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Lipase-catalyzed glycerolysis of olive oil in organic solvent medium: Optimization using response surface methodology

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Abstract−Enzymatic glycerolysis of olive oil for mono- (MG) and diglycerides (DG) synthesis was investigated. Several pure organic solvents and co-solvent mixtures were screened in a batch reaction system. The yields of MG and DG in co-solvent mixtures exceeded those of the corresponding pure organic solvents. Batch reaction conditions of the glycerolysis reaction, the lipase amount, the glycerol to oil molar ratio, the reaction time, and temperature, were studied. In these systems, the high content of reaction products, especially MG (55.8 wt%) and DG (16.4 wt%) was achieved at 40 °C temperature and 0.025 g of lipase with relatively low glycerol to oil molar ratio (2 : 1) within 4 h of reaction time in isopropanol/*tert*-butanol (1 : 3) solvent mixture. Glycerolysis reaction was optimized with the assistance of response surface methodology (RSM). Optimal condition for reaction conversion was recommended as lipase amount 0.025 g, glycerol to oil molar ratio 2 : 1, reaction time 4 h and temperature 40 °C.

Keywords: Monoglycerides, Immobilized *Candida rugosa* Lipase, Glycerolysis, Response Surface Methodology, Olive Oil

INTRODUCTION

Mono-and diglycerides are naturally present as a minor component of animal fat and plant oils and have been widely used as emulsifiers in foods, cosmetics, and pharmaceutical products [1]. These glycerides can be produced by glycerolysis, esterification, hydrolysis of oils and fats through chemical and enzymatic catalysis [2]. Enzymatic process is an advantageous approach because it requires the lowest temperatures and atmospheric pressure and it exhibits high regioselectivity, high catalysis efficiency leading to products with high purity and fewer side products [3]. MG and DG is synthesized during lipase-catalyzed esterification of glycerol and free fatty acids (FFA) with simultaneous removal of water. In the process, the free fatty acids are used as a raw material required in a process of hydrolyzing oil and fats. However, high levels of FFA are needed [4]. In lipase-catalyzed partial hydrolysis, a large amount of FFA is generated and MG and DG yields are relatively low at the end of hydrolysis. Therefore, in view of yielding high productivity, the glycerolysis of fats and oils is more worthwhile than esterification and hydrolysis reactions [5]. Glycerolysis of oils has been conducted with lipases, in organic medium, in solvent-free systems, with free or immobilized lipases, in ionic liquids or using compressed fluids as reaction media [6-10].

In general, the main drawbacks of the low temperature lipase-catalyzed glycerolysis reaction are that it is comprised of three phases: a hydrophobic oil phase, a hydrophilic glycerol phase, and a solid lipase phase. Since lipase has more hydrophilic characteristics, glycerol often binds to the lipase particles, so that the access of oil molecules to the lipase is difficult. In addition, the reaction time is long, synthesis of MG and DG is relatively low, and it is not practical from an industrial point of view. The reuse of enzymes is also difficult in solvent-free systems. Therefore, the organic solvents media is an important solution to reduce the viscosity and to improve the homogeneity, stability and mass transfer limitations of the system. Solvents such as dioxane, *n*-hexane, *n*-heptane, isooctane, acetonitrile, acetone, *tert*-butanol, *tert*-pentanol, or their mixtures are useful in different lipase-catalyzed interesterification reactions [8]. Solvent engineering has been used in lipase-catalyzed glycerolysis of triolein for the selective synthesis of monoolein and diolein, in which the advantage of the use of different binary mixtures of n-hexane and 2-methyl-2-butanol on the production of MG and DG was discussed [11]. Akoh et al. [12] described the synthesis of MG by esterification of glycerol with oleic acid catalyzed with Lipase from *Penicillium camemberti* (Lipase G manufactured by Amano) in hexane. Pawongrat et al. [7] studied the use of *tert*-butyl methyl ether in glycerolysis of tuna oil with Lipase AK from *Pseudomonas fluorescence* for MG formation. Bellot et al. [13] studied the effects of various organic solvents on MG synthesis from esterification of oleic acid with *Rhizomucor miehei* lipase and proposed that an increase in solvent polarity using mixtures of two solvents significantly improves the MG production. Kaewthong and H-Kittikun [14] investigated MG synthesis by *Pseudomonas* sp. lipase-catalyzed glycerolysis of palm olein in organic solvents. The optimal condition for MG synthesis was determined to be in acetone and isooctane mixtures. Li and Ward [15] studied an enzymatic method for synthesis of MG from 1,2-isopropylidene glycerol and n-3 polyunsaturated fatty acid catalyzed with lipase IM-60 from *Mucor miehei* in isooctane and hexane as organic solvents.

Response surface methodology (RSM) is useful for developing, improving, and optimizing the response variable. It defines the rela-

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tionship between the independent variables and the obtained response [16-18]. RSM has been effectively applied to optimize the parameters in lipase-catalyzed reactions [19,20]. The main objective of this work was to investigate the synthesis of MG and DG by glycerolysis of olive oil in the presence of commercial immobilized *Candida rugosa* lipase in organic solvent. Preliminarily, the screening of several pure and mixed solvents with varying polarities in order to evaluate solvents for enzymatic glycerolysis reaction was studied. From the results obtained, process conversion was optimized by evaluating the effect of four process parameters: lipase amount, the glycerol to oil molar ratio, the reaction time, and temperature using central composite rotatable design (CCRD) and RSM analysis. Reaction time and temperature have an effect on the reaction performance, affecting both the initial rate of reaction and the final yield of MG and DG. Lipase amount and molar ratio of glycerol to oil influence mass transfer and reaction rate of glycerolysis reaction. In addition, glycerol can act as effective stabilizer against thermal and affect the system polarity, so that influences the system stability and homogeneity.

MATERIALS AND METHODS

1. Materials

Commercial grade olive oil and glycerol (99.5% purity) were used as substrates for the glycerolysis reactions. The commercial immobilized *Candida rugosa* lipase (immobilized on immobead 150 support) was purchased from Sigma-Aldrich Chemical Co. (Mumbai, India). The organic solvents used in the experiment and analysis were *tert*-butanol, chloroform, ethanol, acetone, toluene, n-octane, acetonitrile, n-hexane, isopropanol and acetic acid were of analytical or, HPLC grade purchased from Merck, Mumbai, India. Tripalmitate, Dipalmitin, DL-α Palmitin, Glyceryl trioleate 1, 2-Diolein, 1, 3-Diolein and 1-Oleoyl-*rac*-glycerol standards were purchased from Sigma Chemical Pvt. Ltd, Mumbai, India.

2. Enzymatic Glycerolysis

The reaction mixtures consisted of 1 g of olive oil, required

Table 1. The CCD matrix used for four independent variables and the comparison between experimental and predicted responses for MG yields

Run	Time (h) X_1	Temperature (^oC) X_2	Amount of lipase (g) X_3	Ratio of glycerol to oil X_4	Yield	
					Experimental	Predicted
1	2	30	0.01	2.5	32.1	31.6
2	2	30	0.02	1.5	38.9	37.9
3	2	50	0.01	1.5	30.7	31.4
4	2	50	0.02	2.5	31.9	31.6
5	4	30	0.01	1.5	41.4	41.9
6	4	30	0.02	2.5	44.9	44.5
7	4	50	0.01	2.5	35.2	36.5
8	4	50	0.02	1.5	40.9	41.6
9	3	40	0.015	2	48.9	48.6
10	3	40	0.015	2	48.3	48.6
11	2	30	0.01	1.5	33.8	33.8
12	2	30	0.02	2.5	35.9	35.7
13	2	50	0.01	2.5	28.6	28.9
14	2	50	0.02	1.5	34.7	34.2
15	4	30	0.01	2.5	39.8	40.1
16	4	30	0.02	1.5	46.8	46.3
17	4	50	0.01	1.5	38.5	38.6
18	4	50	0.02	2.5	39.6	39.5
19	3	40	0.015	2	48.9	48.6
20	3	40	0.015	2	48.5	48.6
21	1	40	0.015	2	34.9	35.6
22	5	40	0.015	2	52.6	51.6
23	3	20	0.015	2	31.8	32.6
24	3	60	0.015	2	26.5	25.3
25	3	40	0.005	2	33.6	32.2
26	3	40	0.025	2	38.2	39.3
27	3	40	0.015	1	42.5	42.4
28	3	40	0.015	3	38.4	38.1
29	3	40	0.015	2	48.8	48.6
30	3	40	0.015	2	48.6	48.6

amount of glycerol, required amount of immobilized *Candida rugosa* lipase, water, required molar ratios of glycerol to oil, required amount of solvents were incubated in a capped flask (50 ml) at the design condition under 600 rpm magnetic stirrer (IKA-RCT S22 digital stirrer) in a water bath equipped with a probe for temperature monitoring. The reaction time, temperature, molar ratio of glycerol to oil and immobilized lipase amount were changed to study their effect on MG and DG production. Aliquots (0.10 ml) of the reaction mixture were taken periodically. The immobilized lipase was removed by centrifugation at 2,900 ×g for 15 min, and the supernatant was used for quantitative or qualitative analysis. All experiments were performed at least three times.

3. Analytical Methods

Mono-, di-, and triglycerides were analyzed by HPLC (Agilent 1100) with a Zorbax C18 column (4.6 m×250 mm, 5 μm), fitted with a refractive index detector and an on-column injector. The following conditions were used: flow rate of 1.0 mL/min, detector temperature 45 °C/min; column temperature of 35 °C/min; the mobile phase n-hexane and isopropyl alcohol (4 : 5 v/v). n-Hexane and isopropyl alcohol were used as a sample dissolving solvent with injection volume of 25 μL. The reaction products were quantified by using standard of MG, DG, TG and FFA to establish the calibration charts. In using these calibration charts, all of the integration results were expressed in terms of the whole amount of MG, DG, TG and FFA, as weight percent of the total sample.

4. Experimental Design

Response surface methodology was used to model and optimize the glycerolysis of olive oil, catalyzed with immobilized *Candida rugosa* lipase. A 5-coded level and 4-factor central composite rotatable design (CCRD) was used to determine the responses (yield of MG and DG). The experimental design was carried out by four independent variables with five levels as shown in Table 1. The independent variables were as follows: reaction time, temperature, lipase amount, and glycerol to oil molar ratio.

5. Statistical Analysis

The results of the experimental design were analyzed by using STATISTICA software from Stat-Soft, Inc., USA. For this study, a total of 30 experiments were necessary to estimate the coefficients. The experimental data was analyzed using a second-order polynomial equation, and the data were fitted into the equation by the response surface regression procedure. The general form of the model equation is

$$Y = b_0 + \sum_{i=1}^{4} b_i X_i + \sum_{i=1}^{4} b_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} b_{ij} X_i X_j \quad (1)$$

where Y is the predicted response value; X_i and X_j represent the independent variables; b_o is the offset term, b_i is the linear coefficients, b_{ii} is the quadratic coefficients and b_{ij} is the cross-product coefficients.

RESULTS AND DISCUSSION

1. Screening of Organic Solvent

The glycerolysis of olive oil with immobilized *Candida rugosa* lipase as biocatalyst was carried out in *tert*-butanol, chloroform, ethanol, acetone, toluene, n-hexane, and isopropanol and their combinations. These organic solvents were selected with different log P values from −0.24 to 4. In this set of experiments, all the reactions were at 40 °C, enzyme amount of 0.015 g, water content in glycerol of 3.5% (w/w), molar ratio of glycerol to oil at 1.5 : 1, and solvent, 4 ml/1g olive oil for 4 h. Hydrophilic solvents (such as isopropanol and *tert*-butanol) yielded higher MG content than hydrophobic solvents (such as chloroform, toluene and *n*-hexane). However, the highest MG yields (46.8 wt%) were found in *tert*-butanol with high hydrophilicity, which has low log P values (0.35). But, higher DG yields (19.3 wt%) were also attained in *n*-hexane, whose log P values of 3.5 (Fig. 1(a)). This is in agreement with the finding of Fu and Vasudevan [21] and confirms that enzymes have selective tolerance to particular organic solvents.

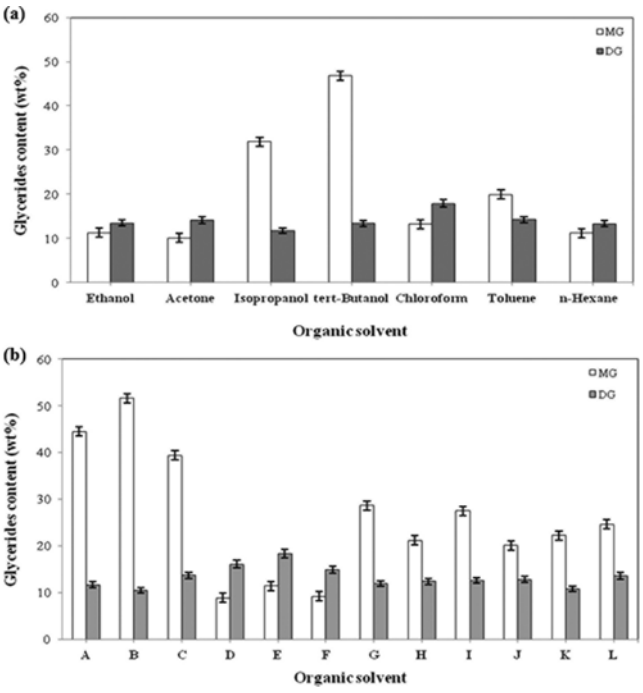

Fig. 1. Effect of (a) pure solvents and (b) solvent mixtures on glycerolysis of olive oil. Reaction conditions: glycerol/olive oil molar ratio, 1.5 : 1; enzyme load, 0.015 g; temperature, 40 °C, reaction time, 3 h; *tert*-butanol, 4 ml. A, isopropanol/*tert*-butanol (1 : 1); B, isopropanol/*tert*-butanol (1 : 3); C, isopropanol/*tert*-butanol (3 : 1); D, acetone/n-hexane (1 : 1), E, acetone/n-hexane (1 : 3); F, acetone/n-hexane (3 : 1); G, isopropanol/n-hexane (1 : 1); H, isopropanol/n-hexane (1 : 3), I, isopropanol/n-hexane (3 : 1); J, *tert*-butanol/n-hexane (1 : 1); K, *tert*-butanol/n-hexane (1 : 3); L, *tert*-butanol/n-hexane (3 : 1).

As shown in Fig. 1(a), high substrate conversion was observed in solvents with a log P value of 0.28 and 0.35, because these solvents have no effect on lipase stability. However, some organic solvents (low log P value) usually strip the essential water off the lipase molecules and then deactivate the biocatalyst and thus influence the stability of lipase during reuse. As an alternative, co-solvent which is mixed with a high log P solvent such as n-hexane and a low log P solvent such as acetone was attempted. Based on this analysis, *tert*-butanol, acetone, n-hexane, and isopropanol were chosen for the mixture to study the effects on MG and DG yields. Laane

et al. [22] reported that there was some influence of the log P value (the logarithm of the partition coefficient in an octanol-water two-phase system) of the organic solvents on the reaction rate.

The log P_{cs} was calculated by following Eq. (2).

$$\log P_{cs}=X_1\log P_1+X_2\log P_2 \quad (2)$$

where, log P_{cs} is the log P of the co-solvent, X_1 and X_2 are the mole fractions of two solvents mixed, log P_1 and log P_2 are the pure solvent values. As shown in Figs. 1(a) and (b), the MG and DG yields were higher in co-solvent than those of pure solvents, and the MG yield in isopropanol and *tert*-butanol mixture was higher than in isopropanol and n-hexane mixture. The highest MG yield (51.6%) was obtained in the *tert*-butanol co-solvent mixture with composition of 25% isopropanol and 75% *tert*-butanol (1 : 3), higher than those of pure isopropanol (31.9 wt%) and *tert*-butanol (46.8 wt%). This result indicates that a mixture of isopropanol and *tert*-butanol (1 : 3) solvent in which the immobilized *Candida rugosa* lipase is stable and optimal for lipase-catalyzed glycerolysis reaction.

Therefore, the log P values of co-solvents mixtures were used to analyze the MG yields in different co-solvent ratios. The MG yield was 39.4 wt% in mixture of isopropanol and *tert*-butanol (3 : 1) with log P_{cs} value 0.29 and 27.5 wt% in a mixture of isopropanol and n-hexane (3 : 1) with log P_{cs} value 1.21. Thus, the log P of a pure organic solvent can be a relationship with the lipase activity, while the log P_{cs} of co-solvent mixture is an average of the log P values of the individual solvent and does not relate to lipase activity. In addition, the enzyme activity is also affected both by functional groups and chemical bonds of solvents [23].

2. Effect of Reaction Parameters on the Production of MG and DG

The effect of reaction parameters, such as the reaction time, temperature, lipase amount, and molar ratio of glycerol to oil on the glycerolysis of olive oil in solvent system was systematically investigated.

The effect of time on the glycerolysis was considered at 40 °C and lipase amount of 0.02 g in isopropanol/*tert*-butanol (1 : 3) solvent system. As shown in Fig. 2, the conversion of TG into MG and DG yields continuously increased with reaction time up to 5 h, but equilibrium was reached after 5 h. No increase in TG conversion and MG and DG yield was observed by increasing the reaction time to 5 h. Since the maximum yield of MG (53.9 wt%) and DG (14.7 wt%) occurred at 5h, the reaction time 5h was then used for the glycerolysis reaction.

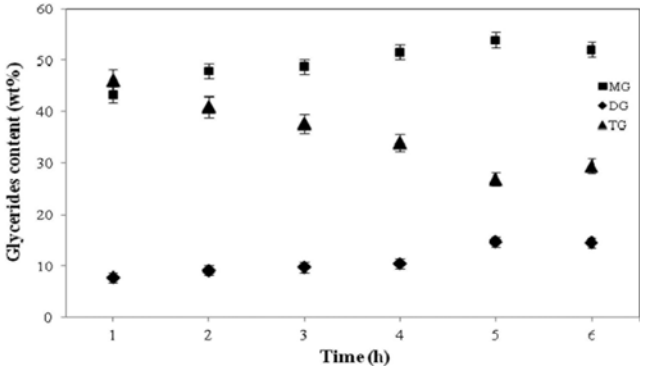

Fig. 2. Effect of time on olive oil glycerolysis at a 1.5 : 1 molar ratio glycerol/oil, 0.02 g lipase/g oil, 40 °C temperature, and 4 ml isopropanol/*tert*-butanol (1 : 3). (■) Monoglycerides; (◆) diglycerides; (▲) triglycerides.

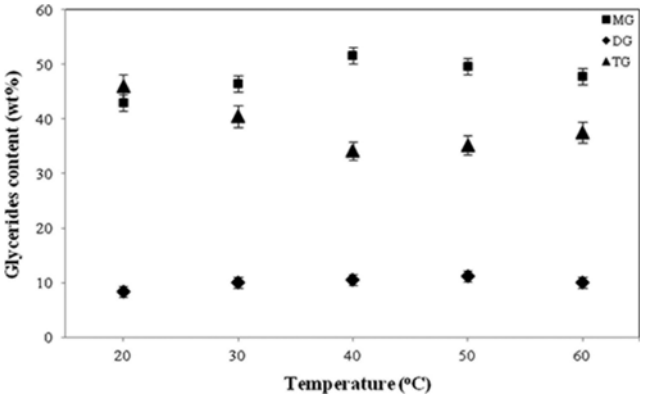

Fig. 3. Effect of temperature on olive oil glycerolysis at a 1.5 : 1 molar ratio glycerol/oil, 0.02 g lipase/g oil, 4 h reaction time and 4 ml isopropanol/*tert*-butanol (1 : 3). (■) Monoglycerides; (◆) diglycerides; (▲) triglycerides.

The glycerolysis of olive oil catalyzed with immobilized *Candida rugosa* lipase was affected by the reaction temperature. The glycerolysis was carried out at 20, 30, 40, 50, and 60 °C, keeping constant the lipase concentration 0.02 g, glycerol to oil molar ratio 1.5 : 1, water content of 3.5% and 4 mL of isopropanol/*tert*-butanol (1 : 3) solvent mixture. The reaction conversion at the initial stage was relatively slow at 20 °C (Fig. 3). An increase in reaction temperature can reduce viscosity of glycerol, enhance solubility and improve substrate diffusion, thus reducing mass transfer limitations and favoring interactions between enzyme and substrates. However, if the reaction temperature is set too high, lipase denaturation can occur. Therefore, an optimum temperature should be selected. The result is shown in Fig. 3. As the temperature was increased, there was a trend in higher synthesis of MG and DG. However, the conversion rate of TG was almost decreasing after 40 °C and yield of MG and DG was decreased. This might be related to the decrease of lipase activity above 40 °C. The optimal temperature was 40 °C with the highest MG (51.6 wt%) and DG (11.2 wt%) yields. Tuter and Aksoy [24] also reported that the enzymatic glycerolysis of palm kernel oil with glycerol led to 31% of MG yield at 40 °C for 24 h of reaction. Yang et al. [25] investigated that the esterification of fatty acids with glycerol catalyzed with Novozyme 435 lipase produced MG and DG at 30-50 °C, but main product is MG.

The effect of lipase amount on glycerolysis of olive oil was conducted at the immobilized *Candida rugosa* lipase load of 0.01, 0.015, 0.02, 0.025, and 0.03 g in 1 g oil. The results are shown in Fig. 4. One can see that the formation of MG and DG increased with an increasing lipase concentration. However, no further increment was obtained beyond 0.025 g lipase. Therefore, a 0.025 g lipase amount was the optimum at which the MG and DG yield was about 55.8 and 16.4 wt%, respectively. It may be because an excess of enzyme concentration in the reaction medium may not always contribute to increase the formation of MG and DG, since high enzyme con-

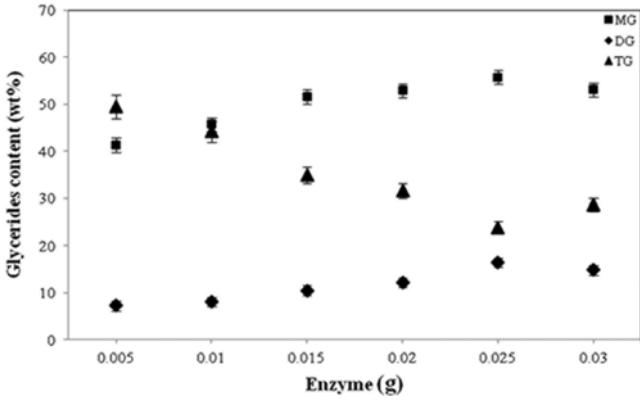

Fig. 4. Effect of lipase amount on product formation in the glycerolysis of olive oil at 40 °C temperature, 1.5 : 1 molar ratio of glycerol/oil, 4 h reaction time, and 4 ml isopropanol/*tert*-butanol (1 : 3). (■) Monoglycerides; (◆) diglycerides; (▲) triglycerides.

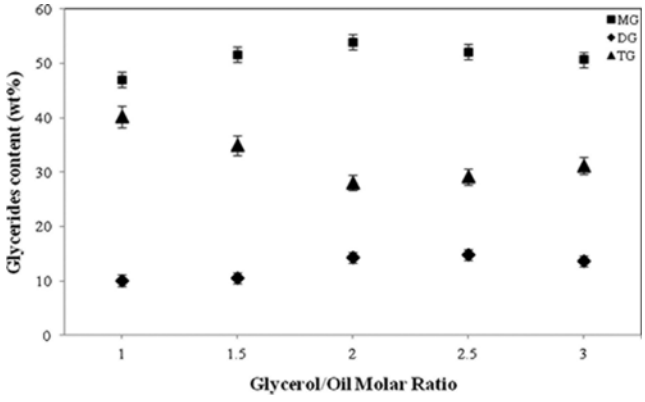

Fig. 5. Effect of glycerol/oil molar ratio on product formation in the glycerolysis of olive oil at 40 °C, 0.02 g lipase/g oil, 4 h reaction time, and 4 ml isopropanol/*tert*-butanol (1 : 3). (■) Monoglycerides; (◆) diglycerides; (▲) triglycerides.

centration may lead to the formation of protein aggregation; thus, enzyme active sites would not be exposed to the substrates in the reaction mixture. Lower activities of enzyme may be reducing the enzyme efficiency, but not enhancing the reaction conversion.

The increase in concentration of glycerol increased the theoretical equilibrium values, which led to an increase in the yield of MG and DG. In addition, glycerol can act as an effective stabilizer against thermal and solvent denaturation. However, the glycerol concentration in reaction mixture will also affect the system polarity, so as to influence the system stability and homogeneity. The reactions were carried out at different molar ratios of glycerol to olive oil: 1 : 1, 1.5 : 1, 2 : 1, 2.5 : 1, 3 : 1 with 0.02 g of lipase at 40 °C. As shown in Fig. 5, the yield of MG and DG increased with an increasing molar ratio of glycerol to oil from 1 : 1 to 2 : 1, and then decreased on further increase of the molar ratio to 2.5 : 1. The optimum ratio must exist due to both effects of glycerol on the reaction equilibrium and the system homogeneity. In this work, glycerol to oil molar ratio at 2 : 1 was selected for the reaction system. Tuter and Aksoy [24] reported the optimum molar ratio of glycerol to palm kernel oil for glycerolysis catalyzed by *Humicola lanuginose* lipase was 2 : 1. Yamane et al. [26] found that the low molar ratio of glycerol to palm olein at 1 : 2, the main product of glycerolysis was MG and DG.

3. Model Fitting

To optimize the process parameters with four variables in reaction system, a statistical experimental design was used with the assistance of RSM. This model is used for evaluating the parameters and their interactions. RSM using CCD was applied to determine the optimal levels of the variables (reaction time, temperature, lipase amount, molar ratio of glycerol to oil), which significantly influenced the reaction conversion. The results obtained from the

Table 2. Analysis of variance (ANOVA) of regression equation for yield of MG

Source of variance	Sum of squares	Degrees of freedom	Mean squares	F-ratio
X_1	383.200	1	383.200	6531.83
X_1^2	43.791	1	43.790	746.43
X_2	81.034	1	81.033	1381.26
X_2^2	662.205	1	662.205	11287.59
X_3	75.970	1	75.970	1294.95
X_3^2	285.459	1	285.458	4865.77
X_4	27.950	1	27.950	476.43
X_4^2	119.644	1	119.643	2039.38
$X_1 X_2$	0.076	1	0.075	1.29
$X_1 X_3$	0.951	1	0.950	16.2
$X_1 X_4$	0.141	1	0.140	2.40
$X_2 X_3$	1.756	1	1.755	29.93
$X_2 X_4$	0.006	1	0.005	0.10
$X_3 X_4$	0.106	1	0.105	1.80
Lack of fit	12.263	10	1.226	20.90
Pure error	0.293	5	0.058	
Total	1457.439	29		

CCD for MG production are presented in Table 1. From the experimental design and result (Table 1), the second-order response functions representing the relationship between MG content and operating parameters are obtained as in Eq. (3):

$$Y=48.66+3.99X_1-1.83X_2+1.77X_3-1.07X_4 -1.26X_1^2-4.91X_2^2-3.22X_3^2-2.08X_4^2-0.24X_1X_2-0.33X_1X_3 -0.08X_1X_4+0.06X_2X_3+0.09X_2X_4-0.01X_3X_4 \quad (3)$$

The corresponding analysis of variance (ANOVA) is presented in Table 2, which indicates the significance of independent variables, square term of the independent variables and first-order interaction terms for each paired combination of independent variables for MG yield. The predicted result along with experimental result is shown in Fig. 6. The predicted values match the experimental values reasonably well with coefficient of determination (R^2) was 0.97 for MG yield. Thus, quadratic regression model, Eq. (3), indicates that the model is satisfactory for the evaluation of such a reaction system.

The effects of each variable on the dependent variable, as well as their interactions can be seen from the Pareto chart as shown in Fig. 7. The order in which bars are displayed corresponds to the order of the size of the effect. The length of each bar in the Pareto chart is proportional to the absolute value of its estimated effect or associated regression coefficient. The chart includes a vertical line that corresponds to the 95% limit, indicating statistical significance. A factor is, therefore, significant if its corresponding bar crosses this vertical line [20]. From the Pareto chart (Fig. 7), a quadratic term of reaction temperature (X_2X_2) is the most significant factor for the MG yield. The highest yield of MG was 51.6 wt% at 40 °C. This result agrees with Fig. 3 in the effect of temperature on the glycerolysis reaction. The linear term of reaction time (X_1) was a significant but considerably low effect on glycerolysis reaction (Fig. 7). The quadratic terms of amount of lipase (X_3X_3), molar ratio of glycerol and oil (X_4X_4), time (X_1X_1) and linear term of temperature (X_2), lipase amount (X_3), molar ratio of glycerol and oil (X_4) were significant but less important.

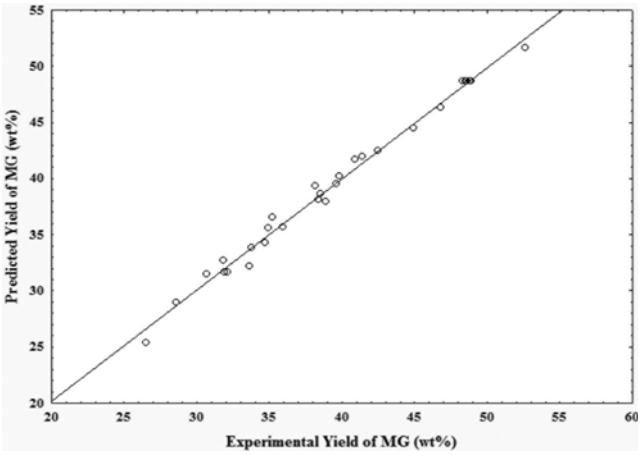

Fig. 6. Correlation between experimental and predicted MG yield.

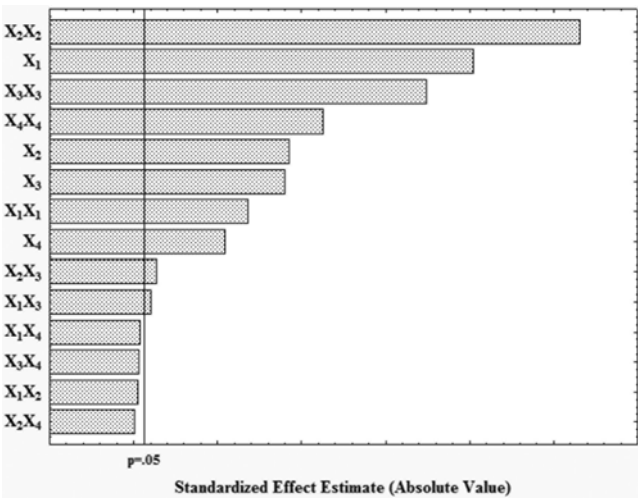

Fig. 7. Pareto chart of standardized effect estimate of MG yield.

Fig. 8(a) shows the three-dimensional surfaces plots of effect of reaction time and lipase amount on the MG yield. It can be seen that enhancing the reaction time from 1 to 5 h could increase the MG yield, but a further increase in time would lead to a slight decrease of the MG content. Also, the effect of lipase amount on MG content was significant. The yield of MG increases with the increase of lipase amount from 0.01 to 0.025 g; however, the yield decreased by further increasing amount of lipase. These results indicate that the long reaction time and large amount of lipase leads to a lower conversion of MG. This result agrees with Fig. 4 in the glycerolysis reaction.

Fig. 8(b) shows the effect of reaction time and temperature and their interactive effects on the MG yield. From analysis of three-dimensional surface plots, the interaction between reaction time and temperature was significant. As can be seen, the MG yield increased with the increase in the temperature from 20 to 40 °C and decreased rapidly above 40 °C. The conversion increased with an increase in the reaction time, although this decreased after 5 h, which also agrees with the result of single factor (reaction time) study (Fig. 2).

Fig. 8(c) shows the effect of lipase amount and reaction temperature on the MG yield. It was observed that the temperature increased from 20 to 40 °C, MG yield increased with the increase in lipase amount (above 0.005 g), which could be attributed to fact that there was low resistance of mass transfer with high concentration of lipase amount in isopropanol/*tert*-butanol (1 : 3) solvent mixture system. In addition, MG yield decline was found at high concentration of lipase (0.03 g) with high reaction temperature (50 °C). The glycerolysis with medium reaction temperature (40 °C) favored maximum MG yield, which agrees with Fig. 3 that lipase was stable in solvent mixture system.

Fig. 8(d) depicts the effect of reaction time and molar ratio of glycerol and oil on the reaction conversion. As indicated, the reaction time had a significance influence on the MG yield. The conversion increased rapidly with the increase of reaction time from 1 to 5 h, and slight conversion decline by further increasing reaction time. The molar ratio of glycerol to oil is one of the most important factors that influence the conversion of TG to MG and DG. The response value increased with increasing molar ratio of glycerol to oil from 1 : 1 to 2 : 1, but a further increase in molar ratio

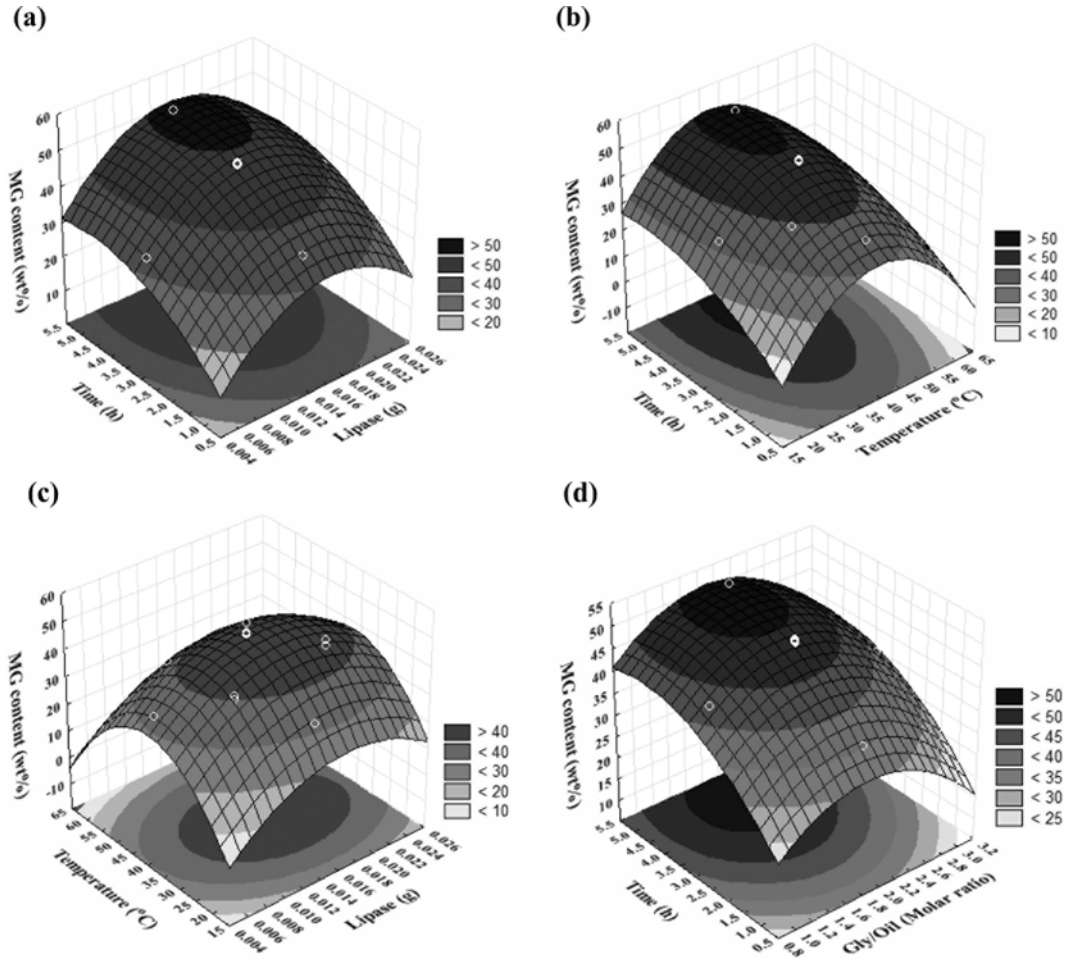

Fig. 8. Response surface plot showing effects of the parameters on MG yield during glycerolysis: (a) At varying reaction time and lipase amount (b) at varying reaction time and temperature (c) at varying reaction temperature and lipase amount and (d) at varying reaction time and glycerol to oil molar ratio.

leads to decrease of the reaction conversion. The reaction with high molar ratio favored the maximum yield of MG, which also agrees well with the result of single factor (molar ratio), as shown in Fig. 5. However, reaction time 4 h, temperature 40 °C, lipase amount 0.025 g and glycerol to oil molar ratio 2 : 1 are suggested as optimal reaction conditions for the enzymatic production of MG and DG after statistical analysis.

CONCLUSION

Enzymatic glycerolysis of olive oil in different organic solvent medium was successfully carried out and the reaction parameters were optimized. The effect of pure solvents and co-solvents mixtures on the glycerolysis was evaluated. A co-solvents mixture, isopropanol and *tert*-butanol at ratio 1 : 3, was optimal and the most suitable organic medium for lipase-catalyzed glycerolysis for MG and DG production. The optimum conditions for MG (55.8 wt%) and DG (16.4 wt%) synthesis under the selected co-solvents mixture were: reaction time 4 h, temperature 40 °C, lipase amount 0.025 g and glycerol to oil molar ratio 2 : 1. Response surface methodology (RSM) was used to optimize the reaction parameters and the optimal condition: lipase amount 0.025 g, glycerol to oil molar ratio 2 : 1, reaction time 4 h and temperature 40 °C.

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