30 wt% MAG, and a maximum of 7 wt% glycerol (8). To fulfill the requirements of the directives or achieve MAG products of even higher purity (90–95%), MAG are often purified from the equilibrium mixture by short-path distillation (9).

Lipase-catalyzed glycerolysis has attracted much interest in recent years (6,10–12). It is believed to be a potentially alternative method to chemical processing; one of the reasons being that a gentler technology with a much lower temperature is required (1,2,7–10,13). The low temperature below 80°C makes production of heat-sensitive MAG with PUFA feasible, which is difficult with the currently used chemical process. Thus, MAG from enzymatic glycerolysis offer industrial potential as ingredients or compounds with improved functionality or a healthier nutritional FA profile.

One of the main drawbacks of the low-temperature enzymatic glycerolysis reaction is that it comprises three phases: a hydrophobic oil phase, a hydrophilic glycerol phase, and a solid enzyme phase. Since enzymes have hydrophilic characteristics, glycerol often binds to the enzyme particles and makes access of the oil molecules to the enzyme difficult (11). As a result, MAG yield is relatively low, and the reaction time required may be impractical from an industrial point of view.

This has led to the use of bioconversions in "nonconventional" media to improve homogeneity and stability as well as to reduce viscosity and mass transfer limitations (1,7,11,14). Examinations of lipase-catalyzed interesterification reactions in different organic solvents such as dioxane, *n*-hexane, *n*-heptane, acetonitrile, acetone, isooctane, *tert*-butanol, and *tert*-pentanol confirm the benefits of nonconventional media (1,4,5,10,11,14).

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Little effort has been made so far to optimize glycerolysis in *tert* pentanol. Addition of solvent lowers the space-time yield compared with a solvent-free system. However, tertiary alcohols, such as *tert*-pentanol, may enhance the reaction efficiency enough to offset the drawback of lowered productivity. High MAG yields after solvent and glycerol removal, as well as a well-preserved PUFA profile of the MAG, are among the benefits that can be obtained by enzymatic processing in solvent system (12). In addition, continuous operation and reusability of the solvent can overcome some of the problems with lowered productivity.

The present study is aimed at optimizing enzymatic glycerolysis in *tert*-pentanol to provide an industrially attractive reaction system for production of heat-sensitive MAG. Batch experiments, in which four process parameters are varied, are carried out and dataare evaluated by modeling with response surface design.

MATERIALS AND METHODS

Materials. Sunflower oil was provided by Aarhus United (Aarhus, Denmark). The sunflower oil was a TAG oil with 97.1% TAG, 2.5% DAG, 0.4% MAG, and a water content less than 0.01%. The FA composition of the sunflower oil was: C14:0, 0.1; C16:0, 6.7; C16:1, 0.2; C17:0, 0.1; C18:0, 3.7; C18:1, 26.3; C18:2, 61.2; C18:3, 0.4; C20:0, 0.3; C20:1, 0.2; C22:0, 0.6; C24:0, 0.2% (w/w). Glycerol was purchased from VWR International Ltd. (Albertslund, Denmark) (purity: 99.5 wt%). Novozymes A/S (Bagsværd, Denmark) supplied the lipase enzyme Novozym® 435. The enzyme is a *Candida antarctica* lipase that is produced by submerged fermentation of a genetically modified *Aspergillus oryzae* microorganism and that is subsequently adsorbed on a macroporous resin. *tert*-Pentanol was provided from Lab-Scan (Dublin, Ireland) (purity: 99%). Diethyl ether was provided from Bie & Berntsen A/S (Rødovre, Denmark) (purity: 99.5%). Chloroform (purity: 99.8%), methanol (purity: 99.8%), and *n*-heptane (purity: 95%) were provided from Lab-Scan. Acetic acid was purchased from VWR International Ltd. (purity: >90%).

Experimental design. The experimental work was designed according to the principle of response surface methodology (RSM) with the assistance of the commercial software, Modde 7.0 from Umetri (Umeå, Sweden). A three-level four-factor fractional experiment with five star points (29 experiments) was carried out. The four factors chosen were: enzyme load (w/w% of sunflower oil), reaction time (min), substrate ratio (glycerol/oil, mol/mol), and solvent amount (vol/wt% of oil). The measured MAG, DAG, and TAG contents were used as the responses. In Table 1 are listed the factors used, the parameter ranges applied, and the responses.

Enzymatic glycerolysis reaction. Enzymatic glycerolysis reactions in *tert*-pentanol were performed as batch experiments. Varied amounts of glycerol and *tert*-pentanol were mixed with 10 g of sunflower oil to obtain different substrate ratios (mol/mol) and solvent amounts (wt% of oil) in the system. Capped flasks containing the reaction mixtures were incubated in a water bath with magnetic stirring (Elektro, Helios) at 50°C. The reactions were initiated by addition of the lipase. After the set reaction time, 1.5 mL of reaction mixture was withdrawn and filtered through a syringe filled with water-repellent cotton to remove enzyme. Samples were flushed with nitrogen to remove air and solvent. All samples were stored at −20°C prior to analysis.

TLC-FID analysis. The lipid profiles (wt% of MAG, DAG, and TAG) were determined with an IATROSCAN MK6 TLC-FID (SES GmbH, Bechenheim, Germany). Silica gel-coated quartz rods (Chromarods-SIII, SESGmbH) were used (12). The dilution medium used was chloroform/methanol (85:15 vol/vol), and solvent was diethyl ether/heptane/acetic acid (35:35:1 by vol). The method is described elsewhere (12).

Theoretical product distribution. The theoretical product distributions of MAG, DAG, TAG, and glycerol at equilibrium conditions after glycerolysis reaction with different molar ratios of glycerol to oil were calculated in Microsoft Excel XP 2003/2002 and were based on binomial random distribution with probability calculations of ester formation between mole FA and mole hydroxyl groups (OH) calculated as $p_{ester} = {}^{n}FA/{}^{n}OH$. The mol% glycerol in the mixture with no esters had a b(0|3;p_{ester}) distribution. The mol% MAG in the mixture with one ester formed had a b(1|3;p_{ester}) distribution. The mol% DAG in the mixture with two esters formed had a b(2|3;p_{ester}) distribution. The mol% TAG in the mixture with two esters formed had a b(3|3;p_{ester}) distribution.

Statistical analysis. The experimental data were analyzed by means of RSM with Modde 7.0. Second-order coefficients were generated by regression with backward elimination. Responses were initially fitted to the factors by multiple regressions. The fit of the model was evaluated by the determination coefficients R^2 and Q^2 and the ANOVA. The insignificant coefficients were eliminated and the model was finally refined. The quadratic response surface model was fitted to the following equation:

$$Y = \hat{a}_0 + \sum_{i=1}^{4} \hat{a}_i x_i + \sum_{i=1}^{4} \hat{a}_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \hat{a}_{ij} x_i x_j \quad [1]$$

Y indicates the response variables (MAG, DAG, and TAG content, wt%), x_i (i = 1–4) the i^{th} independent variable, β_0 the intercept, β_i the first-order model coefficient, β_{ii} the quadratic coefficient for the i^{th} variable, and β_{ij} the linear model coefficient for the interaction between factor i and j (15).

RESULTS AND DISCUSSION

Preliminary evaluation of parameters. Preliminary studies were conducted to evaluate the ranges applied of selected parameters for RSM experiments. The MAG contents were evaluated during the time course of glycerolysis in the *tert*-pentanol system at different substrate ratios and solvent amounts (Fig. 1).

According to binomial distribution of MAG, DAG, TAG, and glycerol at equilibrium conditions after glycerolysis reaction with different molar ratios of glycerol to oil, increased MAG content was expected with increased molar ratio

TABLE 1
Factors for the Experiments Carried Out Based on a Four-Factor, Three-Level Surface Response Design and the Responses Achieved After Analysis on TLC-FID

	Factors[a]				Responses[b]		
Experiment no.	Enz.	Sol.	Sub.	Time	MAG	DAG	TAG
1	10	300	4	60	51.96	14.94	33.10
2	20	300	4	60	67.01	21.04	11.95
3	10	500	4	60	44.31	13.57	42.13
4	20	500	4	60	66.93	19.11	13.96
5	10	300	6	60	37.80	8.64	53.55
6	20	300	6	60	59.18	14.23	26.60
7	10	500	6	60	37.95	8.78	53.27
8	20	500	6	60	67.55	14.03	18.42
9	10	300	4	120	63.11	19.88	17.00
10	20	300	4	120	70.48	22.84	6.68
11	10	500	4	120	65.47	16.94	17.58
12	20	500	4	120	74.13	20.36	5.51
13	10	300	6	120	62.93	10.45	26.62
14	20	300	6	120	41.20	11.07	47.72
15	10	500	6	120	67.02	12.80	20.18
16	20	500	6	120	76.37	14.56	9.07
17	5	400	5	90	33.65	12.94	53.41
18	25	400	5	90	66.88	30.25	2.88
19	15	200	5	90	65.66	15.36	18.97
20	15	600	5	90	64.92	16.37	18.71
21	15	400	3	90	57.36	24.70	17.93
22	15	400	7	90	77.46	15.10	7.44
23	15	400	5	30	35.14	9.71	55.15
24	15	400	5	150	69.90	17.06	13.04
25	15	400	5	90	69.37	17.71	12.92
26	15	400	5	90	71.35	17.44	11.21
27	15	400	5	90	65.73	21.64	12.64
28	15	400	5	90	63.83	17.07	19.10
29	15	400	5	90	67.08	17.16	15.76

[a]Enz. = enzyme dosage (w/w of oil amount), Sol. = solvent amount (vol/wt of oil), Sub. = substrate ratio glycerol/oil (mol/mol), and Time in minutes.
[b]Responses of MAG, DAG, and TAG are calculated as weight percentages of the oil phase (MAG + DAG + TAG = 100%).

(Fig. 2A). Surprisingly, increased MAG formation was not seen with increased substrate ratio at ratios higher than six (all yielded MAG contents of approximately 80%) (Fig. 1). According to the binomial distribution calculations, an optimal MAG content, based on the complete product mixture including glycerol, was achieved at a substrate ratio (glycerol to oil) of 5 (mol/mol) (Fig. 2B). Hence, it was preferable to choose a substrate ratio close to 5. This explains why a glycerol to oil ratio of less than 7 (mol/mol) was suitable for further investigations. Equilibrium conditions were achieved after 120 min for all substrate ratios (Fig. 1). There was a tendency toward longer reaction time needed with increasing substrate ratios before equilibrium conditions were reached (Fig. 1). The intention was to set up a reaction system with shorter reaction time, but equilibrium should preferably be reached before the reaction was stopped. Thus, reaction times up to 150 min were selected to achieve maximal MAG formation.

Increasing the solvent amount seemed to enhance the formation of MAG (Fig. 1). This could be due to changes in the polarity of the system caused by the solvent. A greater amount of polar solvents might be a factor for increasing the catalytic activity because of the high polarity of the enzymes. Thus, variations in the amounts of solvents were of interest.

Calculated vs. experimentally achieved values. Experimental data with a reaction time of 90 min (Table 1) were compared with theoretical calculated equilibrium values (Fig. 2C). Experimental data agreed well with calculated values, showing that equilibrium indeed was reached after 90 min. Thus, only very short reaction times were required, confirming the greater efficiency of the *tert*-pentanol system compared with other enzymatic glycerolysis systems such as solvent free, solid-phase systems, where much longer reaction times typically are required (5,13).

Model fitting. Modeling of factors and responses was performed by RSM to predict the highest possible content of MAG. The underlying results for the models are listed in Table 1. A central composite rotatable design is generally the best design for response surface optimization (15). The best-fitting model was determined by regression and backward elimination. According to the models, the generated MAG, DAG, and TAG contents were affected by first-order variables (main effects) as well as second-order variables (interactions). All model coefficients (β) and probability values (P) were below

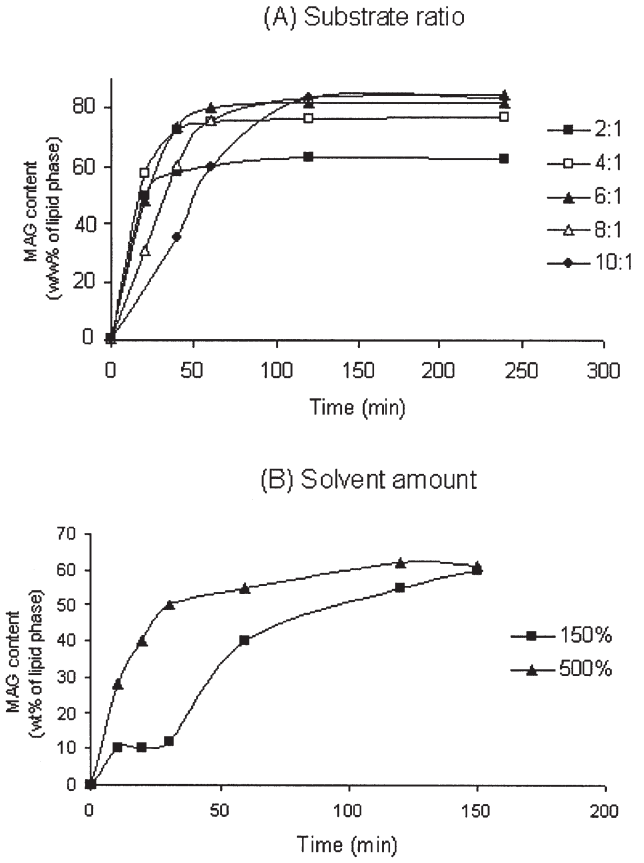

FIG. 1. Time course for MAG content during glycerolysis reaction in *tert*-pentanol system with: (A) varying substrate ratios of glycerol/oil (mol/mol), and (B) different solvent amounts. Reaction conditions: Enzyme dosage 30 wt% of oil, reaction temperature, 50°C. (A) Solvent amount, 200% (vol/wt of oil); (B) glycerol/oil ratio 5 (mol/mol).

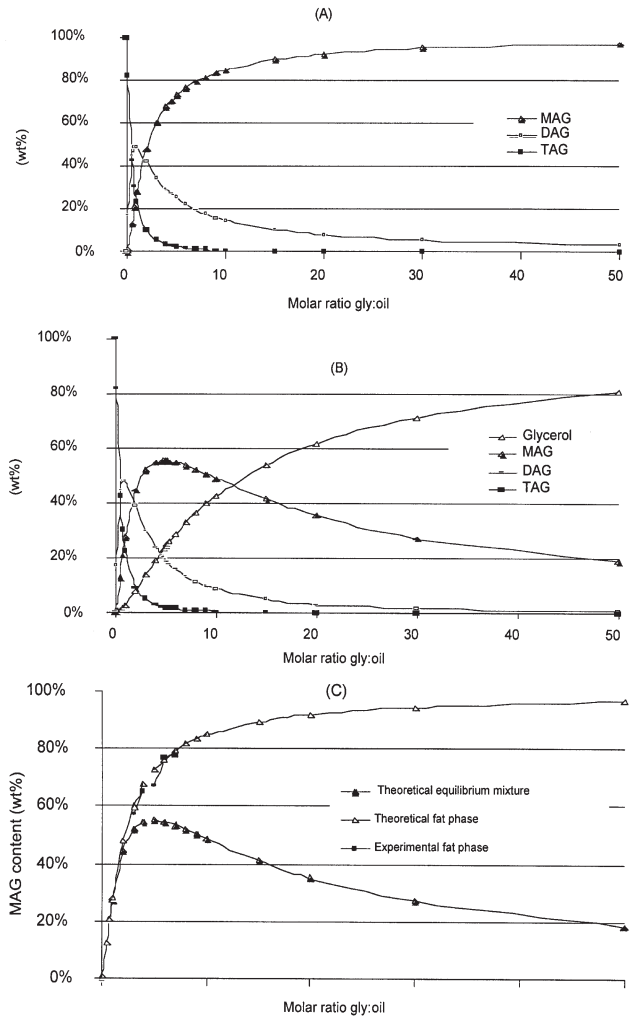

FIG. 2. Theoretical calculated product distribution at equilibrium conditions after glycerolysis reaction with different molar ratios of glycerol to oil. (A) Distribution between MAG, DAG, and TAG (fat phase), (B) distribution in the complete product mixture (fat phase and glycerol), and (C) theoretical and experimental MAG content in fat phase compared with the theoretical MAG content in complete product mixture including glycerol. Theoretical calculations are based on binomial random distribution with probability calculations of ester formation between mole FA and mole hydroxyl groups (OH), expressed as weight percentages.

0.05 after the models were refined (Table 2). ANOVA demonstrated that models were satisfactory with a coefficient of determination (R^2) for MAG, DAG, and TAG contents of 0.89, 0.88, and 0.92, respectively. The observed and predicted values were sufficiently correlated except for no. 14 (Fig. 3). No. 14 was treated as an outlier and eliminated; thereafter there was no lack of fit according to ANOVA. The successful fits indicate that models represent an actual relationship of reaction parameters within the ranges selected. It should be noted that the polynomials were only a statistical empirical model in the selected ranges and may not be true beyond the ranges of factors.

Main effects of parameters. The major influence of parameters can be evaluated from plots of main effects on MAG, DAG, and TAG contents. Enzyme amount and reaction time were the only two factors tested that significantly influenced MAG, DAG, and TAG content at the same time (Table 2). The first-order coefficients that had a positive effect on the MAG content had the opposite effect for the second-order coefficients. This indicates, surprisingly, a nonlinear relationship between factors and responses with optima. Increased enzyme load resulted in increased MAG content, until an optimal MAG content of 75% was obtained with an enzyme load of 20 wt% (Fig. 4). The MAG content was improved by longer reaction time until an optimum was obtained after 120 min with a MAG content of 76%. Instead of optima, equilibrium conditions with constant amounts of MAG, DAG, and TAG were expected after a certain reaction time and enzyme dosage in the selected enzyme range. The spreads in data at an enzyme dosage of 25% and reaction time of 150 min are relatively high and do not significantly differ from optimum enzyme dosage of 20% and reaction time of 120 min (Fig. 4). Thus, the decrease in MAG content with a reaction time longer than 120 min and an enzyme dosage higher than 20% are ascribed to spread in the data.

Substrate ratio had only a minor influence on glycerolysis (Table 2), in accordance with preliminary results (Fig. 1). The effect of substrate ratio was only significant on DAG content,

TABLE 2
Multiple Linear Regression Coefficients (β) and *P*-Values Describing the Influences of Different Parameters on the MAG, DAG, and TAG Content[a]

	MAG		DAG		TAG	
Term	Regression coefficient (β)	*P*-value[b]	Regression coefficient (β)	*P*-value[b]	Regression coefficient (β)	*P*-value[b]
Constant	68.04	4.09×10^{-19}	18.31	7.99×10^{-16}	13.65	1.25×10^{-6}
Enzyme	7.96	4.06×10^{-7}	3.03	9.44×10^{-7}	–10.99	7.09×10^{-9}
Solvent	0.52	0.63	–0.33	0.45	–0.19	0.87
Substrate	0.79	0.46	–2.77	3.27×10^{-6}	1.98	9.34×10^{-2}
Time	7.91	4.43×10^{-7}	1.51	2.25×10^{-3}	–9.41	7.91×10^{-8}
Enz*Enz	–3.93	6.9×10^{-4}	0.49	0.23	3.44	3.43×10^{-3}
Solvent*Solvent	–0.17	0.86	–0.94	2.70×10^{-2}	1.11	0.29
Time*Time	–3.37	2.59×10^{-3}	–1.56	8.05×10^{-4}	4.93	1.28×10^{-4}
Enz*Time	–3.30	2.10×10^{-2}	–0.43	0.43	3.73	1.47×10^{-2}

[a]Content values reported are based on weight percentages of MAG + DAG + TAG.
[b]*P*-values are defined as the smallest level of significance leading to rejection of the null hypothesis. The main effect of each factor (linear and quadratic) and the interaction effects are statistically significant when *P*-value is <0.05.

whereas the effect on MAG and TAG was insignificant (Table 2). Since the distribution of the three components MAG, DAG, and TAG are related to each other, the substrate ratio was expected to have a significant influence on MAG and TAG as well. However, no obvious explanation was found for the differences in significance level. Surprisingly, more glycerol did not produce more MAG, as expected from binomial distribu-

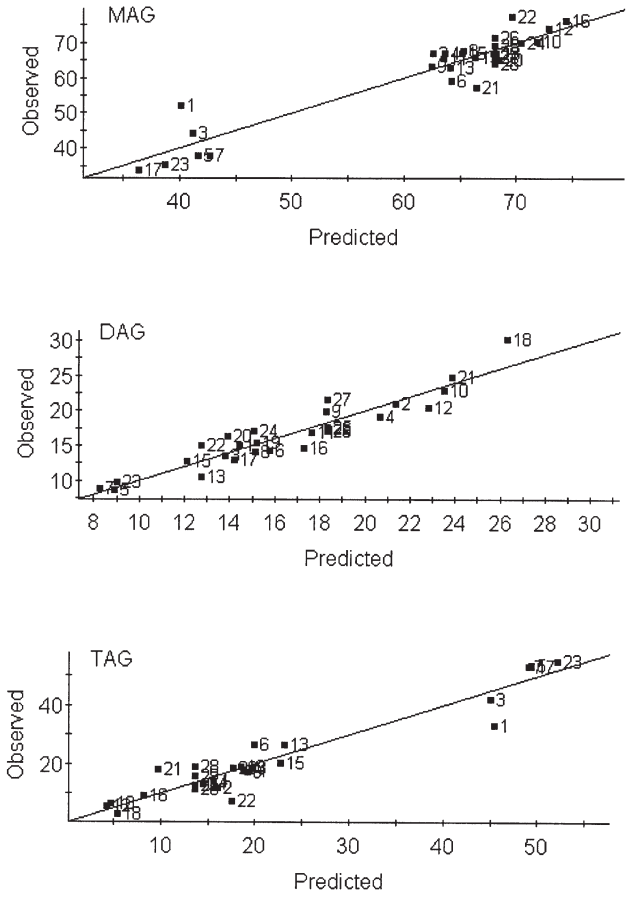

FIG. 3. Relationship between observed responses and results predicted by the developed model for MAG, DAG, and TAG content (w/w of lipid phase). Numbers inside the figures are experimental setting numbers. The solid line represents a linear regression line.

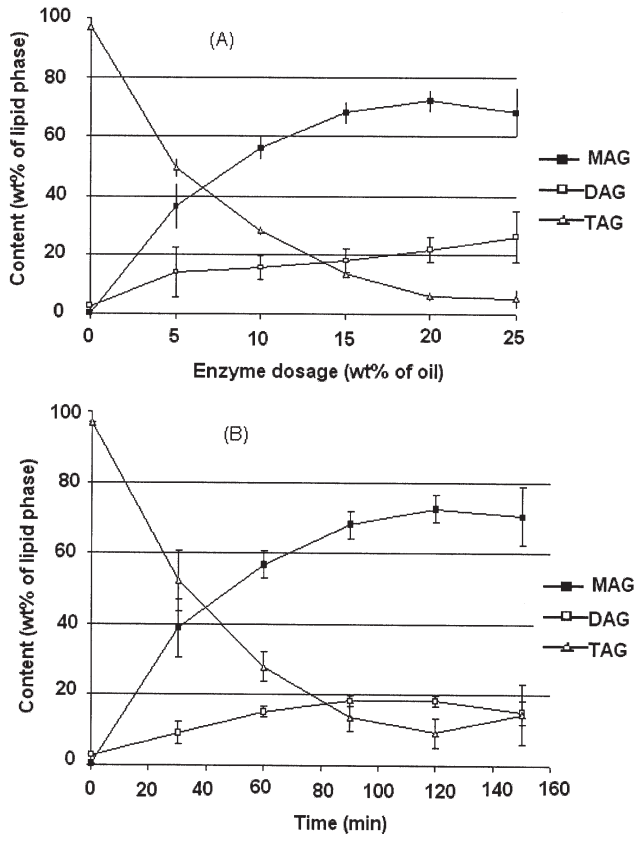

FIG. 4. Main effect of (A) enzyme dosage and (B) reaction time on MAG, DAG, and TAG formation at constant level of substrate ratio (glycerol to oil of 5 mol/mol), solvent amount (400 vol/wt of oil), and reaction temperature (50°C). Enzyme dosage in (B) was 15 (wt% of oil) and reaction time in (A) was 150 min. Error bars represent SD.

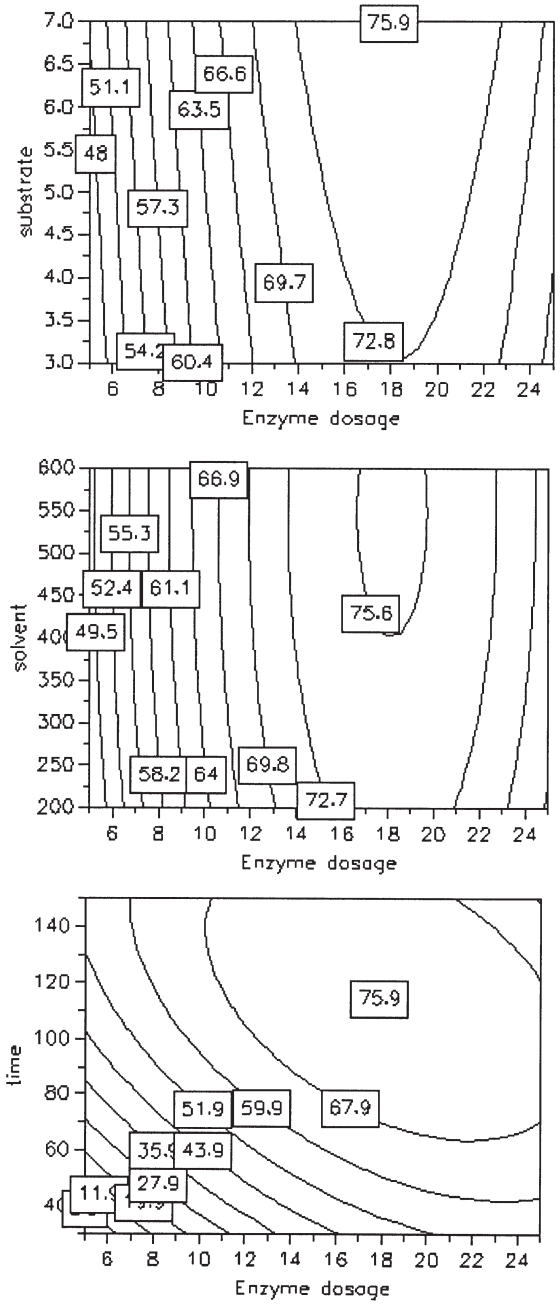

FIG. 5. Contour plots of MAG at optimized conditions. Enzyme dosage 18 wt%, solvent amount 500%, substrate ratio 7:1, and reaction time 115 min.

tion calculations (Fig. 2). No obvious explanation was found. The improved contact between glycerol and oil, caused by the solvent, might lead to higher efficiency, even at relatively low glycerol to oil ratios. Increasing the amount of glycerol changes the polarity of the system and renders plausible certain influences on the system stability and homogeneity. Apparently, an increased glycerol to oil ratio influenced the reaction in such a way that the expected stochiometric positive effects were neutralized.

The solvent amount was not crucial in batch reactions since the effect on MAG, DAG, and TAG contents was insignificant (Table 2). The effect of solvent amount was therefore neglected in the range tested (200–600% vol/wt). As a result, the system was not sensitive to reduced conversion with decreased solvent amount, either for reduced homogeneity or stability with reduced solvent amount. The conclusion with sufficient amount of solvent medium at both low and high solvent concentrations was not in complete agreement with preliminary results, in which the increasing amount of solvent affected the MAG content (Fig. 1). A slightly lower solvent amount of 150% was used in preliminary investigations. This indicates a lower critical solvent amount of approximately 200% before the polarity/homogeneity was affected negatively.

Optimization. According to the models generated, MAG, DAG, and TAG contents were influenced not only by first-order variables but also by second-order variables and parameter interactions. The complex relationship between reaction parameters and responses can be well evaluated by contour plots giving good predictions of optimized conditions. Several optimal combinations are available to obtain the highest MAG content. Contour plots between different parameters were generated for MAG formation. A pattern with high effect of enzyme dosage and reaction time and little effect of solvent amount and substrate ratio was seen (Fig. 5). The highest possible MAG content that could be established in this system was predicted to be 76 wt%, requiring an enzyme load of 18 wt%, substrate ratio of 7:1 (mol/mol), solvent amount of 500% (vol/wt of oil), and reaction time of 115 min. Verification experiments under optimized reaction conditions were conducted, and the results agreed well with the range of predictions.

Predicted optimal conditions must be interpreted in the context of industrial operations. The use of solvents increases expense, an extra process step for solvent removal is needed, and extra attention to safety issues is required as well. Accordingly, a solvent amount as low as possible is advantageous from an industrial point of view. A compromise in the solvent amount could easily be made without a dramatic reduction in the predicted MAG content (Fig. 5). A reduction of the solvent amount from 600 to 200% caused only a 3% reduction in the MAG content. A solvent amount lower than the predicted optimum is therefore recommended.

To fulfill the direct requirements of a maximum of 7 wt% glycerol in final products, the surplus of glycerol in the equilibrium mixture should be removed after the reaction. The predicted optimal glycerol to oil ratio of 7 theoretically provides excessive quantities of nonreacted glycerol in the equilibrium mixture of 42% (Fig. 2B). Removal of the excess glycerol is a main drawback from an industrial point of view because extra processing is required and it lowers the space-time yield of MAG in the product mixture. Since the influence of the substrate ratios on MAG content achieved was insignificant, a substrate ratio lower than the predicted optimum could easily be considered. A reduction of the substrate ratio from 7 to 3 caused only a 3% reduction in the MAG content (Fig. 5). Theoretically, this leads to a reduction in the nonreacted glycerol content from 42 to 24 wt% in the equilibrium mixture (Fig.

2B). However, at the same time, the DAG content increased from 15 to 25 wt% in the mixture (Table 1, Fig. 2B) and a high molar ratio of glycerol to oil allowed greater conversion of TAG to MAG and reduced the amount of DAG (Fig. 2). Although the increased DAG content did not affect the fulfillment of the directives of at least 70 wt% MAG + DAG and 30% MAG, it definitely had a negative impact on MAG purity and most likely on the product quality in general. Thus, the molar ratio of glycerol to oil should be very carefully selected depending on the required profile for the final product. To produce the highest possible MAG content in the lipid phase after solvent and glycerol removal, a high molar ratio of glycerol to oil was advantageous. On the other hand, to reach the highest MAG content in the complete product mixture (including glycerol, a molar ratio of approximately 5 was more appropriate. Industrial cost–benefits analysis must be performed to evaluate the optimized balance between product characteristics achieved, and the required processing.

The *tert*-pentanol system was successfully optimized through RSM. The empirical model developed satisfactorily expressed the MAG, DAG, and TAG formed during glycerolysis with regard to selected parameters. The high MAG content predicted showed an efficient glycerolysis system, even though some compromises in solvent amount and molar ratio were considered. The potential of *tert*-pentanol as a suitable solvent for low-temperature glycerolysis reaction was confirmed by the efficient optimized system that was developed.

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