Critical Temperature for Production of MAG by Esterification of Different FA with Glycerol Using *Penicillium camembertii* Lipase

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ABSTRACT: Production of MAG by a lipase-catalyzed reaction is known to be effective at low temperature. This phenomenon can be explained by assuming that synthesized MAG are excluded from the reaction system because MAG, which have low m.p., are solidified at low temperatures. Consequently, MAG are efficiently accumulated and do not serve as the precursor of DAG. If this hypothesis is correct, the critical temperature for MAG production, defined as the highest temperature at which DAG synthesis is repressed, should depend on the m.p. of the MAG. Esterification of FFA with glycerol using *Candida rugosa*, *Rhizopus oryzae*, and *Penicillium camembertii* lipases produced MAG efficiently at low temperatures. However, *Candida* lipase showed very low esterification activity at high temperatures (>20°C), and *Rhizopus* lipase produced not only MAG but also DAG even at low temperatures. Meanwhile, *P. camembertii* lipase catalyzed synthesis of MAG only from FFA and glycerol at low temperatures, although the enzyme catalyzed synthesis of DAG from MAG in addition to synthesis of MAG at high temperatures. We thus studied the effect of temperature on esterification of C_{10}–C_{18} FFA with glycerol using *Penicillium* lipase as a catalyst and determined the critical temperatures for production of MAG. The critical temperature for production of each MAG showed a linear correlation with m.p. of the MAG, which supported the hypothesis. In addition, because the m.p. of MAG are estimated from that of the constituent FA, the optimal temperature for production of MAG can be predicted from the m.p. of the FFA used as a substrate.

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KEY WORDS: Critical temperature, esterification, FFA, lipase, MAG, *Penicillium camembertii*.

MAG are well-known and widely used as emulsifiers in the manufacture of processed foods. Owing to their ability to form complexes with amylose or proteins, MAG are also used as conditioning agents to modify starch- and protein-containing products. In addition, they also can be utilized to modify physical characteristics of fats by controlling their crystal polymorphism (1), or as building blocks for the synthesis of structured TAG (2–4).

MAG are produced industrially by chemical glycerolysis of fats and oils under high temperatures of 210–240°C (5,6). However, high temperatures cause the formation of undesirably flavored and colored by-products and limit the synthesis of MAG with unstable FA. Therefore, lipase-catalyzed reactions have attracted much attention, particularly in the production of MAG containing functional FA (7–16).

Several papers have indicated that the yield of MAG in enzyme syntheses is increased by lowering the reaction temperature (3,11,12,16). This phenomenon can be explained by assuming that MAG solidified at low temperatures are excluded from the reaction mixture. But the hypothesis has not been verified. If this is correct, the critical temperature, defined as the highest temperature at which MAG are produced efficiently, should depend on the m.p. of the MAG. We thus studied the effect of reaction temperature on the production of MAG by esterification of various FFA with glycerol using *Penicillium camembertii* mono- and diacylglycerol lipase (referred to as *Penicillium* lipase). This paper indicates that the hypothesis is valid, and that the critical temperature can be estimated from the m.p. of FFA used as a substrate.

MATERIALS AND METHODS

Materials. Capric acid (C_{10}; purity, >99%), lauric acid (C_{12}; >99%), myristic acid (C_{14}; >99%), oleic acid ($C_{18:1}$; 91%), linoleic acid ($C_{18:2}$; 98%), and glycerol were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). An α-linolenic acid ($C_{18:3}$) preparation composed of 77.6 wt% α-linolenic acid, 20.8 wt% linoleic acid, and 1.3 wt% oleic acid was prepared by urea adduct fractionation of linseed oil FFA (Yashiro Co. Ltd., Osaka, Japan) (17). In brief, a mixture of 150 g linseed oil FFA, 300 g urea, 1500 mL ethanol, and 120 mL water was heated at 65°C to completely dissolve all components. The mixture was gradually cooled to 5°C over *ca*. 10 h. After removal of the resulting precipitate, 1700 mL of 0.1 N HCl was added to the filtrate. Nonadducted FFA were extracted three times with 400 mL *n*-hexane, and the solvent was then removed by evaporation. A FFA mixture containing CLA (named FFA-CLA) was a commercial product (CLA-80; Rinoru Oil Mills Co. Ltd., Tokyo, Japan) composed of 33.1 wt% 9*cis*,11*trans*-CLA, 33.9 wt% 10*t*,12*c*-CLA, 0.9 wt% 9*c*,11*c*-CLA, 1.4 wt% 10*c*,12*c*-CLA, 1.8 wt% other CLA isomers, 6.7 wt% palmitic acid, 2.7 wt% stearic acid, and 17.4 wt% oleic acid. The other chemicals were of analytical grade.

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Lipases. Lipases from *P. camembertii* and *Rhizopus oryzae* were gifts from Amano Enzyme Inc. (Aichi, Japan). *Penicillium* lipase was dissolved in deionized water at a concentration of 200 mg/mL [10,000 units (U)/mL], and the solution or a dilution of it was used for the esterification reactions. *Rhizopus* lipase was also dissolved in deionized water at a concentration of 200 mg/mL (9,800 U/mL), and the diluted solution was used for the reaction. The activity of *Penicillium* lipase was measured by titrating FFA liberated from monoolein (Tokyo Chemical Industry) with 50 mM KOH as described previously (15): Hydrolysis was conducted at 30°C in a 5-mL mixture of 0.5 mL monoolein, 50 mM Na-acetate buffer (pH 5.6), and 10 mM $CaCl_2$ with stirring for 30 min. For measurement of the activity of *Rhizopus* lipase, olive oil (Wako Pure Chemical Industry Ltd., Osaka, Japan) was used as a substrate, and the hydrolysis was performed under conditions similar to those for *Penicillium* lipase. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol FFA per minute.

Reactions. A large-scale esterification of FFA-CLA with glycerol was conducted at 5 or 30°C in a 1-L four-necked round-bottomed flask containing 111.3 g CLA, 182.7 g glycerol, and 6 mL *Penicillium* or *Rhizopus* lipase solution (5,000 U/mL). The mixture was agitated at 250 rpm with an impeller to form a homogeneous reaction mixture. A small-scale reaction was performed in a 50-mL vessel at temperatures ranging from −15 to 55°C depending on the FFA. The reaction was started with stirring at 500 rpm on a magnetic stirrer. After the stirring became difficult, owing to solidification of the reaction mixture, the mixture was left at the temperature without stirring. A typical reaction mixture was 10 g in total weight and contained 1 mol FFA, 5 mol glycerol, and 100 U/g-mixture of *Penicillium* lipase with 2 wt% of water originating from the enzyme solution. The degree of esterification was expressed as a ratio (mol%) of the amount of FA esterified to the total FA in the reaction mixture.

Purification of MAG. FFA and acylglycerols in the reaction mixture (*ca*. 10 g) were first extracted with 40 mL ether at 40°C, and the solvent was then evaporated. The extracts were dissolved in 20 mL *n*-hexane and were applied to a silica gel column (35 g; 20 × 250 mm; Merck, Darmstadt, Germany). After DAG and FFA were eluted with a 200-mL mixture of *n*-hexane/ethyl acetate (80:20, vol/vol), MAG were eluted with *n*-hexane/ethyl acetate (50:50, vol/vol). Organic solvents in the MAG fraction were removed with an evaporator.

Analyses. About 1 g of the reaction mixture was taken at time intervals and was used for analysis of the acid value and acylglycerol composition. The contents of MAG, DAG, TAG, and FFA were analyzed by a TLC/FID analyzer (Iatroscan MK-5; Iatron Laboratories Inc., Tokyo, Japan). A reaction mixture was dissolved in *n*-hexane at a concentration of 2 wt%, and sodium sulfate was added to the solution. One microliter of the solution was spotted on a silica gel rod, and the components were developed with a mixture of *n*-hexane/ethyl acetate/acetic acid (90:10:1, by vol).

The m.p. of the purified MAG were determined according to the AOCS standard open tube m.p. (slip m.p.) method (18) with some modifications. A hematocrit capillary tube (1.5 × 75 mm; Hirschmann Laborgeräte GmbH & Co., Eberstadt, Germany) was dipped in the liquid or melted sample so that the sample rose about 10 mm. The lower end of the capillary tube was slowly lifted up to create about 0.5 mm of empty space at the bottom of the tube. The sample was chilled at once on ice for samples with higher m.p. or dry ice for samples with m.p. lower than 15°C until solidified by holding the end of the tube that contained the sample. The tube was held in a refrigerator at 5°C (for samples with m.p. higher than 15°C) or in a freezer at −20°C (for samples with m.p. lower than 15°C) overnight. The slip m.p. of the MAG, except for $C_{18:2}$- and $C_{18:3}$-MAG, were measured according to the procedure described in the AOCS Official Method (18). For measurement of the m.p. of $C_{18:2}$- and $C_{18:3}$-MAG, cold acetone (−30°C), instead of water, was used as a heating medium.

RESULTS AND DISCUSSION

Production of MAG-CLA from FFA-CLA and glycerol using several lipases. Rhizopus and *Penicillium* lipases synthesized MAG-CLA by an efficient esterification of FFA-CLA with glycerol at 5°C. Typical time courses are shown in Figure 1. The reaction with *Rhizopus* lipase at 5°C produced MAG-CLA

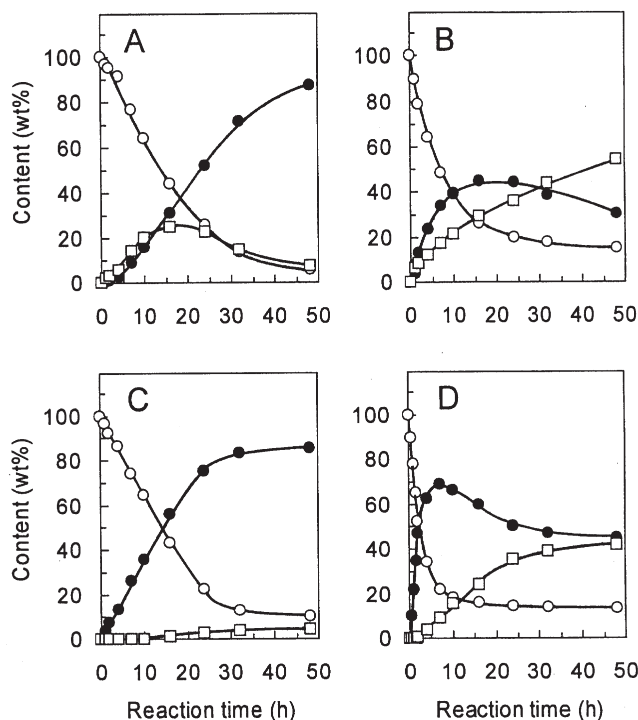
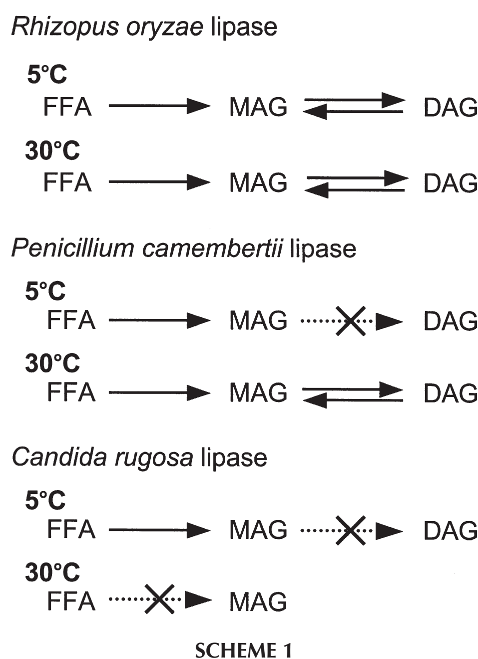


FIG. 1. Production of MAG-CLA with lipases from *Rhizopus oryzae* and *Penicillium camembertii*. A mixture of 294 g FFA-CLA/glycerol (1:5, mol/mol) and 6 mL lipase solution was agitated at 5 or 30°C. The amounts of *Rhizopus* and *Penicillium* lipases were 50 and 100 units (U)/g-mixture, respectively. (A) Reaction at 5°C with *Rhizopus* lipase; (B) reaction at 30°C with *Rhizopus* lipase; (C) reaction at 5°C with *Penicillium* lipase; (D) reaction at 30°C with *Penicillium* lipase. ○, Content of FFA; ●, MAG; □, DAG.



SCHEME 1

and DAG-CLA in the early stage of the reaction, and the content of DAG-CLA decreased after 16 h (Fig. 1A). An efficient accumulation of MAG-CLA after 16 h at 5°C could be explained by the glycerolysis of DAG-CLA occurring faster after 16 h than esterification of MAG-CLA. On the other hand, at 30°C, MAG-CLA was synthesized and was converted to DAG-CLA (Fig. 1B). Even in the late stage, the content of DAG-CLA increased, indicating that esterification of MAG-CLA was faster than glycerolysis of DAG at 30°C. The outlines of these reactions are shown in Scheme 1.

When esterification of FFA-CLA with glycerol was conducted at 5°C using *Penicillium* lipase, only MAG-CLA was synthesized, showing that esterification of MAG-CLA scarcely occurred at 5°C (Fig. 1C). Meanwhile, the reaction at 30°C synthesized MAG-CLA and DAG-CLA, and the content of DAG-CLA increased even in the late stage (after 10 h) as observed in the reaction at 30°C with *R. oryzae* lipase (Fig. 1D, Scheme 1).

Our previous study indicated that *C. rugosa* lipase also produced MAG-CLA efficiently at 5°C, but that the enzyme showed very low activity at higher temperatures (>20°C), owing to the attraction to the glycerol layer of water bound to the enzyme molecule (Scheme 1) (16).

The aim of this study was to determine the critical temperatures for production of MAG of various FA. *Candida* lipase is therefore not suitable because of poor esterification activity at high temperature. In addition, *Rhizopus* lipase catalyzed not only esterification of FFA with glycerol but also esterification of MAG and glycerolysis of DAG, even at low temperatures. Meanwhile, *Penicillium* lipase catalyzed only esterification of FFA with glycerol at low temperatures. We thus selected this lipase to analyze the critical temperatures.

Definition of critical temperature. Esterification of FFA with glycerol using *Penicillium* lipase produced MAG and DAG at 30°C, but only MAG at 5°C. The highest temperature at which only a very low amount of DAG is accumulated in the reaction mixture is referred to as the critical temperature for MAG production.

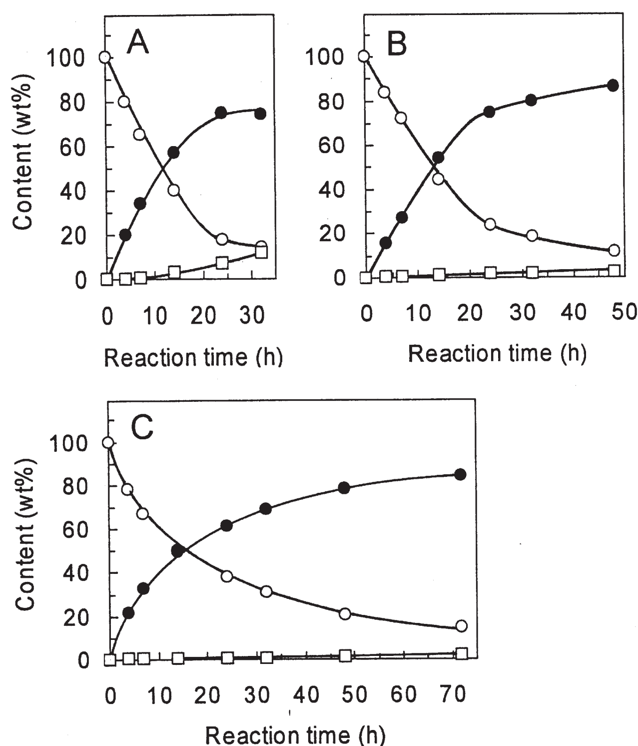


FIG. 2. Critical temperature for the production of MAG of capric acid by *Penicillium* lipase. A mixture of 9.8 g capric acid/glycerol (1:5, mol/mol) and 0.2 mL lipase solution was stirred at 15, 20, and 25°C. (A) Reaction at 25°C with a 100 U/g-mixture of lipase; (B) at 20°C with 100 U/g lipase; (C) at 15°C with 200 U/g lipase. ○, Content of FFA; ●, MAG; □, DAG. See Figure 1 for abbreviation.

Since MAG of different FA solidify at different temperatures, the critical temperatures for their production are assumed to be different. The reaction temperature was first decreased at 10°C intervals and then at 5°C intervals when it was close to the critical temperature. As an example, an approach for determining the critical temperature for production of MAG-C_{10} is shown in Figure 2. The esterification of capric acid with a fivefold molar excess of glycerol was first conducted at 35°C with a 100 U/g-mixture of *Penicillium* lipase. After 24 h, the degree of esterification reached 82%, and the contents of MAG and DAG were 59.9 and 25.9 wt%, respectively (the percentage of MAG to acylglycerols, 70 wt%). The reaction temperature was then decreased to 25°C. The percentage of MAG to acylglycerols after 32 h was 86 wt% at 81% esterification (Fig. 2A). When the reactions were conducted at 20°C, the degree of esterification reached 85% and the percentage of MAG to acylglycerols, 97 wt% (Fig. 2B). The reaction temperature was further decreased to 15°C. Because the velocity of esterification was very slow, a 200 U/g-mixture of lipase was used. After 72 h, the degree of esterification reached 85%, and the percentage of MAG/acylglycerols was 98 wt% (Fig. 2C). In this study, a

TABLE 1
Reaction Velocity, Degree of Esterification, and Ratio of MAG/Acylglycerols in the Esterification of Various FA with Glycerol by *Penicillium camembertii* Lipase

FFA	Temperature (°C)	Reaction velocity[a] (μmol/h/U)	Reaction time (h)	Esterification (%)	MAG/acylglycerols[b] (wt%)
C_{10}	15[c]	2.41	72	81	98
	20[d]	3.87	48	85	97
	25[d]	4.72	32	81	86
C_{12}	25[d]	1.92	96	80	99
	30[d]	4.34	32	82	96
	35[d]	6.43	24	81	68
C_{14}	35[c]	2.47	96	69	99
	40[c]	3.42	48	80	97
	45[d]	9.67	24	84	89
$C_{18:1}$	–5[c]	1.19	96	74	99
	0[c]	2.77	32	82	98
	5[d]	4.22	24	81	92
$C_{18:2}$	–15[e]	0.24	120	82	98
	–10[c]	0.88	60	85	97
	–5[c]	3.04	32	81	93
$C_{18:3}$	–15[e]	0.19	120	82	98
	–10[c]	1.13	60	87	98
	–5[c]	2.93	32	80	92
CLA	0[c]	2.33	32	84	99
	5[d]	3.64	48	86	95
	10[d]	5.57	24	81	93

[a]Reaction velocity is expressed as the amount of FA esterified per 1 h with 1 unit (U) of the lipase in the early stage of reaction.
[b]Weight percentage of MAG based on the amount of total reaction products, MAG and DAG.
[c]Reaction was conducted at the temperature indicated with stirring in a mixture of 9.8 g FFA/glycerol (1:5, mol/mol) and 0.2 mL water originating from the lipase solution using a 200 U/g-mixture of the lipase.
[d]The reaction was performed using a 100 U/g-mixture of the lipase.
[e]The reaction was performed using a 400 U/g-mixture of the lipase.

critical temperature for the production of MAG was defined as the highest temperature at which the reaction produced 95 wt% of MAG based on the total amount of acylglycerols (MAG and DAG) at >80% esterification. Hence, the critical temperature for production of MAG-C_{10} was determined to be 20°C.

Critical temperatures for the production of MAG of various FA. Capric acid, lauric acid, myristic acid, oleic acid, linoleic acid, α-linolenic acid, and FFA-CLA were esterified at various temperatures with *Penicillium* lipase. Table 1 shows the initial velocity in each reaction and the content of MAG based on total content of acylglycerols. From these results, critical temperatures for the production of MAG of capric acid, lauric acid, myristic acid, oleic acid, linoleic acid, α-linolenic acid, and FFA-CLA were determined to be 20, 30, 40, 0, −10, −10, and 5°C, respectively.

Relationship between the m.p. of MAG and critical temperature for MAG production. The reaction mixture from the esterification of each FA with glycerol at critical temperature became a solid after a certain reaction time. At temperatures higher than the critical temperature, on the other hand, the physical state of the reaction mixture tended to be softer and became more liquid at the optimal reaction temperature (about 45°C), which could be determined from the initial esterification velocity at various temperatures (data not shown).

The critical temperature for the production of MAG from each FFA may correlate with the solidification temperature of the reaction mixture, which depends on the m.p. of the corresponding MAG. MAG synthesized at their critical temperatures were purified by silica gel column chromatography, and their m.p. were then measured: The m.p. of MAG-C_{10} was 49.3°C; MAG-C_{12}, 58.3°C; MAG-C_{14}, 65.3°C; MAG-$C_{18:1}$, 30.7°C; MAG-$C_{18:2}$, 13.3°C; MAG-$C_{18:3}$, 14.5°C; and MAG-CLA, 34.9°C. The critical temperatures for the production of MAG from each FFA were plotted against the m.p. of the corresponding MAG (Fig. 3A). The plots revealed a linear correlation ($Y = 0.95X - 26$, $R^2 = 0.98$), strongly indicating that most of the MAG produced during the esterification at the critical temperature was solidified and excluded from the reaction system. Accordingly, further esterification of MAG to yield DAG was unlikely to take place, resulting in very low levels of DAG accumulation.

Estimation of critical temperature from FFA. If the optimal reaction temperature can be predicted from the components in the reaction mixture, optimization of the reaction conditions will be facilitated. The m.p. of the FFA used as substrates were therefore measured according to the procedure by which the m.p. of MAG were determined: m.p. of C_{10} FFA, 31.1°C; C_{12} FFA, 43.7°C; C_{14} FFA, 53.8°C; $C_{18:1}$ FFA, 11.2°C; $C_{18:2}$, –5.5°C; $C_{18:3}$, −10.8°C; FFA-CLA, 10.0°C. The m.p. of each MAG was plotted against the m.p. of the corresponding FFA (Fig. 3B). The plots afforded a linear correlation ($Y = 0.83X + 22$, $R^2 = 0.98$). Consequently, the critical temperature for MAG

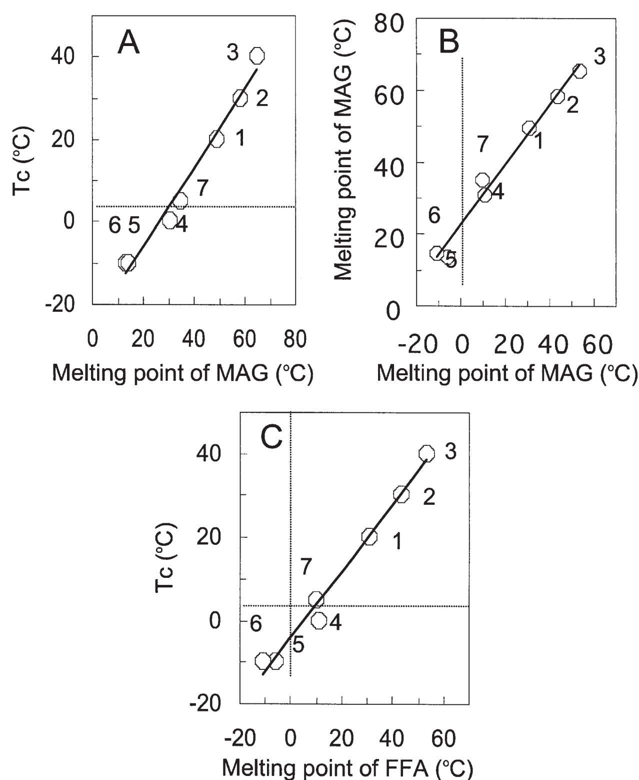


FIG. 3. Correlations among critical temperature (Tc) for the production of MAG from FFA and glycerol and m.p. of corresponding MAG and FFA. (A) Correlation between critical temperature and m.p. of MAG; (B) correlation between m.p. of MAG and FFA; (C) correlation between critical temperature and m.p. of FFA. 1, Capric acid; 2, lauric acid; 3, myristic acid; 4, oleic acid; 5, linoleic acid; 6, α-linolenic acid; 7, FFA-CLA.

production and the m.p. of the corresponding FFA bear a linear correlation ($Y = 0.80X - 4.5$, $R^2 = 0.98$) (Fig. 3C). It was concluded that the optimal temperature for production of MAG from FFA and glycerol with *Penicillium* lipase can be estimated from the m.p. of the FFA (or the FFA mixture) used as reactant.

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