American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Food Technology

ISSN 1557-4571
DOI: 10.3923/ajft.2016.143.151

Research Article

Optimized Transesterification for Diacylglycerol in Rapeseed Oil Using Response Surface Methodology Basing on FT-IR Spectroscopy

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Abstract

Objective: Glycerolysis of rapeseed oil was catalyzed with lipozyme TL IM lipase to produce diacylglycerol (DG). **Methodology:** To optimize DG yield, the effect of five-level four factors and their reciprocal interactions on product yield through Response Surface Methodology (RSM) was evaluated. Thirty individual experiments were performed to investigate reaction temperature, reaction time, enzyme loading and substrate mass ratio. **Results:** Well-fitting model was established through multiple regression with backward elimination for DG yield ($R^2 = 0.8388$). The optimal conditions established include 60°C reaction temperature, 8 h reaction time, 16% enzyme loading and 0.07 substrate mass ratio (glycerol to rapeseed oil), resulting to 82.4% DG yield. **Conclusion:** The experimental data showed good validation with the predicted value. Transesterification of rapeseed oil and glycerol was monitored by using Fourier transform infrared (FT-IR) spectroscopy.

Key words: Diacylglycerol, glycerolysis, response surface methodology, lipozyme TL IM, FT-IR

Received: March 20, 2016 Accepted: April 30, 2016 Published: June 15, 2016

Citation: Xiuzhu Yu, Yandie Yang, Lirong Xu, Zhong Zhao and Rui Zhang, 2016. Optimized transesterification for diacylglycerol in rapeseed oil using response surface methodology basing on FT-IR spectroscopy. Am. J. Food Technol., 11: 143-151.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diacylglycerol (DG), a natural component of glycerides, comprises up to 10% (w/w) various fats and oils[1]. The DG occurs in two isoforms, i.e., 1,2-(or 2,3)-diacyl-sn-glycerol (1,2-DG) and 1,3-diacyl-sn-glycerol (1,3-DG)[2]. The beneficial health effects of DG on animals and humans have been studied. The DG has similar digestibility and energy value to triacylglycerol (TG); however, DG can also decrease postprandial lipid levels[3-5]. Producing DG by using edible fats and oils has gained increasing attention because of their improved physical and chemical properties.

The DG can be enzymatically produced through esterification[6-8], glycerolysis[9,10] and partial hydrolysis[11-13]. High-purity DG can be easily obtained through enzymatic esterification. Kim and Lee[14] obtained a maximum DG concentration of 80.5% when the pressure was decreased to 3 mmHg. Moreover, Guo and Sun[15] obtained a yield of 92% to 96% 1,3-DG using a vacuum-driven N_2 bubbling protocol[15]. However, this processing DG involves multiple steps and thus considered costly[16]. Partial hydrolysis is advantageous because it is a one-step reaction, but controlling the degree of hydrolysis in this process is difficult. Considering time, space and cost efficiency, glycerolysis of oil is a more straightforward approach, which directly produces DG through one-step reaction[17]. Several studies on glycerolysis of oil have been documented, in which DG contents[18-22] vary from 40-70%. Yamane *et al.*[23] obtained a high DG content of 90% by incubating the mixture (hydrogenated beef tallow and glycerol) at three different temperatures. However, this process is complex and time consuming. To obtain high DG yield using a simple process, the present work focused on optimizing DG yield. Lipase-catalyzed glycerolysis of rapeseed oil in a solvent-free system was the synthesis strategy in this research. The solvent-free system can minimize the negative effects on enzyme stability or thermodynamic equilibrium caused by organic solvents[24].

For optimization, Response Surface Methodology (RSM) was used to evaluate multiple parameters individually or in combination with response variables[25]. The relationships between factors (reaction temperature, reaction time, enzyme loading and substrate mass ratio) and response (DG yield) were determined. The optimal condition was also established using Central Composite Design (CCD) and RSM analysis.

Fourier transform infrared (FT-IR) spectroscopy is a fast, simple, sample-nondestructive and efficient tool for qualitative and quantitative analyses of various food products[26,27]. The FT-IR spectroscopy is potentially useful for on-line monitoring. The IR spectra exhibit corresponding changes with the appearance of monoglycerides (MGs), DGs and Free Fatty Acids (FFA) in fat samples. These compounds contain the -OH group, which exhibits a distinct absorption in the IR spectra[28]. Hence, FT-IR spectroscopy can be used to rapidly determine the TG content of a reaction system. Thus far, several studies have used Attenuated Total Reflectance (ATR)-based FT-IR to monitor enzymatic interesterification[28,29], but using FT-IR to determine TG content has not been reported. A stainless steel mesh attached to an FT-IR sample holder was used for the first time to determine edible oil oxidation[30]. The mesh-IR cell is simple, demountable and reusable and thus can be used to easily monitor glycerolysis reactions. The proposed method provides the foundation for on-line monitoring of glycerolysis reaction through FT-IR spectroscopy.

MATERIALS AND METHODS

Materials: Lipozyme TL IM (*Rhizomucor miehei* lipase immobilized on microporous anion-exchange resins) was supplied by Novozymes A/S (Bagsvaerd, Denmark). Rapeseed oil was obtained from a local market. All other reagents used were of high purity and commercially available unless otherwise noted.

General procedure for enzymatic reaction: Lipozyme TL IM was used as a catalyst for the glycerolysis of glycerol with rapeseed oil. The reaction was performed in 50 mL triangular flask. Different concentration of enzyme (10-18%, w/w by weight of rapeseed oil) was added into the flask containing rapeseed oil and glycerol. The reaction mixture was incubated in a temperature-controlled magnetic stirrer (Shanghai Naji Scientific Instrument Co., Ltd., China) at 300 rpm and specified different temperatures for optimization.

Lipozyme TL IM was activated at 40°C for 1 h in an oven before using[29]. At the end of the reaction, the immobilized lipase and glycerol were removed by centrifugal separation. The reaction products were stored at -20°C for further analysis.

FT-IR analysis: The FT-IR spectra of all the samples were recorded using an FT-IR Vetex70 spectrometer (Bruker, Germany). Each spectrum was recorded from the co-addition of 16 scans at a resolution of 4 cm^{-1}. The spectrum of each sample was obtained with oil films by applying the oil onto 100 mesh stainless steel wire cloth. The background spectrum was obtained using the instrumental and environmental conditions similar to those applied for other samples.

The sample and background spectra were based on absorbance. To obtain comparable variables, we transferred raw FT-IR spectra data to OMNIC software and then standardized.

Analysis of glycerolysis reaction products: The reaction mixtures were separated through Thin-Layer Chromatography (TLC) on silica gel GF 254 plates (Shenghai, Qingdao, China) using a solvent mixture system of n-hexane/diethyl ether/acetic acid (70:30:1, v/v/v). After development, the plate was dried. Acylglycerol spots were visualized with iodine.

The DG yield was quantitatively analyzed using TLC-vis spectrophotometric method, which has been described by Bao and Zhang's method for TG content determination[31], with some modifications. To measure the DG yield of the reaction products, the sample solution was prepared in the following procedure: The developed plate which was developed in qualitative analysis was visualized at 105°C for 5 min. After the spots were covered with the ethanol solution of phosphomolybdic acid (10%), the DG spots were removed, eluted with 4.0 mL of distilled water, centrifuged and then diluted to 5.0 mL with distilled water. The blank solution was prepared using the similar method with a blank plate. The absorption spectrum of the solution was obtained within the range of 400-800 nm with a UV-2550 spectrophotometer (Shimadzu, Japan) and λ_{max} was determined based on the absorption spectrum. The absorption spectrum illustrated that the λ_{max} was 700 nm. The absorbance of each sample solution was determined at λ_{max} using the spectrophotometer. The DG obtained through silica gel chromatography was used to construct the standard curve. The DG yield was expressed as a weight percentage in the sample. All measurements were performed in triplicate.

Experimental design and statistical analysis: Design-Experiment software 8.0.6 (Stat-Ease, USA) was used for experimental design and regression of experimental data. A four-factor five-level CCD was adopted in this study. The CCD is a suitable design for sequential experiments to obtain appropriate information for "Lack of fit" testing without using a large number of design points[32]. The investigated factors were reaction temperature (°C), reaction time (h), enzyme loading (%, w/w by weight of rapeseed oil) and substrate mass ratio (glycerol/rapeseed oil) (Table 1).

Table 1: Coded and actual levels of factors used in the experimental design

	Coded levels					
Factors	Symbols	-2	-1	0	1	2
Reaction temperature (°C)	X_1	55.00	60.00	65.00	70.00	75.00
Reaction time (h)	X_2	6.00	8.00	10.00	12.00	14.00
Enzyme loading (%)	X_3	10.00	12.00	14.00	16.00	18.00
Mass ratio (glycerol/rapeseed oil)	X_4	0.04	0.05	0.06	0.07	0.08

RESULTS AND DISCUSSION

Determination of TG conversion: Figure 1 illustrates the standardized preprocessed IR spectra of different TG conversion samples within the region of 4500-400 cm^{-1}.

The glycerolysis reaction involves the exchange of acyl groups between glycerol and rapeseed oil. The FT-IR monitoring of the glycerolysis reaction is based on the absorbance of the characteristic functional groups in the lipid products after the removal of glycerol prior to analysis. The change in absorbance at 3473 cm^{-1}, which is attributed to the OH groups in MG, DG and FFA was monitored. The TG conversion involves the increase in the OH moiety, resulting in the increase in the peak height at 3473 cm^{-1}. The absorbance at 3473 cm^{-1} increased with the increase in TG conversion.

The 98 of oil samples were prepared by mixing rapeseed oil with reaction solution (TG had been removed first) to span a TG content range of 0-100% (68 of oil samples were used to calibrate and the other 30 of oil samples were used to validate). The calibration equation of TG content was obtained as Eq. 1:

$$y = -0.0142x+1.5257 \quad (1)$$

where, x is the TG content (%) and y is the absorbance at 3473 cm^{-1}) with R^2 = 0.9937. For the validation,

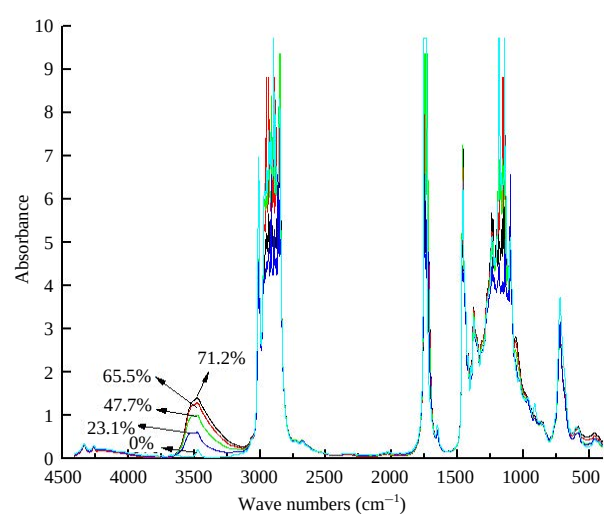

Fig. 1: Spectra of different TG conversion samples

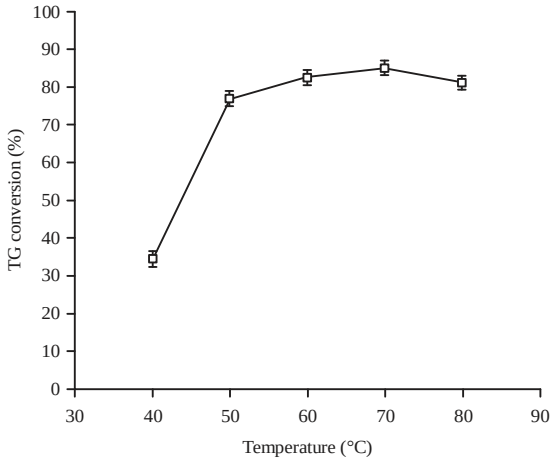

Fig. 2: Effect of temperature on TG conversion

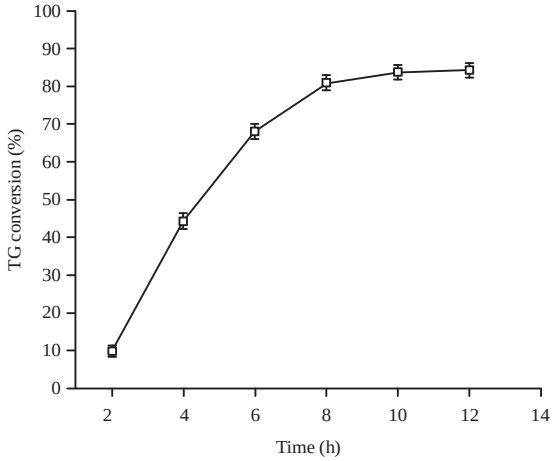

Fig. 3: Effect of reaction time on TG conversion

the concurrence between the actual TG content and predicted TG content (calculated using Eq. 1) can be evaluated using the following regression equation:

$$y = 1.0128x+0.0877$$

where, x is the actual value (%) and y is the predicted value (%) with R^2 = 0.9978. This indicates that this method can be employed for the determination of TG content.

The reaction solutions were determined at the same condition and TG contents were calculated using Eq. 1. The TG conversion was obtained from the following Eq. 2:

$$\text{TG conversion} = 1 - x_i \quad (2)$$

where, x_i is the TG content of each reaction solution, %.

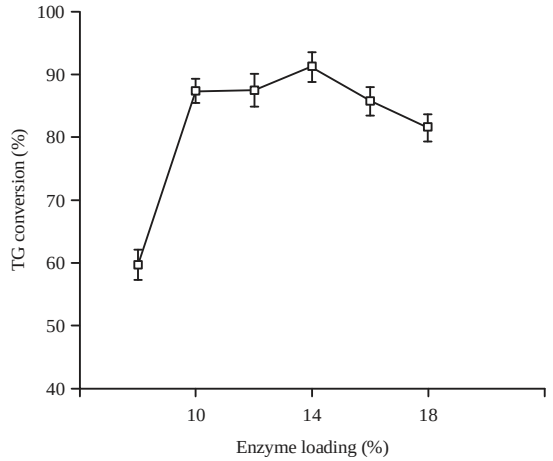

Fig. 4: Effect of enzyme loading on TG conversion

Effect of reaction temperature on TG conversion: The effect of reaction temperature on TG conversion was investigated from 40-80°C and the results are presented in Fig. 2. No substantial effect was observed when the reaction temperature was increased from 60-80°C. However, -60°C was considered to be appropriate temperature for the reaction.

Effect of reaction time on TG conversion: The effect of reaction time on TG conversion is illustrated in Fig. 3. The TG conversion marginally increased when the reaction time was increased. Hence, the reaction time of 10 h was selected for the process.

Effect of enzyme loading on TG conversion: The effect of enzyme loading on TG conversion is shown in Fig. 4. Enzyme loading significantly affected the conversion of TG, which slowly increased when the experiment was carried out at the lowest enzyme loading of 10%. However, at high enzyme loading (>14%), TG conversion slightly decreased because of high mass transfer resistance. The results suggested that high enzyme loading was unnecessary.

Effect of mass ratio on TG conversion: The results of varying the mass ratio (glycerol/rapeseed oil) by different proportions from 0.04-0.08 are shown in Fig. 5. The TG conversion was significantly affected by mass ratio. At higher mass ratio, maintaining mixture homogeneity can be difficult because of the higher viscosity of the mixture. From these experiments, a ratio 0.07 was considered to be adequate for the process.

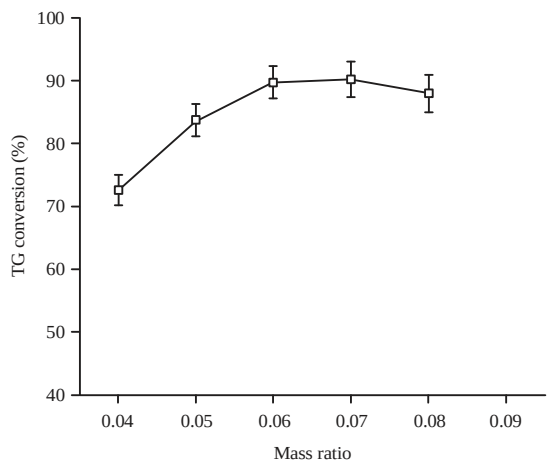

Fig. 5: Effect of mass ratio on TG conversion

Table 2: Experimental design and results of the CCD design

		Variable level			
					Response (wt %)
Trial	Temperature (°C)	Time (h)	Enzyme (%)	Mass ratio	$DG_{(yield)}$
1	70	12	16	0.05	68.85
2	65	10	18	0.06	72.75
3	70	8	16	0.05	67.31
4	60	8	16	0.07	82.03
5	70	8	12	0.05	63.83
6	65	10	14	0.06	74.45
7	70	8	12	0.07	50.13
8	60	12	16	0.05	67.94
9	60	12	12	0.07	63.83
10	65	10	14	0.06	74.84
11	60	12	16	0.07	79.36
12	65	10	14	0.08	77.19
13	65	10	10	0.06	42.86
14	65	10	14	0.06	73.28
15	70	8	16	0.07	78.44
16	60	8	12	0.07	77.19
17	65	6	14	0.06	69.98
18	65	14	14	0.06	77.34
19	60	8	16	0.05	67.31
20	70	12	12	0.05	69.38
21	70	12	12	0.07	58.00
22	75	10	14	0.06	73.11
23	65	10	14	0.06	83.30
24	65	10	14	0.04	59.52
25	60	8	12	0.05	69.02
26	70	12	16	0.07	83.05
27	65	10	14	0.06	73.25
28	55	10	14	0.06	68.11
29	65	10	14	0.06	74.33
30	60	12	12	0.05	70.28

Determination of DG yield: The absorbance of each solution was obtained at 700 nm. The linear correlativity between the content of DG and the absorbance of the solution was very high (R^2 = 0.9931) when the DG content ranged from 10-90 μg mL^{-1}. This relationship can be described using the following Eq. 3:

Table 3: Coefficients of the model and ANOVA for the quadratic model for DG yield

Source	Sum of squares	Df	Mean squares	F-value	p-value
Model	2005.93	11	182.36	8.51	<0.0001
X_1	32.60	1	32.60	1.52	0.2332
X_2	16.92	1	16.92	0.79	0.3859
X_3	730.52	1	730.52	34.11	<0.0001
X_4	167.75	1	167.75	7.83	0.0119
X_1X_2	71.02	1	71.02	3.32	0.0853
X_1X_3	99.95	1	99.95	4.67	0.0445
X_1X_4	47.64	1	47.64	2.22	0.1532
X_3X_4	349.97	1	349.97	16.34	0.0008
X_1^2	21.06	1	21.06	0.98	0.3345
X_3^2	463.47	1	463.47	21.64	0.0002
X_4^2	57.34	1	57.34	2.68	0.1192
Residual	385.52	18	21.42		
Lack of fit	311.81	13	23.99	1.63	0.3090
Pure error	73.70	5	14.74		
Total	2391.45	29			

$$y = 0.0091x+0.0983 \quad (3)$$

where, y is the absorbance of the solution and x is the content of DG).

An appropriate amount (set depending on TG conversion) of the reaction solution was dispersed with a specific amount of n-hexane to ensure that the solution contained 10-90 μg mL^{-1} DG. The absorbance of the solution was determined at 700 nm and the DG yield of the reaction solution was calculated using Eq. 3. All analyses were performed in triplicate.

Model fitting: The experimental value of DG yield ($DG_{(yield)}$) is presented in Table 2. A quadratic model was used to fit the experimental data. Each term in the selected model is considered significant at α level of 0.05.

The data obtained was subjected to regression analysis using the second-order regression equation. The coefficient of the regression equation was calculated using Design-Experiment 8.0.6 software with the following Eq. 4:

$$DG_{(yield)} = 75.43-1.16x_1+0.84x_2+5.52x_3+2.64x_4+2.11x_1x_2+ 2.5x_1x_3-1.73x_1x_4+4.68x_3x_4-0.87x_1^2-4.07x_3^2-1.43x_4^2 \quad (4)$$

where, x_i is the coded value of each factor.

The results of the second-order regression model fitting using analysis of variance (ANOVA) are presented in Table 3. The regression model provided an accurate description of the experimental data, indicating the successful correlation among the four independent parameters that affect DG yield. To measure the accuracy of the suggested model fit with the experimental data, the parameters R^2, p-value, F-value and lack of fit were used[33].

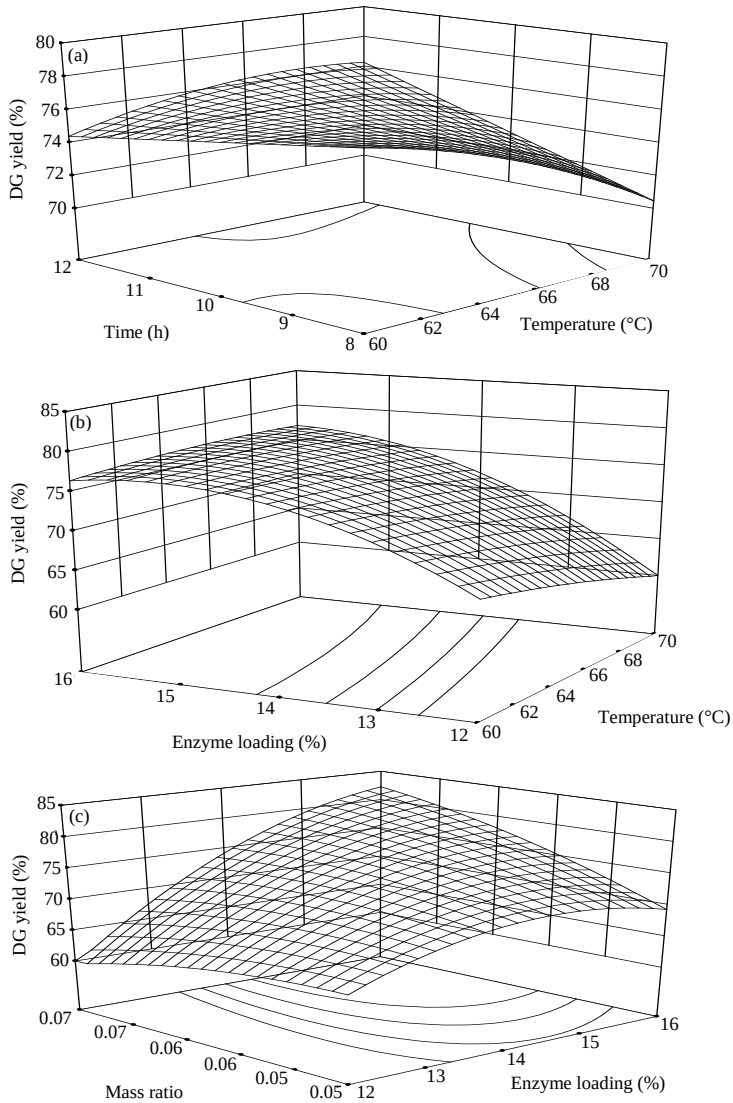

Fig. 6(a-c): Three-dimensional plot between any two parameters for the DG yield, (a) Enzyme loading = 14% and mass ratio = 0.06, (b) t = 10.05 h and mass ratio = 0.06 and (c) T = 65 °C and t = 10.05 h

As shown in Table 3, the regression model for $DG_{(yield)}$ was statistically accurate with a significance level of $p<0.0001$ and without significant lack of fit ($p>0.05$). The accuracy of the model was evaluated using the coefficient of determination (R^2). The value of R^2 was 0.8388, indicating that the model adequately represented the actual relationships among the selected reaction factors. Thus, the well-fitting model for $DG_{(yield)}$ was successfully established.

Furthermore, the conclusions obtained from Table 3 were presented as follows. (1) Independent variable enzyme loading (x_3) was the most significant factor that affected DG yield ($p<0.0001$), (2) DG yield was also affected by the independent variable mass ration (x_4) and the quadratic term of enzyme loading (x_3^2). Moreover, significant interactions were detected between reaction temperature and enzyme loading (x_1x_3) as well as enzyme loading and mass ratio (x_3x_4) and (3) The following factors exhibited no significant effect on DG yield within the designed intervals: Independent variable of reaction temperature (x_1) and reaction time (x_2), quadratic terms of reaction temperature (x_1^2) and mass ratio (x_4^2) and interactive terms of reaction temperature and reaction time (x_1x_2) as well as reaction temperature and mass ratio (x_1x_4).

Analysis of response surfaces: The main and interactive effects of the four factors on DG yield are shown in Fig. 6.

Reaction conditions: The effects of reaction temperature and reaction time on DG yield are shown in Fig. 6a. At reaction temperature higher than 66°C, the DG yield slightly increased with prolonged reaction time of 8-12 h. By contrast, if the reaction temperature was lower than 64°C, the DG yield remained stable with prolonged reaction time. Thus, the reaction with high reaction temperature (70°C) and the longer time produced the maximum DG yield.

Figure 6b shows the main and interactive effects of reaction temperature and enzyme loading on DG yield. The interaction between reaction temperature and enzyme loading was significant as indicated by the analysis on the response surface plots. At high temperature (>66°C), DG yield increased with the increase in enzyme loading. However, DG yield decreased at low temperature (<64°C) and high amount of enzyme loading. This finding can be attributed to the high mass transfer resistance at high enzyme loading and low temperature in the oil system (solvent-free system)[34,35].

Figure 6c shows the main and interactive effects of enzyme loading and mass ratio on DG yield. At low enzyme loading (<13%), increasing the substrate mass ratio from 0.05-0.06 could increase the DG yield. However, further increase in mass ratio slightly decreased the DG yield. At high enzyme loading (>15%), DG yield increased with the increase in substrate mass ratio. This phenomenon can be explained by the following reasons. At low enzyme loading, a high amount of glycerol could lead to the high mass transfer resistance caused by the viscosity of glycerol in the solvent-free system, thus decreasing the DG yield. Therefore, a high conversion could be obtained by combining appropriate substrate mass ratio and enzyme loading.

The high DG yield could be attributed to the following: Lipozyme TL IM, which was activated in an oven at 40°C for 1 h before use and the four important parameters that affect the glycerolysis reactions.

Obtaining optimal conditions and model verification: Within the experimental range, the optimal conditions for lipase-catalyzed synthesis of DG were predicted using the optimization function of Design-Expert Software. The results showed no significant effect on DG yield after 8 h. Thus, from an industrial point of view, reaction time was optimized at the lowest possible level to produce high DG yield. The present study also demonstrated that DG yield did not significantly increase at reaction temperatures ranging from 60-70°C. Hence, reaction temperature was optimized at the lowest possible level. Considering the solution derived from the design and cost efficiency, the optimal reaction was presented conditions for obtaining maximum DG yield. The reaction conditions included the following: Reaction temperature, 60°C; reaction time, 8 h; enzyme loading, 16% and substrate mass ratio, 0.07. The predicted DG yield obtained from Eq. 4 was 83.56%. This DG yield wan higher than several studies reported[18-22]. However, Yamane *et al.*[23] obtained a high DG content of 90% by incubating the mixture (hydrogenated beef tallow and glycerol) at three different temperatures. The DG yield is less than this study, but our process is more simple and convenience to operate[29].

CONCLUSION

The RSM was successfully applied to optimize the reaction parameters for lipase-catalyzed-conversion of DG in a solvent-free system. One set of quadratic model was obtained to describe the relationship between the parameters (reaction temperature, reaction time, enzyme loading and substrate mass ratio) and the response (DG yield). Three-dimensional surface plots were used to analyze the conditions for obtaining maximum DG yield. To control the processing cost, optimum conditions were established (60°C, reaction time of 8 h, enzyme loading of 16% and substrate mass ratio of 0.07). Under the optimized conditions, the average value of DG yield was 82.39%, the experimental value obtained was consistent with the predicted data.

Moreover, FT-IR spectroscopy and TLC-vis spectrophotometry were successfully applied to determine TG content, thus establishing a foundation for the monitoring of the glycerolysis reaction.

ACKNOWLEDGMENTS

This research was financially supported by Key Science and Technology Program of Shaanxi Province, China (2016NY-159).

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