Chapter 3

RESULTS AND DISCUSSION

1. Commercial lipase for MAG production

Hydrolytic activity of commercial lipases was analyzed. Palm olein in isooctane was used as the substrate. The results are shown in Table 7. Lipase PS from *Pseudomonas* sp. and lipase AY and OF from *Candida rogosa* gave high specific activity with 189.63, 241.91 and 162.39 U/mg protein, respectively. Lipase OF from *Candida rugosa* and lipase LP from *Chromobacterium viscosum* gave high hydrolytic activity with 33.94 and 32.32 U/mg enzyme, respectively. Lipase PL, D and M, however, had low activity.

2. Physical and chemical properties of palm olein

Composition and properties of palm olein as the substrate were analyzed. The results are shown in Table 8. Molecular weight of palm olein that calculated from sponification value was 838.22. Compositions of palm olein are shown in Figture 8. TAG (96.07 %) was the major composition in palm olein. The major fatty acid in palm olein included palmitic acid and oleic acid (Figure 9). Furthermore, some stearic acid and linoleic acid were found in palm olein. The results were similar to the results that reported by Maclellan (1983).

Table 7 Commercial lipases for glycerolysis of palm olein

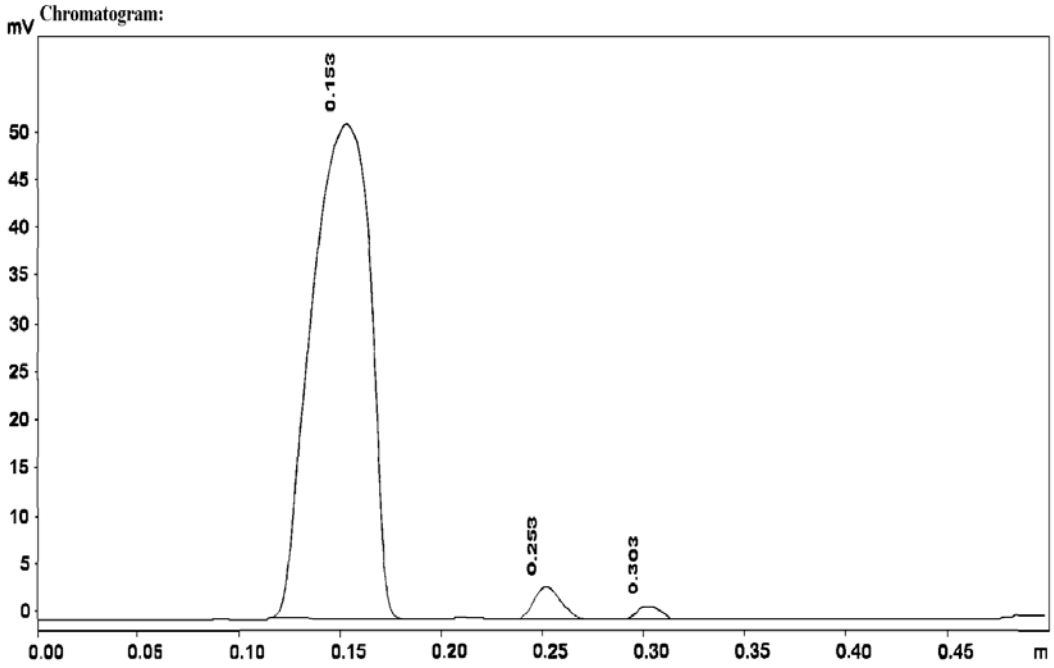
Enzyme	Activity U/mg enzyme	Protein Mg/mg enzyme	Specific activity U/mg protein
AK (*Pseudomonas fluorescens*)	5.35±0.02[a]	0.18±0.005	29.07
LP (*Chromobacterium viscosum*)	32.32±0.84	0.74±0.000	43.56
PL (*Alcaligenes* sp.)	2.67±0.08	0.25±0.004	10.82
PS (*Pseudomonas* sp.)	10.24±0.09	0.05±0.001	189.63
AY (*Candida rugosa*)	5.32±0.06	0.02±0.003	241.91
OF (*Candida rugosa*)	33.94±0.44	0.21±0.003	162.39
D (*Rhizopus delemar*)	14.04±0.45	0.91±0.008	15.47
F (*Rhizopus oryzae*)	8.25±0.03	0.31±0.002	26.54
M (*Mucor javanicus*)	2.29±0.07	0.16±0.006	14.07

[a] Mean ± standard deviation from triplicate determination

Table 8 Composition and properties of palm olein

Properties	This study	Maclellan (1983)
Sponification value	200.88	190.1-201.7
Iodine value	73.92	36.1-60.6
Acid value	0.57	
MW	838.22	
Composition (%)		
TAG	96.07	
FFA	-[a]	
1,3-DAG	2.84	
1,2(2,3)-DAG	1.09	
MAG	-[a]	
Fatty acid composition (%)		
Lauric acid ($C_{12:0}$)	-[a]	0.1-1.1
Myristic acid ($C_{14:0}$)	-[a]	0.9-1.4
Palmitic acid ($C_{16:0}$)	43.70	37.7-47.7
Palmitoleic acid ($C_{16:1}$)	-[a]	0.1-0.4
Stearic acid ($C_{18:0}$)	2.19	4.0-4.8
Oleic acid ($C_{18:1}$)	45.76	40.7-43.9
Linoleic acid ($C_{18:2}$)	8.35	10.4-13.4
Linolenic acid ($C_{18:3}$)	-[a]	0.1-0.6
Arachidic acid ($C_{20:0}$)	-[a]	0.2-0.5

[a] – could not detect



Peak No	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area %
1	TAG	0.153	0.115	0.182	54079	51.52	96.073
2	1,3-DAG	0.253	0.238	0.273	1600	3.34	2.842
3	1,2(2,3)-DAG	0.303	0.290	0.320	611	1.38	1.085
					56289	56.24	100.000

Condition:

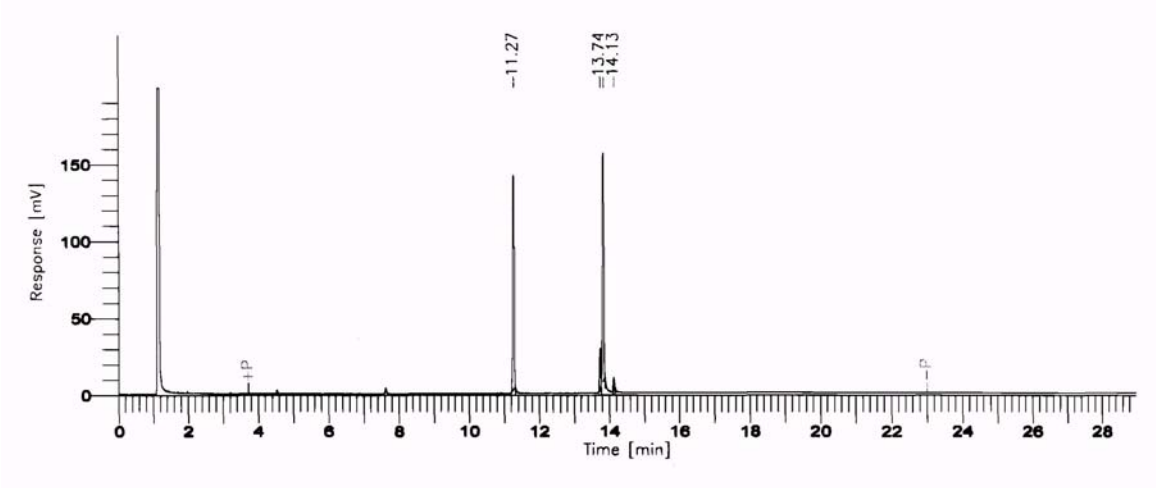
Stationary phase : CHROMAROD-SIII

Mobile phase : benzene : chloroform : acetic acid (50:20:0.7)

Gas flow : H_2 160 mL/min, Air 2.0 L/min

Scanning speed : 30 s/scan

Figure 8 TLC/FID chromatogram of palm olein



Peak #	Name	Time (min)	Area (μV.s)	Height (μV)	Norm Area (%)	BL	Area (%)
1	Palmitic acid	11.268	342721.15	140999.74	0.00	BB	43.70
2	Linoleic acid	13.738	65462.88	28177.76	0.00	BV	8.35
3	Oleic acid	13.821	358915.13	155175.16	0.00	VB	45.76
4	Stearic acid	14.129	17169.82	8313.56	0.00	BB	2.19
			784268.98	333666.22	0.00		100.00

Condition:

Column : Optima-5-0.25 μm 25 m x 0.25 mm

Carrier gas : Helium at 1.65 mL/min

Temp : 150 °C (40 °C/min, 0.5 min), 170 °C (5°C/min), 195°C (10°C/min) and 215°C (9.5 min)

Injector temp : 250 °C

Detector : FID at 250 °C

Figure 9 GC chromatogram of palm olein

3. Selection of commercial lipase for MAG production

Nine commercial lipases were screened for their ability to produce MAG though glycerolysis of palm olein at 30 °C in batch system. The results are shown in Figure 10. Lipase LP, lipase PL, lipase D, lipase F and lipase PS gave relatively high yield of MAG with 49.16, 48.16, 40.99, 35.33 and 32.67 %, respectively. In the other hand, lipase OF, lipase AK, lipase AY and lipase M gave low yield of MAG (< 3 %). Only 1-MAG was obtained, 2-MAG concentration being less than the sensitivity of the analysis method (less than 1 %). Many researchers found that lipase from *Chromobacterium viscosum* (Thude *et al*., 1997; Kamlangdee and Yamane, 1996) and *Pseudomonas* sp. (Chag *et al*., 1991; McNill and Yamane, 1991; Hongpattarakere, 2001) gave high yield of MAG. Chang *et al*. (1991) found that although in all experiments the amounts of hydrolytic activity were equivalent, their glycerolysis activities were quite inconsistent. Bornscheuer and Yamane (1994) found that in the solid-phase lipase-catalyzed glycerolysis of triolein, high concentration of MAG were obtained with bacterial lipases. Yeast and mold lipases were unsuitable for this reaction because of the rapid inactivation in the reaction mixture. However, after the product was produced, the reaction mixture become solid and further continuous production was impossible. Therefore, five commercial lipases that gave high yield of MAG were screened again at high temperature (45 °C). The results are shown in Figure 11 lipase LP and lipase PS gave the high yield of MAG with 30.15 and 28.05 %, respectively. According to the result of heat stability at 45 °C (Figure 12). Lipase LP and lipase PS were suitable for MAG production at 45 °C. When cost of enzyme was taken into account, lipase LP was not suitable for commercial production because it was more expensive. Thus, lipase PS was chosen for glycerolysis of palm olein in this work.

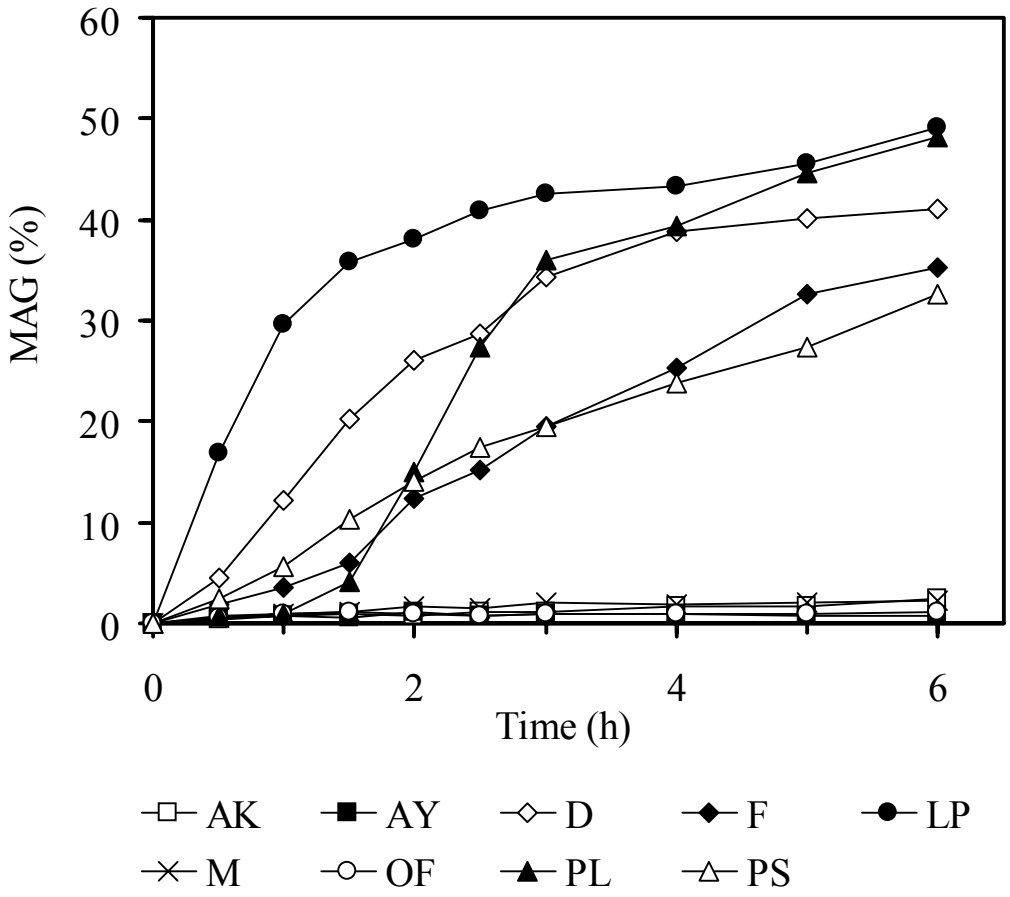


Figure 10 Glycerolysis of palm olein by lipases at 30 °C

The reaction mixture contained 15 g palm olein, 4.46 g glycerol, 0.18 g water and 500 U lipase. The reaction was carried out at 300 rpm and 30 °C.

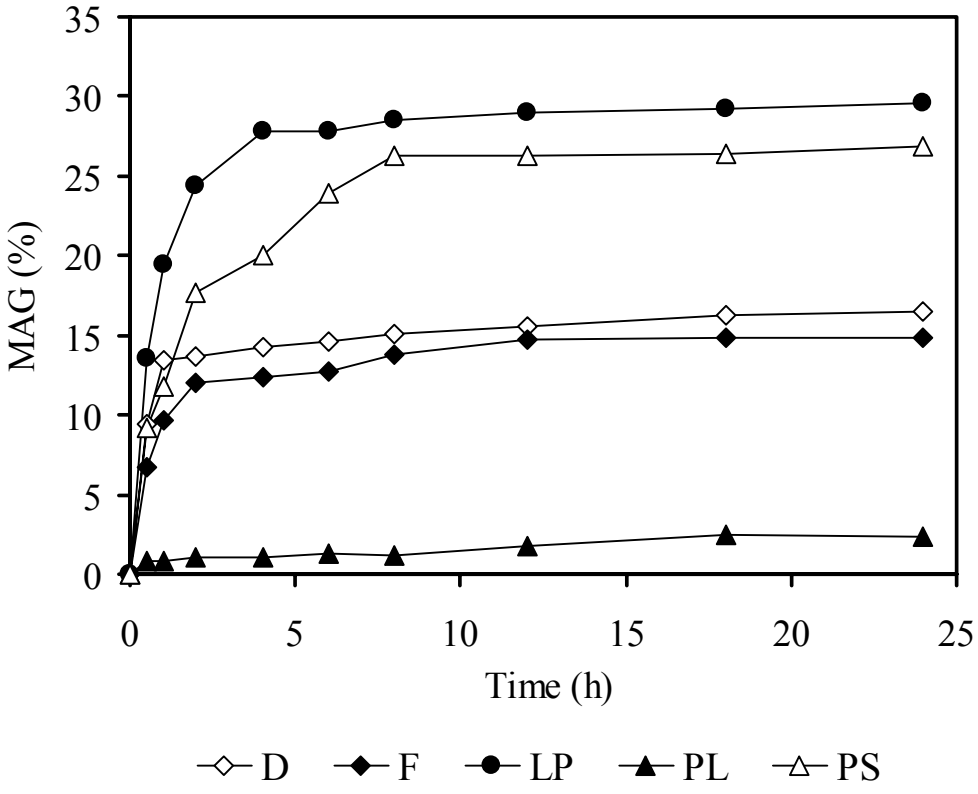


Figure 11 Glycerolysis of palm olein by lipases at 45 °C

The reaction mixture contained 15 g palm olein, 4.46 g glycerol, 0.18 g water and 500 U lipase. The reaction was carried out at 300 rpm and 45 °C.

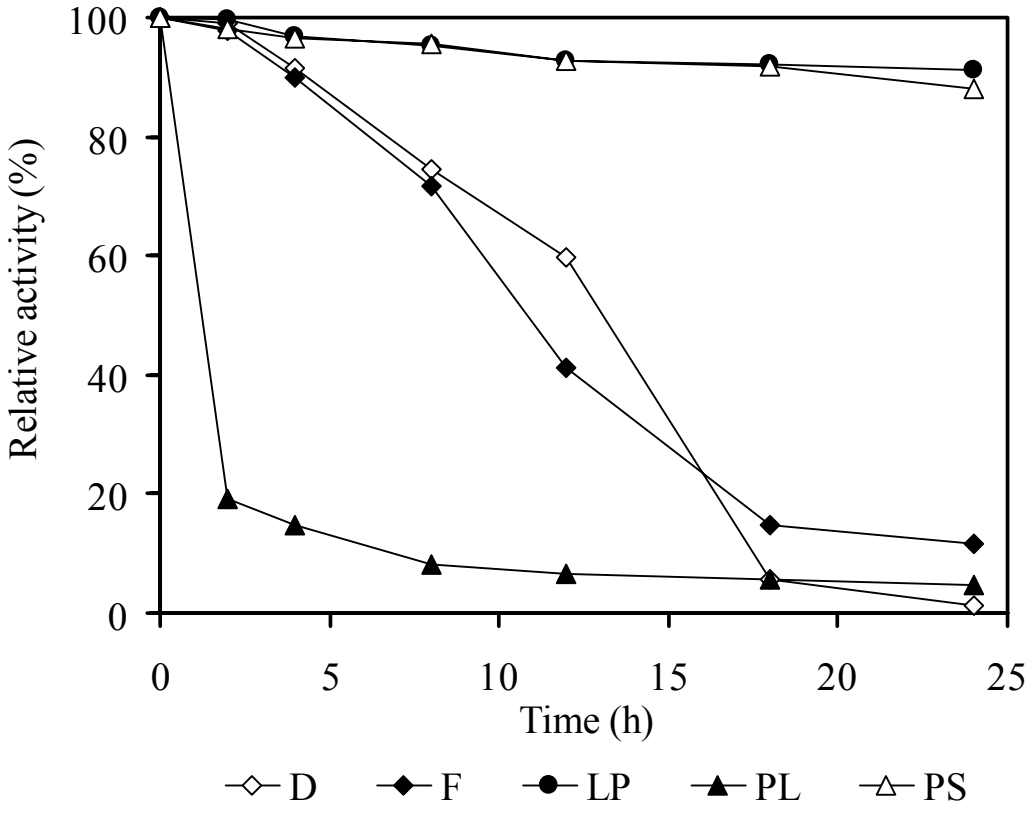


Figure 12 Thermostability of soluble lipase at 45 °C

4. Selection of support to immobilize lipase

Lipase PS was immobilized on different solid supports by physical adsorption. Table 9 shows the enzyme adsorption on the support particles. Accurel EP100 (<200 μm) displayed the best immobilized yield for adsorption and gave the highest immobilized activity with 0.37 U/mg support. Brady *et al*. (1988) reported that hydrophobic microporous materials such as Accurel provided better performance for immobilized lipases. Person *et al.* (2002) also found that adsorption on Accurel EP100 was the best method to immobilize lipase. Furthermore, Kimura *et al*. (1983) immobilized lipases on different inorganic and organic supports and found that the hydrophobic materials such as polypropylene and Celgard 2500 exhibited a higher activity in hydrolysis of

olive oil. Therefore, it was concluded that hydrophobic, microporous polymeric material is superior adsorbents for lipase immobilization. When the lipase PS immobilized on Accurel EP100 (<200 μm) was used for MAG production. It gave the highest yield of MAG with 20.74 % at 24 hours after incubation at 45 °C (Figure 13). In contrast, Rosu *et al.* (1997) found that lipase immobilized on $CaCO_3$ powder and celite exerted good activity and $CaCO_3$ displayed the best capacity for adsorption. Khare and Nakajima (2000) also showed that the lipase from *Rhizopus japonicus* immoblized on celite had more transesterification activity than its free form. However, Accurel EP100 (<200 μm) was suitable support for immobilizing lipase PS.

Table 9 Hydrolytic activity of immobilized lipase PS on various supports

Supports	Immobilized activity (U/mg support)	Immobilized yield (%)
Accurel EP100 (<200 μm)	0.368±0.004[a] a[b]	37.16±0.391 a
Accurel EP 100 (200-400 μm)	0.305±0.001 b	31.10±0.149 b
$CaCO_3$	0.008±0.000 e	0.79±0.044 e
Celite	0.035±0.000 d	3.56±0.027 d
Silica gel	0.064±0.001 c	6.42±0.121 c
Activated charcoal	0.004±0.000 e	0.36±0.019 e

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

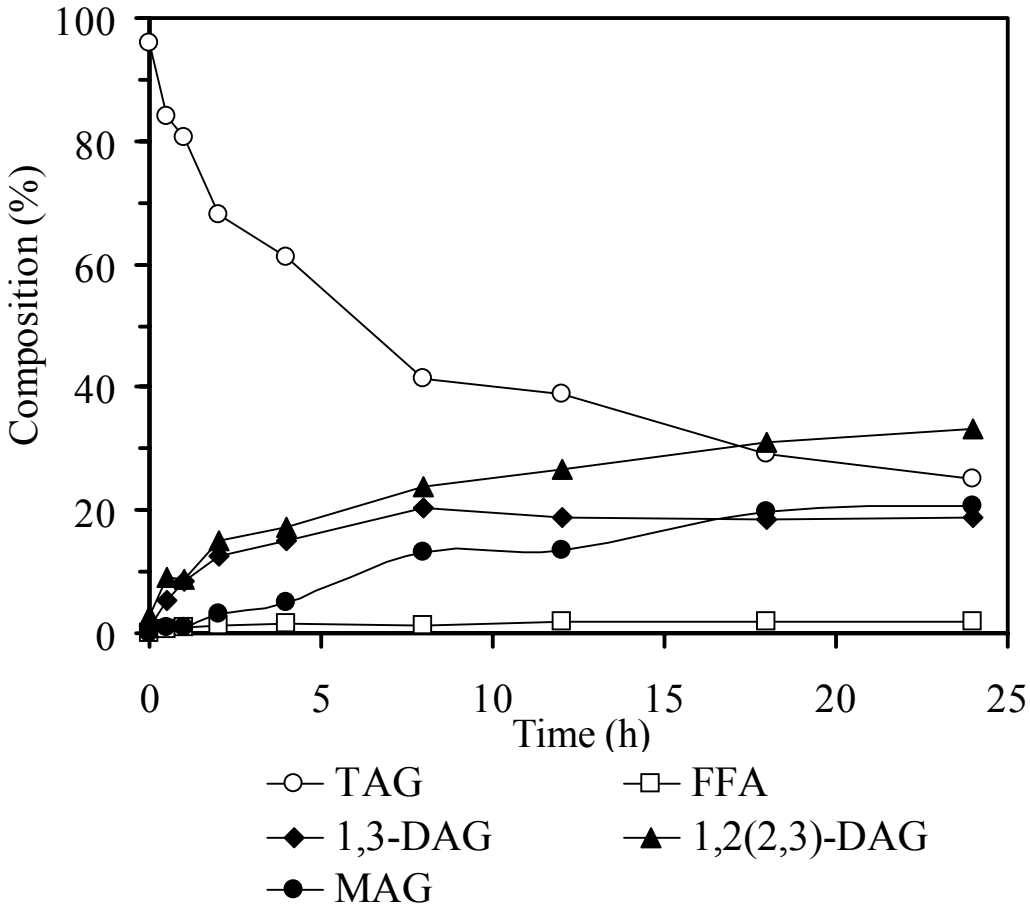


Figure 13 The composition of the reaction mixture during glycerolysis of palm olein with glycerol by lipase PS immobilized on Accurel EP100 (<200 μm)

The reaction mixture contained 15 g palm olein, 4.46 g glycerol, 0.18 g water and 0.2 g immobilized lipase PS (0.37 U/mg immobilized enzyme). The reaction was carried out at 300 rpm and 45 °C.

5. Optimal condition for enzyme immobilization

Accurel EP100 (<200 μm) was chosen to immobilize lipase PS. The parameters that effect on immobilization were investigated.

5.1 Effect of enzyme loading

The effect of enzyme loading on immobilization on Accurel was determined. The results are shown in Table 10. The hydrolytic activity of immobilized enzyme increased with increasing enzyme loading. On the other hand, the immobilized yield decreased with increasing the concentration of enzyme. These profiles could be due to limitation of substrate diffusion toward the surface and into the pore of the support because of its microporous nature. At high enzyme loading, steric hindrances caused by the excessive packing of the enzyme might occur. Moreover, the lipase molecules would penetrate and be immobilized to binding sites in the matrix pores, in sites inaccessible to the substrate. When the immobilized activity and immobilized yield were considered it was found that the concentration of enzyme with 50 U/mL was suitable for immobilized lipase PS on Accurel.

Table 10 Effect of enzyme loading on immobilization of lipase PS with Accurel

Enzyme concentration U/mL	Immobilized activity (U/mg support)	Immobilized yield (%)
5	0.049±0.000[a] e[b]	97.20±0.241 a
10	0.081±0.001 d	80.41±0.761 b
50	0.217±0.000 c	42.92±0.048 c
100	0.353±0.001 b	34.99±0.044 d
150	0.458±0.002 a	28.75±0.079 e

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

5.2 Effect of temperature

The effect of temperature on immobilized lipase PS on Accurel was investigated. The results are shown in Table 11. Variation of the immobilization temperature (4, 25 and 30 °C) had minimal effect on immobilized activity and immobilized yield. It might be caused by the short immobilization time and the lipase PS has high stability. In all cases the immobilized activity and immobilized yield were approximately 0.23 U/mg support and 44 %, respectively. A similar observation was made by Prumduang (2000), the immobilization temperature had no effect on immobilized lipase LP on celite. Accordingly, Sungpud (1999) found that immobilized lipase OF on Accurel at 4 and 25 °C gave a similar immobilized yield. However, temperature at 4 °C was suitable for immobilized lipase OF on Accurel (Phichaiyute, 1997).

Table 11 Effect of temperature on immobilization of lipase PS with Accurel

Temperature (°C)	Immobilized activity (U/mg support)	Immobilized yield (%)
4	0.234±0.003[a] a[b]	44.78±0.523 a
25	0.233±0.002 a	44.67±0.476 a
30	0.230±0.002 a	43.94±0.424 a

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

5.3 Effect of immobilization time

The effect of time on immobilization lipase PS on Accurel was studied. The results are shown in Table 12. The immobilized activity and yield were increased with time from 5 to 30 min. However, the time was longer than 30 min no effect on immobilized activity and yield were observed. A similar observation was made by Montero *et al.* (1993), after a 1-min incubation at room temperature with the polypropylene support, as much as 64 % of the initial activity disappeared from the solution, while after 30 min, nearly all the lipase activity was apparently adsorbed on the support. Thus, 30 min was enough to immobilize lipase PS on Accurel. However, 6 h (Phichaiyute, 1997) and 8 h (Sungpud, 1999) were suitable for immobilized lipase OF on Accurel.

Table 12 Effect of time on immobilization of lipase PS with Accurel

Immobilization time (min)	Immobilized activity (U/mg support)	Immobilized yield (%)
5	0.223±0.001[a] c[b]	43.29±0.167 c
10	0.220±0.002 c	43.25±0.359 c
15	0.223±0.002 c	43.29±0.335 c
20	0.228±0.002 b	44.40±0.311 b
30	0.233±0.002 a	45.38±0.355 a
60	0.234±0.001 a	45.53±0.145 a
120	0.234±0.001 a	45.51±0.215 a
240	0.234±0.001 a	45.50±0.193 a

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

6. Properties of immobilized lipase PS (IM-PS)

6.1 Optimum temperature

Optimum temperature for hydrolytic activity of IM-PS was studied. The results are shown in Figure 14. It was found that the optimum temperature of IM-PS was between 45 and 55 °C. The results obtained for immobilized form was almost similar to those for free form of lipase PS. Montero *et al.* (1993) suggested that the immobilization promotes a shift in the temperature profiles, which could arise from a lower restriction in the diffusion of the substrate and products at higher reaction temperatures.

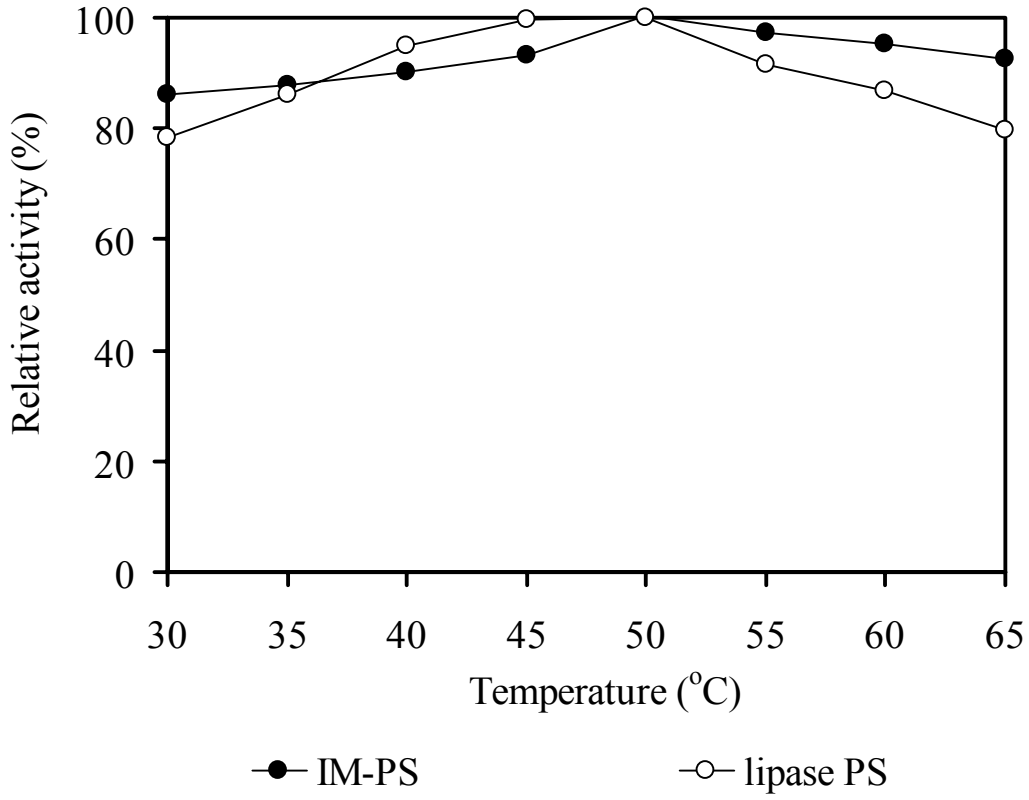


Figure 14 Effect of temperature on hydrolysis activity of lipase PS and IM-PS

6.2 Thermostability

Thermostability of IM-PS was studied at 45 °C (Figure 15). It was found that more than 90 % of the immobilized activity remained after incubated at 45 °C for 24 h. Furthermore, the immobilized lipase is slightly more stable than free form. Montero *et al.* (1993) found that *Candida rugosa* lipase immobilized on Accurel was apparently more stable than the soluble enzyme at temperature above 40 °C. Brady *et al.* (1988) found that after 120 days of storage on the shelf in a glass jar, the lipase that immobilized on HDPE (high-density polyethylene) Accurel powder lost less than 10 % of its original activity. When continuous hydrolysis in PBR and CSTR, half-life of 157 and 237 h were obtained, respectively.

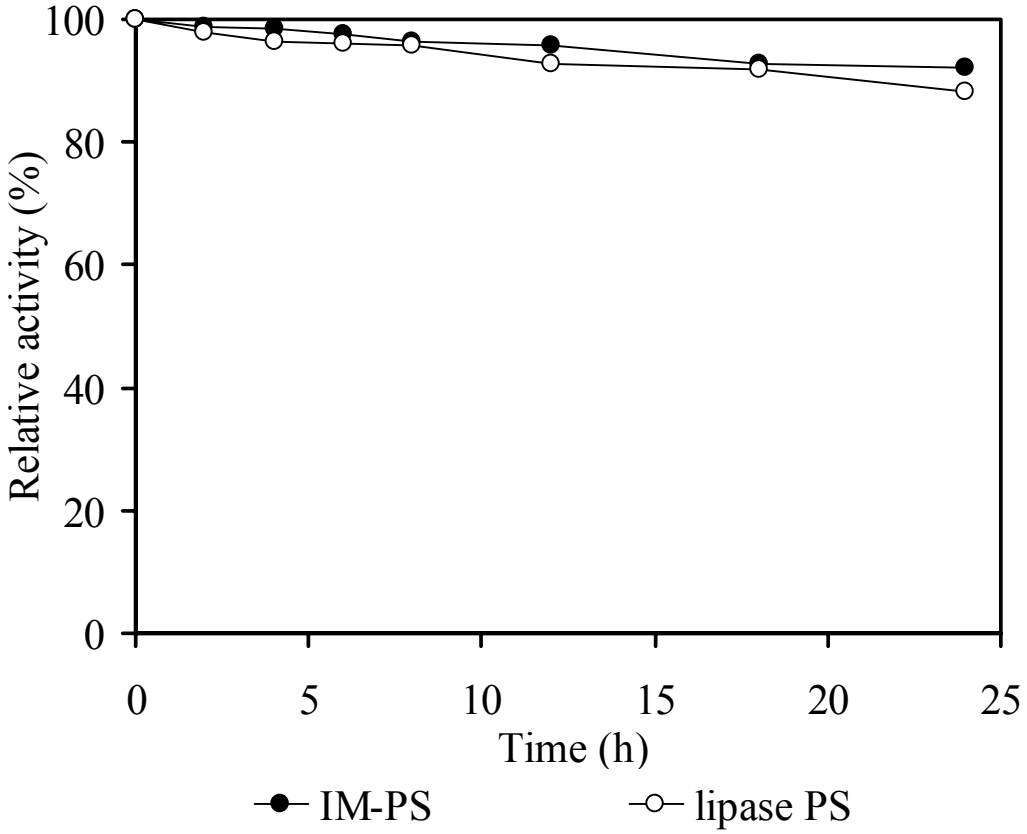


Figure 15 Thermostability of lipase PS and IM-PS at 45 °C

7. Continuous glycerolysis

7.1 Continuous glycerolysis in CSTR

The continuous glycerolysis of palm olein with glycerol were done in CSTR (4.5 cm ID, 6.0 cm height) that showed in Figure 6. The results are shown in Figure 16. After 24 h batch operation, the continuous operation was started. It was observed that some TAG was still remained in the process. The MAG yield was slightly stable at the level about 14 %. A productivity of 1.07 x 10^{-2} g MAG/U.day was obtained. Surprisingly, DAG [1,2(2,3)-DAG and 1,3-DAG] was the major product (46.4 %) in this reaction. However, the low content of FFA (< 2 %) was obtained in this reaction.

7.2 Continuous glycerolysis in PBR

Continuous monoglyceride production using IM-PS was performed in PBR reactor (Figure 7). The results are shown in Figure 17. The average yield of MAG was about 14 % and was slightly decreased with increasing the operation time. A productivity of 1.05 x 10^{-2} g MAG/U.day was obtained. A similar observation was made by Stevenson *et al.* (1993) during continuous glycerolysis of tallow with immobilized lipase in PBR. A yield of MAG approximately 17-19 % was obtained. Furthermore, Garcia *et al.* (1996) studied on continuous glycerolysis of butter oil in the absence of solvent using fixed-bed reactor at 55 °C and at reactor space time approaching 10 h. Under these condition, a product mixture containing about 22 % MAG was obtained. The high DAG yield [1,2(2,3)-DAG and 1,3-DAG] and FFA yield about 45 and < 2%, respectively were observed in PBR as well as in CSTR.

In solvent free system, substrates consisted of palm olein and glycerol have high viscosity, the reaction mixture was mixed difficulty. The pressure drops were occurred, especially in packed-bed column of PBR. Because of high substrate viscosity, IM-PS in CSTR could not mobile freely. So, IM-PS was destroyed by magnetic bar. Moreover, when intermediates and products were

produced in the reaction, some of these became solid and clogged in CSTR and PBR. Therefore, the continuous glycerolysis of palm olein in the solvent free system is not suitable to produce in both CSTR and PBR.

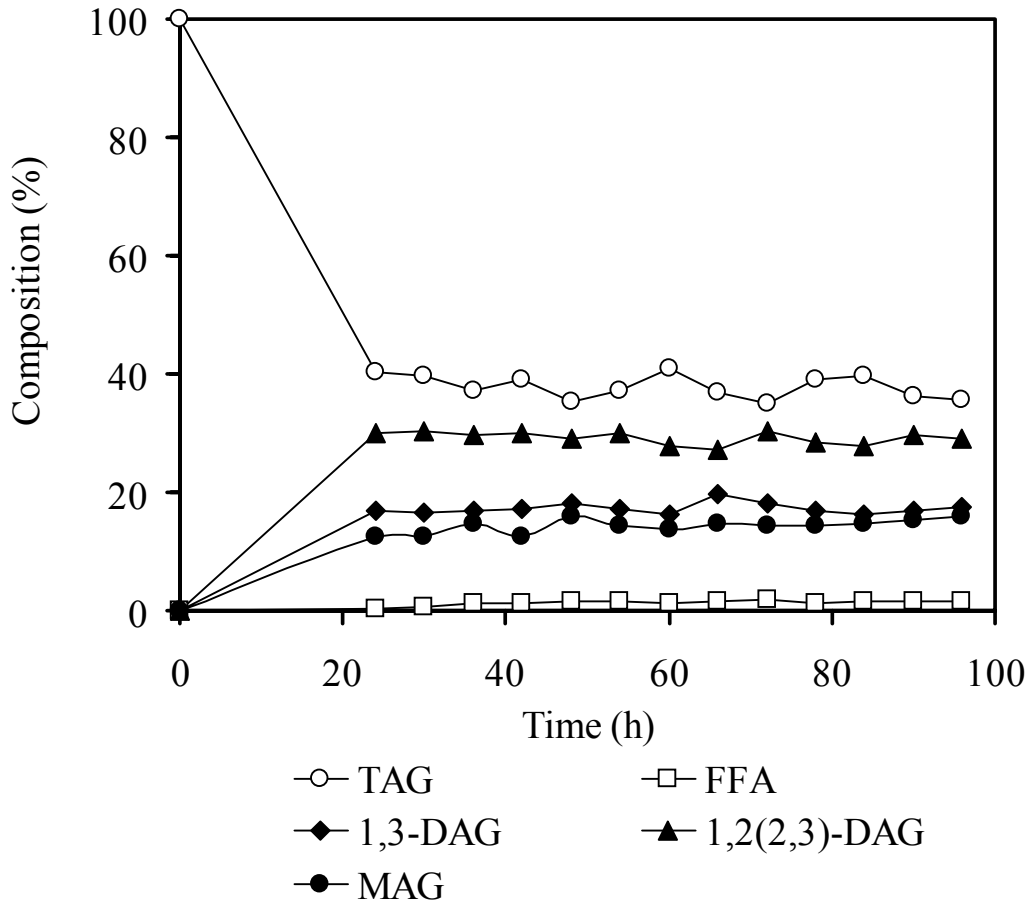


Figure 16 Continuous glycerolysis of palm olein with glycerol by IM-PS in CSTR at 45 °C

The amount of IM-PS used was 1.5 g (0.34 U/mg). Substrates consisted of glycerol to palm olein molar ratio of 2.7:1 and 4 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min.

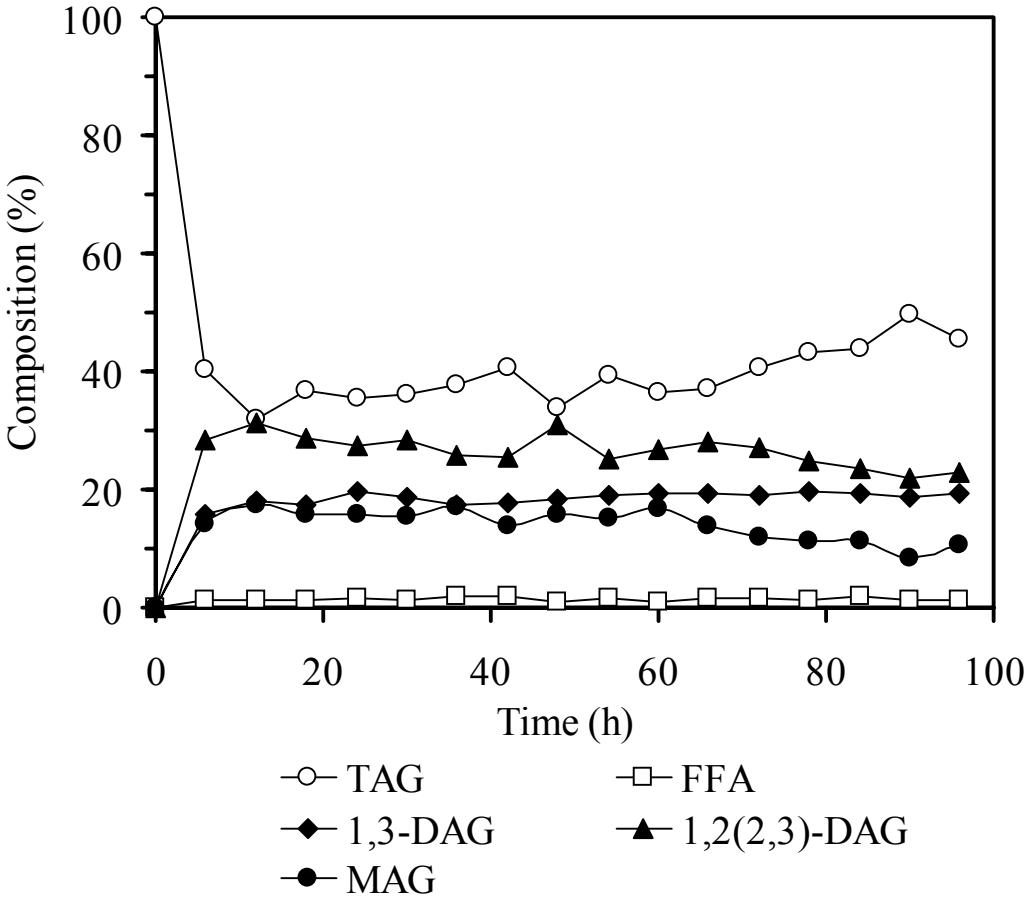


Figure 17 Continuous glycerolysis of palm olein with glycerol by IM-PS in PBR at 45 °C

The amount of IM-PS used was 1.5 g (0.34 U/mg). Substrates consisted of glycerol to palm olein molar ratio of 2.7:1 and 4 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min.

8. Optimization of MAG production in solvent system

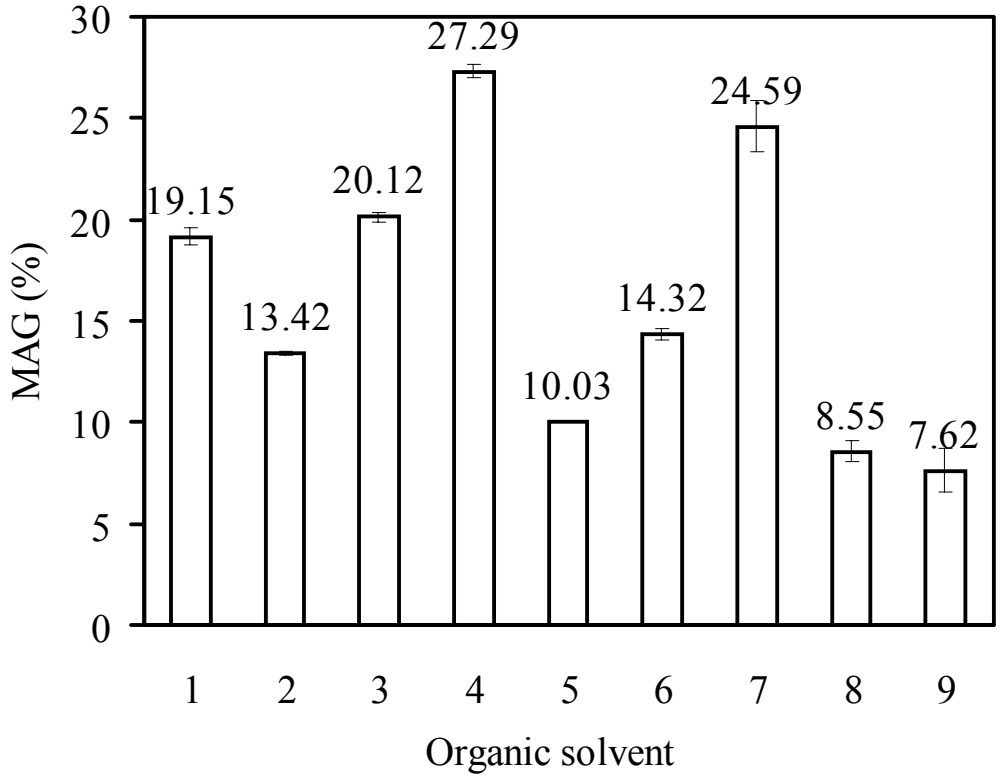
Because solvent free system has many problems when using in continuous production, the solvent system was investigated for continuous MAG production. To carry out bioconversion of lipophilic compounds effectively, it is essential to introduce organic solvents into the reaction system. The use of organic solvents can improve the poor solubility in water of

substrates or other reaction components of hydrophobic nature. However, organic solvents produce various physicochemical effects on enzyme molecules, and these effects differ depending on the kinds of organic solvents and enzymes used. Therefore, glycerolysis in organic solvent system for MAG production was investigated.

8.1 Effect of solvent type on MAG production

To select the most suitable solvent for glycerolysis reaction system, an effect of organic solvents on the catalytic activity of lipase was examined. The glycerolysis of palm olein by IM-PS was carried out in acetone, hexane and isooctane and combination of these solvents. The results are shown in Figure 18 and 19. It was found that acetone gave higher yield than isooctane and hexane. It gave the MAG yield of 19.15 % at 24 h. Furthermore, acetone/isooctane mixture (3:1, v/v) gave the best result for MAG production (27.29 %) at 24 h. Therefore, acetone/isooctane mixture (3:1,v/v) was suitable for MAG production. Chang *et al*. (1991) used isooctane as organic solvent for continuous glycerolysis of olive oil in CSTR. Holmberg and Osterberg (1988) found that isooctane gave a considerably better yield of MAG than both *n*-hexane and *n*-octane when MAG was produced in microemulsion. Li and Ward (1993) also found that a higher extent of esterification was observed in isooctane and hexane as compared to other solvents but more polar solvents, such as benzene and acetone were unsuitable for the synthetic reaction. Kosugi and Tanaka (1990) suggested that the addition of a nonpolar solvent such as isooctane slightly increased the lipolysis by the immobilized lipase from *Penicillium fluorescens*. Fukui *et al.* (1990) also suggested that water-miscible or hydrophilic organic solvents tend to inactivate the enzyme while hydrophobic solvents are known to flavor the enzyme. In particular, isooctane is an excellent solvent for the enzymatic reaction catalysed by lipase. From various organic solvents tested for immobilized lipase activity from *Candida rugosa*, isooctane showed the highest activity in reverse phase system.

Moreover, Yang and Rhee (1992) used isooctane as organic solvent for continuous hydrolysis reaction in PBR and found that the immobilized lipase was more stable in isooctane than in hexane.



1) acetone
2) acetone/isooctane 1:3,v/v)
3) acetone/isooctane (1:1,v/v)
4) acetone/isooctane (3:1,v/v)
5) acetone/hexane (1:3,v/v)
6) acetone/hexane (1:1,v/v)
7) acetone/hexane (3:1,v/v)
8) isooctane
9) hexane

Figure 18 Effect of organic solvents on MAG production by IM-PS

The reaction mixture contained 20 mL of 30 %(w/v) palm olein in organic solvents and 1.78 g glycerol with 4 %(w/w) water. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

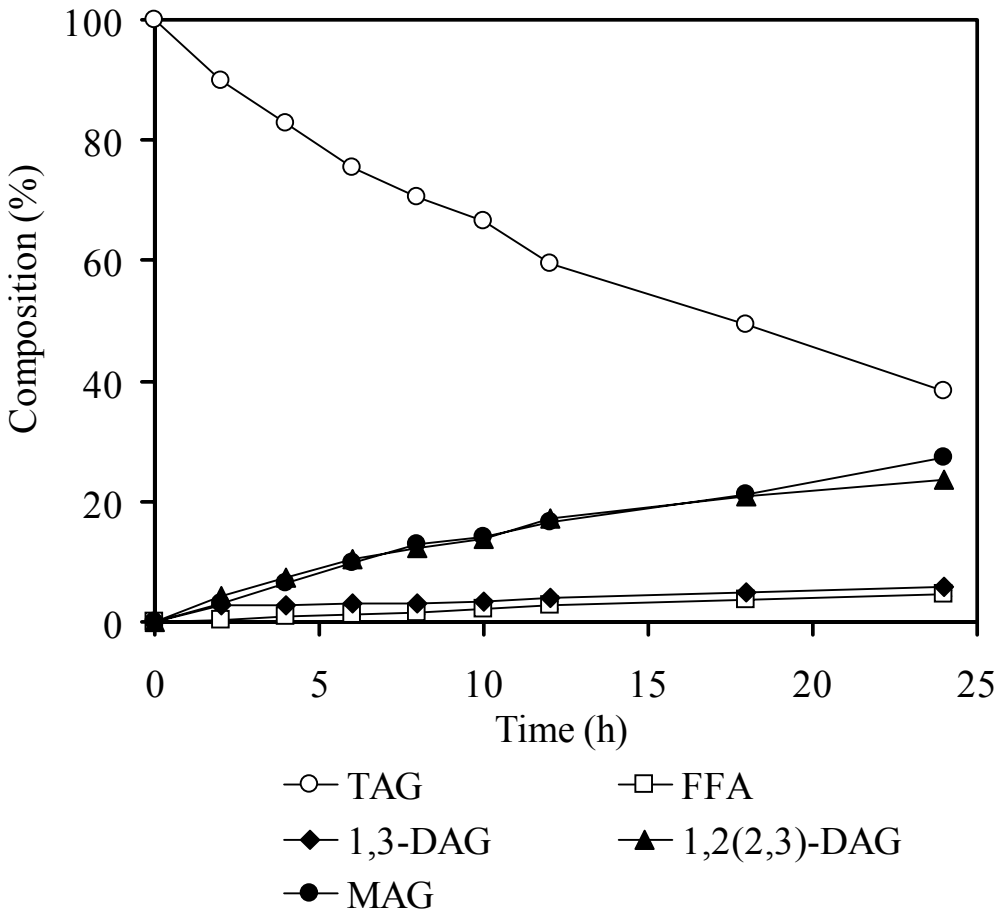


Figure 19 Time course of glycerolysis by IM-PS in acetone/isooctane mixture (3:1,v/v)

The reaction mixture contained 20 mL of 30 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 4 %(w/w) water. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

8.2 Stability of IM-PS in acetone/isooctane mixture (3:1,v/v)

Stability of IM-PS in acetone/isooctane mixture (3:1,v/v) was studied at 4 and 45 °C. The results are shown in Figure 20. It was found that more than 90 and 80 % of the hydrolytic activity remained after incubated for 24 h at 4

and 45 °C, respectively. However, Fukui *et al.* (1990) found that benzene was better for lipase stability while gave a moderate result for lipase activity. Kang and Rhee (1989a) suggested that the immobilized lipase activity in a reverse phase system decreases as the polarity of solvent increases. Kwon *et al.* (1995) reported that the enzyme was stabilized by the substrate in a two phase reaction system (isooctane-water); the half life of the enzyme was 10 h without the substrate and 20 h with 30 % olive oil at 30 °C. Besides, Yang and Rhee (1991) suggested that the operational half life of immobilized lipase was extended as the substrate concentration was increased and found that glycerol obviously acts as an effective stabilizer against thermal and solvent denaturation as well.

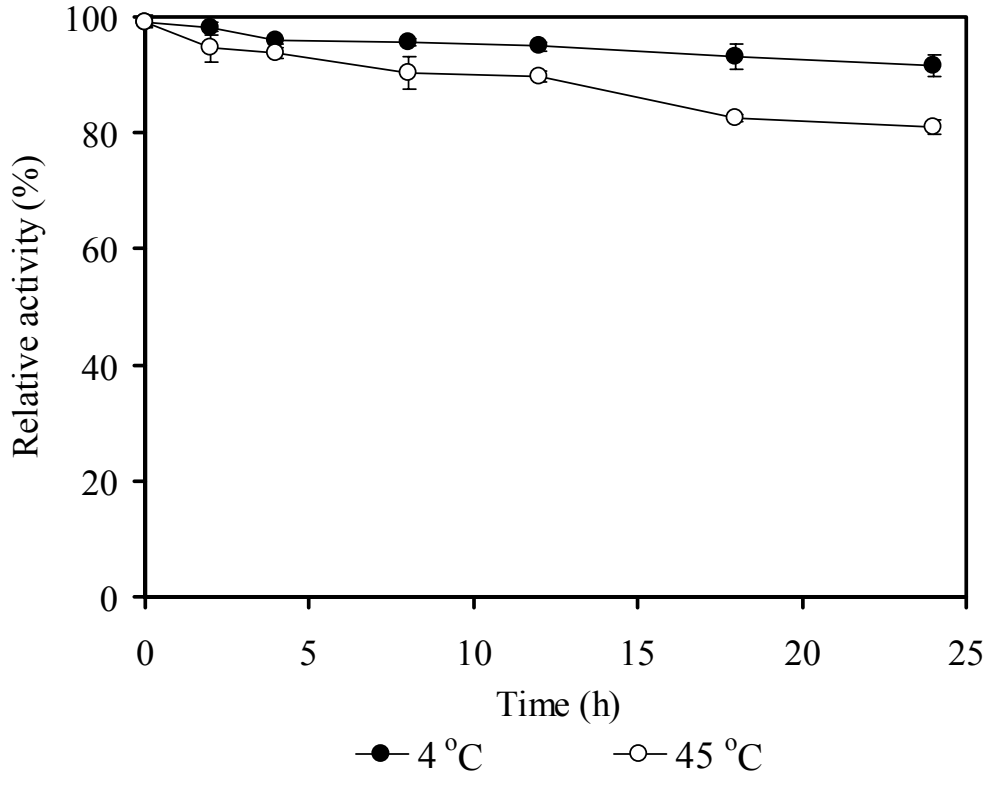


Figure 20 Stability of IM-PS in acetone/isooctane mixture (3:1,v/v)

8.3 Effect of initial water content on MAG production

Initial water content in the range of 4-12 %(w/w) was investigated for MAG production. The results are shown in Figure 21 and 22. Using 4 %(w/w) of initial water content in glycerol gave the lowest yield of MAG, but initial water content with 6-12 %(w/w) in glycerol gave higher yield of MAG. An initial water content at 10 %(w/w) in glycerol gave the highest yield of MAG with 32.3 %. However, at higher than 10 % water content in glycerol the yield of MAG dropped gradually. Such drop might be due to hydrolysis occur at too high water content. FFA content at equilibrium depended on the water concentration in the glycerol phase. Increasing the water concentration in glycerol phase increased FFA content (Yamane *et al.*, 1994). Therefore, initial water content at 10 %(w/w) in glycerol was the best water content for MAG production. A similar result was obtained by Garcia *et al.* (1996) for glycerolysis of butter oil. Yang and Parkin (1994) also found that the yield of MAG formation by glycerolysis of butter oil using a gel-entrapped lipase increased with increasing water content up to 10 % in glycerol. Furthermore, the yield of MAG is considerably reduced when 12 % water in glycerol was used for glycerolysis of beef tallow (McNeill *et al.*, 1990).

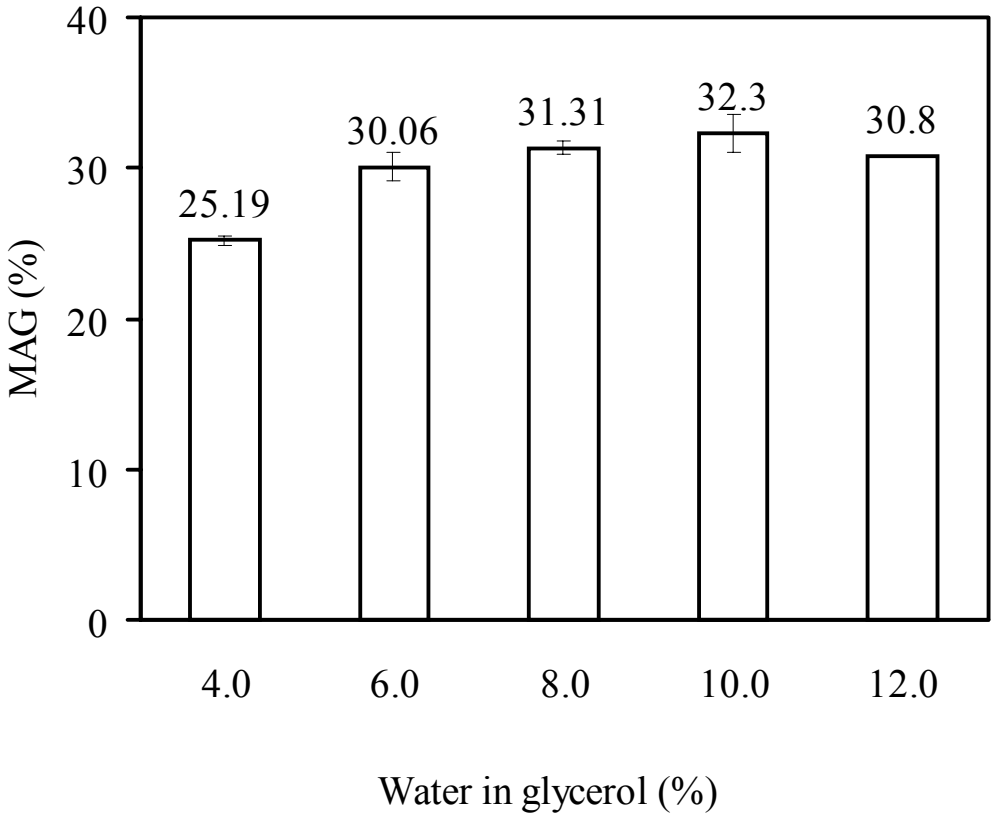


Figure 21 Effect of initial water content on MAG production by IM-PS

The reaction mixture contained 20 mL of 30 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with various amounts of water. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

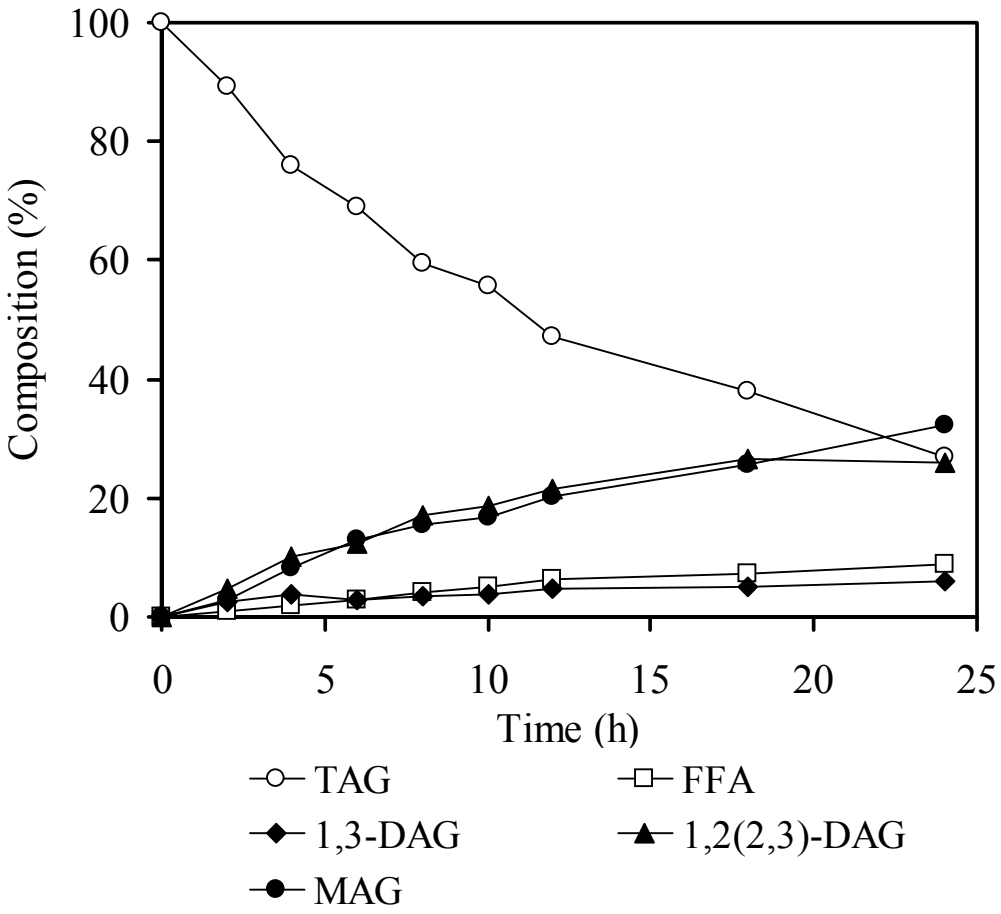


Figure 22 Time course of glycerolysis by IM-PS in acetone/isooctane mixture (3:1,v/v) using 10 %(w/w) water in glycerol

The reaction mixture contained 20 mL of 30 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

8.4 Effect of the molar ratio of glycerol to palm olein on MAG production

The effect of the molar ratio of glycerol to palm olein in the reaction mixture on glycerolysis was investigated. The results are shown in Figure 23 and 24. The yield of MAG increased with increasing the concentration of glycerol. When the molar ratios of glycerol to palm olein were between 2.7:1 and 3.5:1, high yields of MAG were obtained. The yield of MAG was independent of the molar ratios of glycerol to palm olein at 2.7:1 or greater. It may be caused by the reaction having excess glycerol. The molar ratio of glycerol to palm olein with 2.7:1 was chosen for further studies. The main products of glycerolysis was DAG when using low glycerol to triglyceride molar ratio (1:2) (Yamane *et al.*, 1994). Brady *et al*. (1988) suggested that glycerol enhanced the stability of enzyme. Moreover, Yang and Rhee (1991) also suggested that glycerol obviously acts as an effective stabilizer against thermal and solvent denaturation as well.

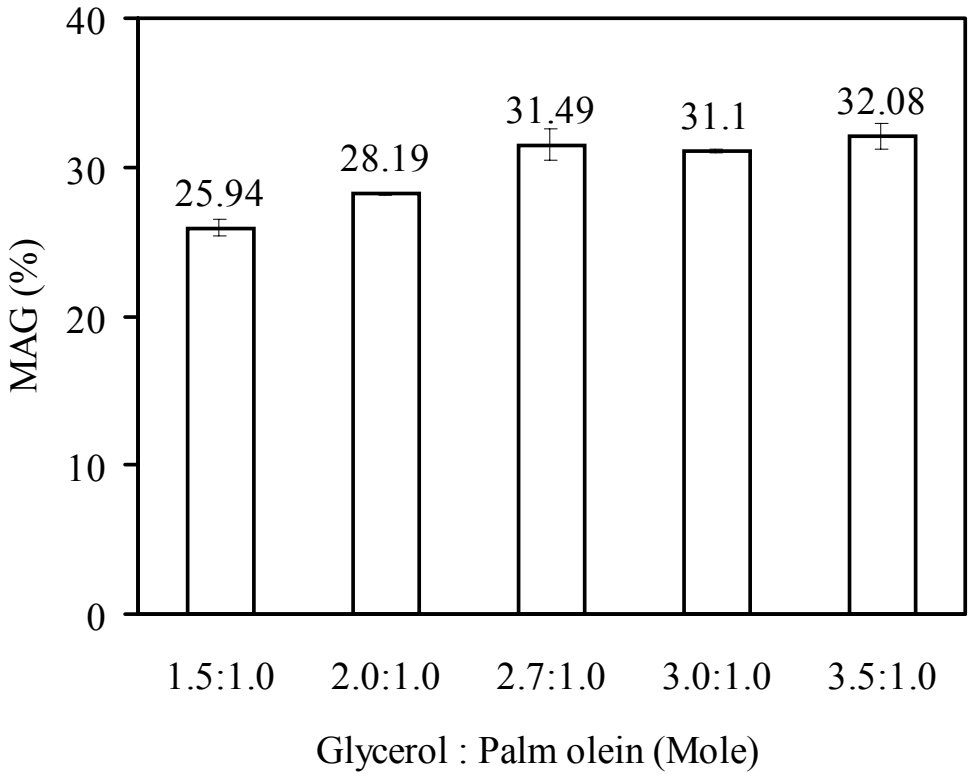


Figure 23 Effect of the molar ratio of glycerol to palm olein on MAG production by IM-PS

The reaction mixture contained 20 mL of 30 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), 0.178 g water and various amounts of glycerol. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

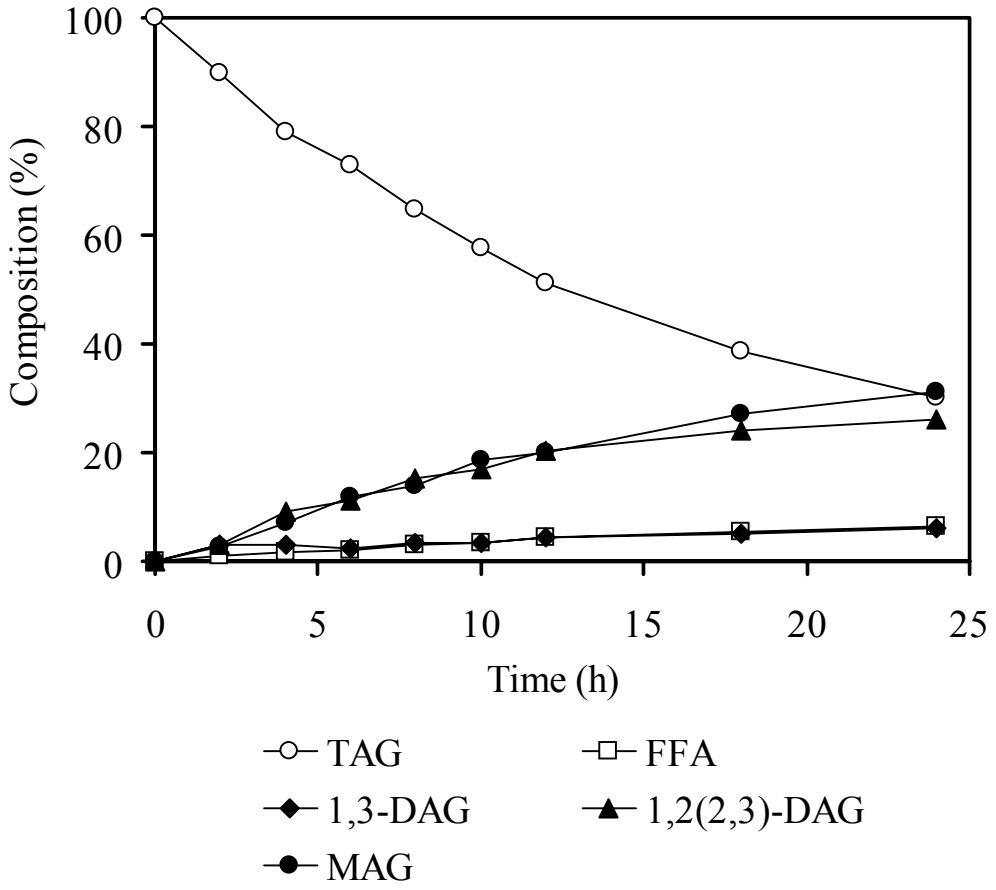


Figure 24 Time course of glycerolysis by IM-PS in acetone/isooctane mixture (3:1,v/v) using the molar ratio of glycerol to palm olein with 2.7:1

The reaction mixture contained 20 mL of 30 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

8.5. Effect of palm olein concentration in acetone/isooctane mixture (3:1,v/v) on MAG production

In order to select an initial substrate (palm olein) concentration, an effect of palm olein concentration in mixed organic solvents on MAG production was investigated. The results are shown in Figure 25 and 26. It was found that the MAG yield increased with decreasing the concentration of palm olein. However, when the concentration of palm olein was lower than 10 %(w/v), the yield of MAG was slightly decreased. It may be due to two reasons: (1) the solvent inhibits the enzyme activity at low substrate concentration, and (2) addition of solvent decreases the amount of available substrate at the interface between the solvent and glycerol and hence decreases the MAG yield. The concentration of 10 %(w/v) palm olein or 8:1 (molar ratio of glycerol to palm olein) in acetone/isooctane mixture (3:1,v/v) gave the best yield of MAG with 43.68 % for 24 h incubation. On the other hand, Holmberg *et al.* (1989) used 5 % palm oil in isooctane for glycerolysis. When the reaction was run at 37 °C for 24 h, 1.4 mol MAG per mol starting TAG was obtained. Kang and Rhee (1989b) suggested that substrate binding to lipase can reduce the conformational changes of the lipase and preserve the activity of immobilized lipase. The operational half life was extended as the substrate concentration was increased. But, it was difficult to use substrate concentration higher than 20 % because there was a substantial pressure-drop (Yang and Rhee, 1991).

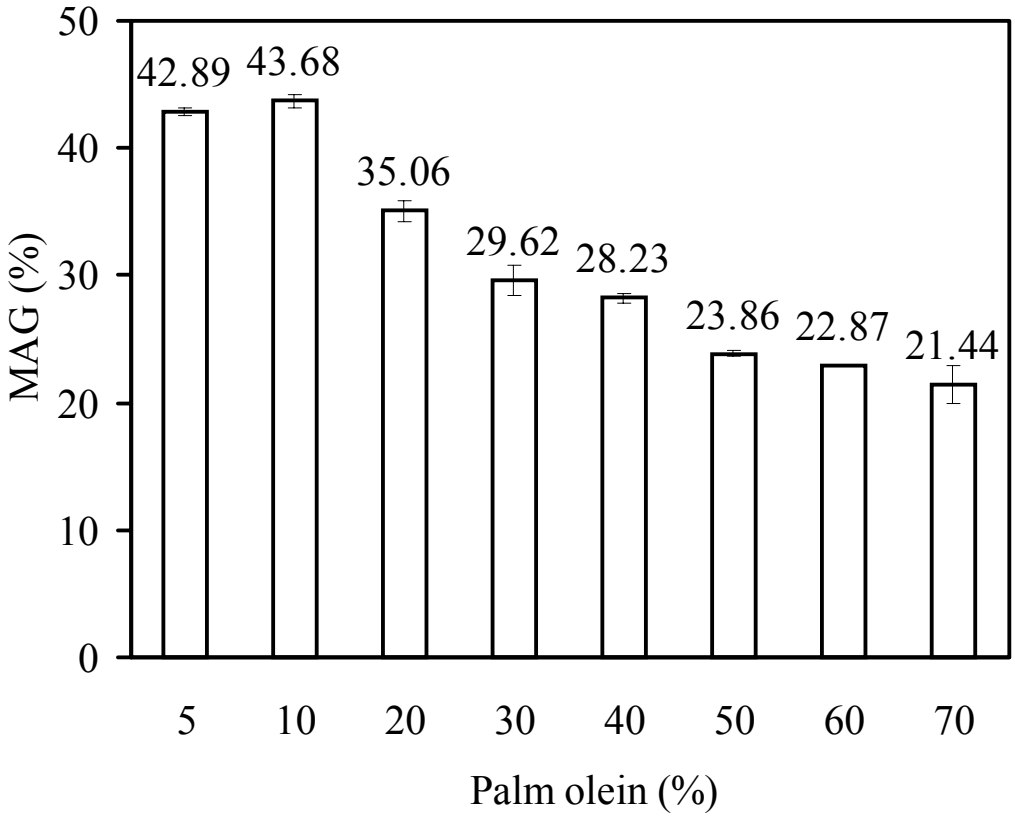


Figure 25 Effect of palm olein concentration on MAG production by IM-PS

The reaction mixture contained various amounts of palm olein in 20 mL of acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

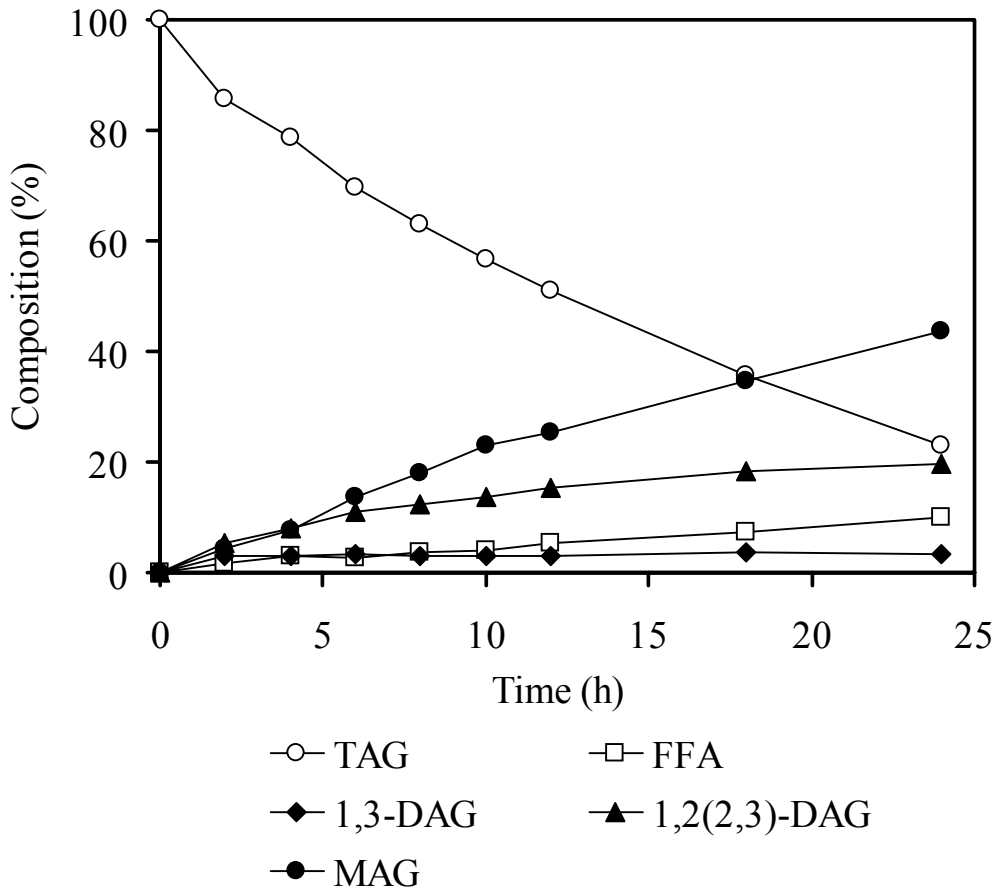


Figure 26 Time course of glycerolysis by IM-PS in acetone/isooctane mixture (3:1,v/v) using 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v)

The reaction mixture contained 20 mL of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 0.6 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

8.6 Effect of IM-PS loading on MAG production

An effect of IM-PS loading on MAG production was determined. The results are shown in Figure 27 and 28. When increasing the amount of IM-PS in the reaction mixture, MAG production was also increased. However, at 60 % IM-PS, no benefit came from increasing enzyme loading. Therefore, the amount of immobilized lipase with 50 % of palm olein was the best suitable MAG production.

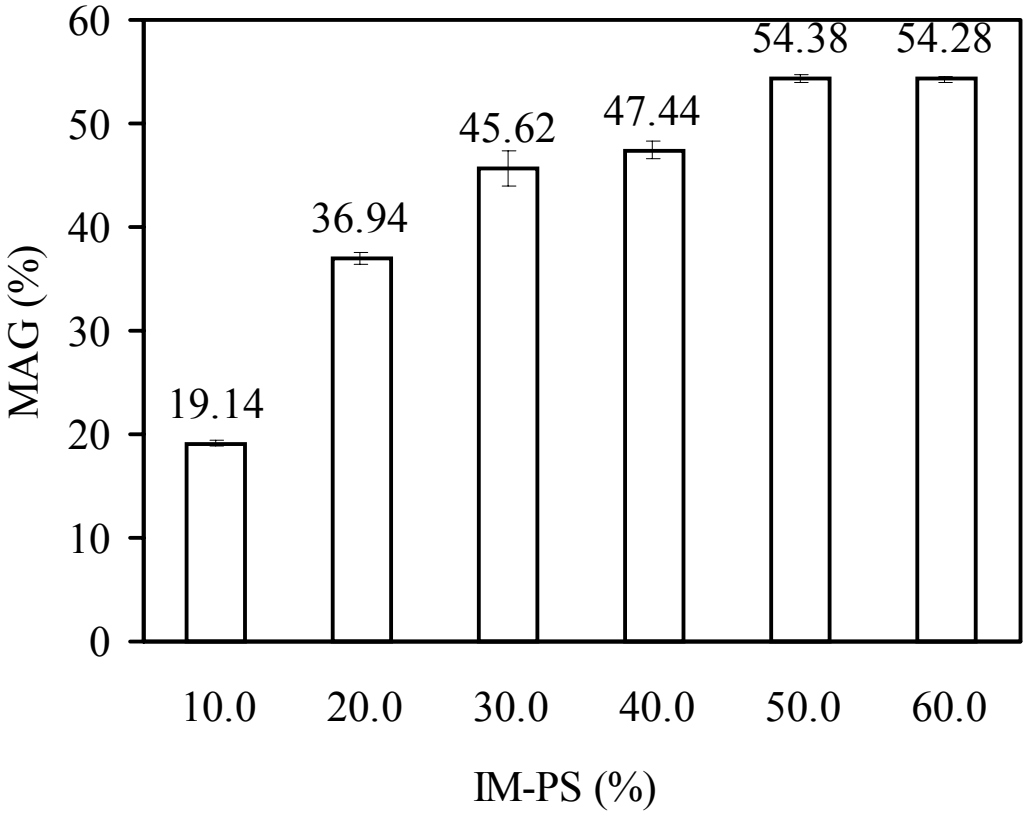


Figure 27 Effect of IM-PS loading on MAG production

The reaction mixture contained 20 mL of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was varied. The reaction was carried out at 300 rpm and 45 °C for 24 h.

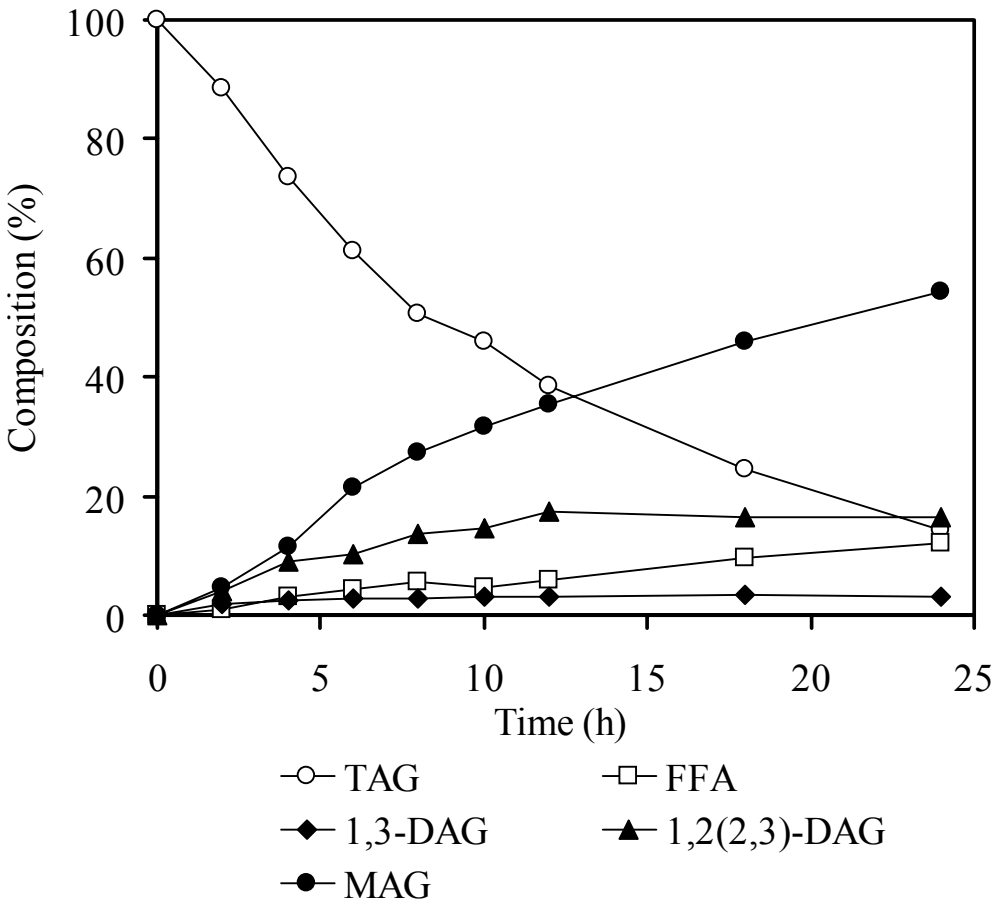


Figure 28 Time course of glycerolysis by IM-PS in acetone/isooctane mixture (3:1,v/v) using 50 %(w/w) IM-PS of palm olein

The reaction mixture contained 20 mL of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 1 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

8.7 Effect of temperature on MAG production

An effect of temperature on MAG production was studied. The temperature was controlled at 25-55 °C for MAG production. The results are shown in Figure 29 and 30. When the temperature was controlled in the range of 25-45 °C, MAG production increased with increasing temperature. This result is a consequence of an increase in the reaction rate as temperature increases. When increasing temperature from 45 to 55 °C the yield of MAG was decreased because IM-PS was not heat stable over 45 °C. Therefore, temperature at 45 °C was suitable for MAG production and it was used through out further studies.

9. MAG production at optimal condition

The optimum conditions for MAG production included, 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), 8:1 molar ratio of glycerol to palm olein with 10 %(w/w) water and 50 %(w/w) IM-PS of palm olein. The temperature was controlled at 45 °C. The results are shown in Figure 31. Under optimal conditions, the yield of MAG with 55.75 % was obtained at 24 h incubation and 11.74 % of TAG was remained. Furthermore, using both lipase PS and IM-PS gave the same yield of MAG (3.8×10^{-3} g MAG/U.day) (Figure 32).

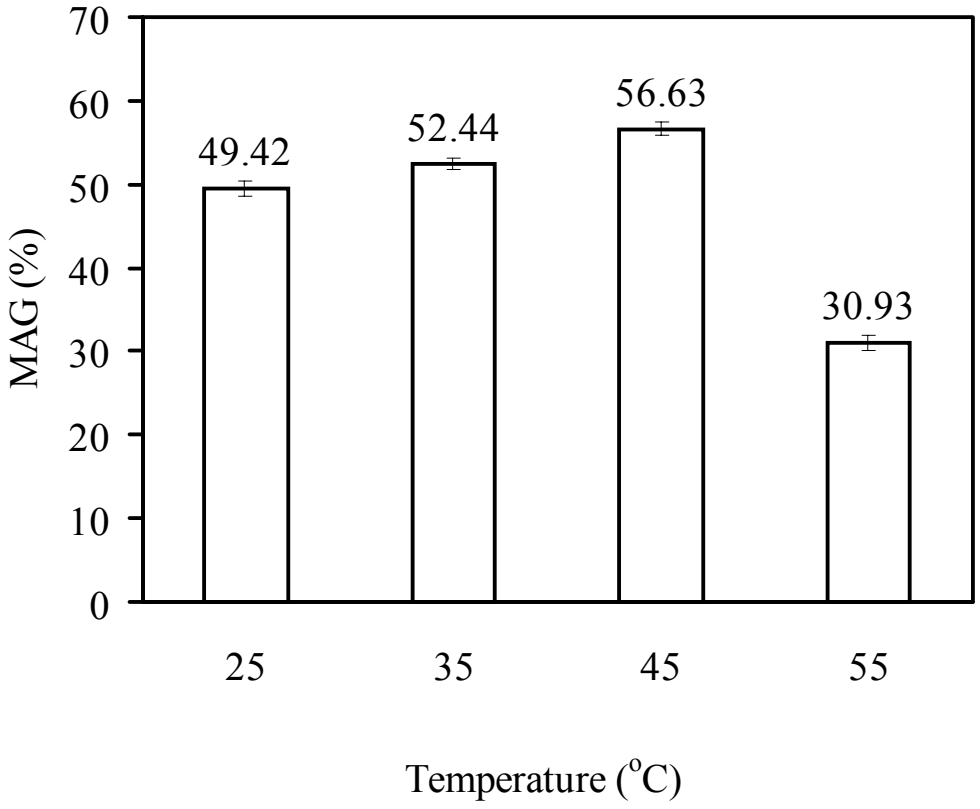


Figure 29 Effect of temperature on MAG production by IM-PS

The reaction mixture contained 20 mL of 10 %(w/v) of palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 1 g (0.33 U/mg). The reaction was carried out at 300 rpm are varied temperatures for 24 h.

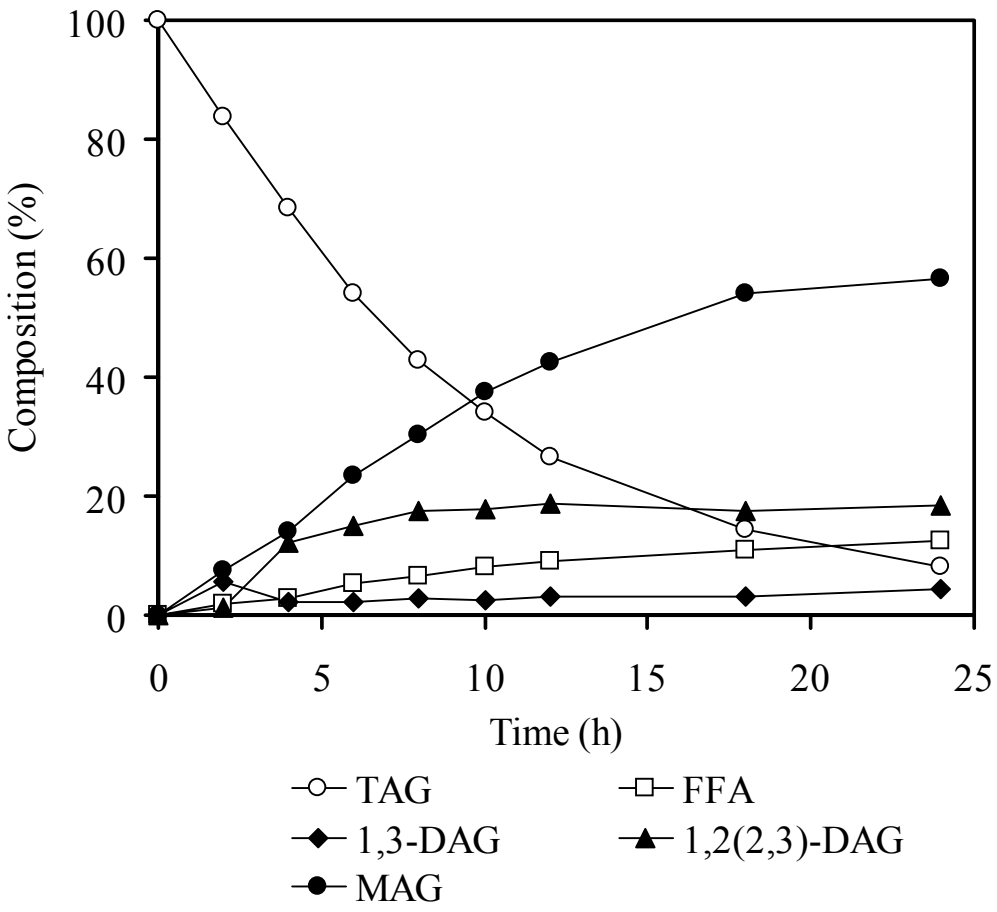


Figure 30 Time course of glycerolysis by IM-PS in acetone/isooctane mixture (3:1,v/v) at 45 °C

The reaction mixture contained 20 mL of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 1 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

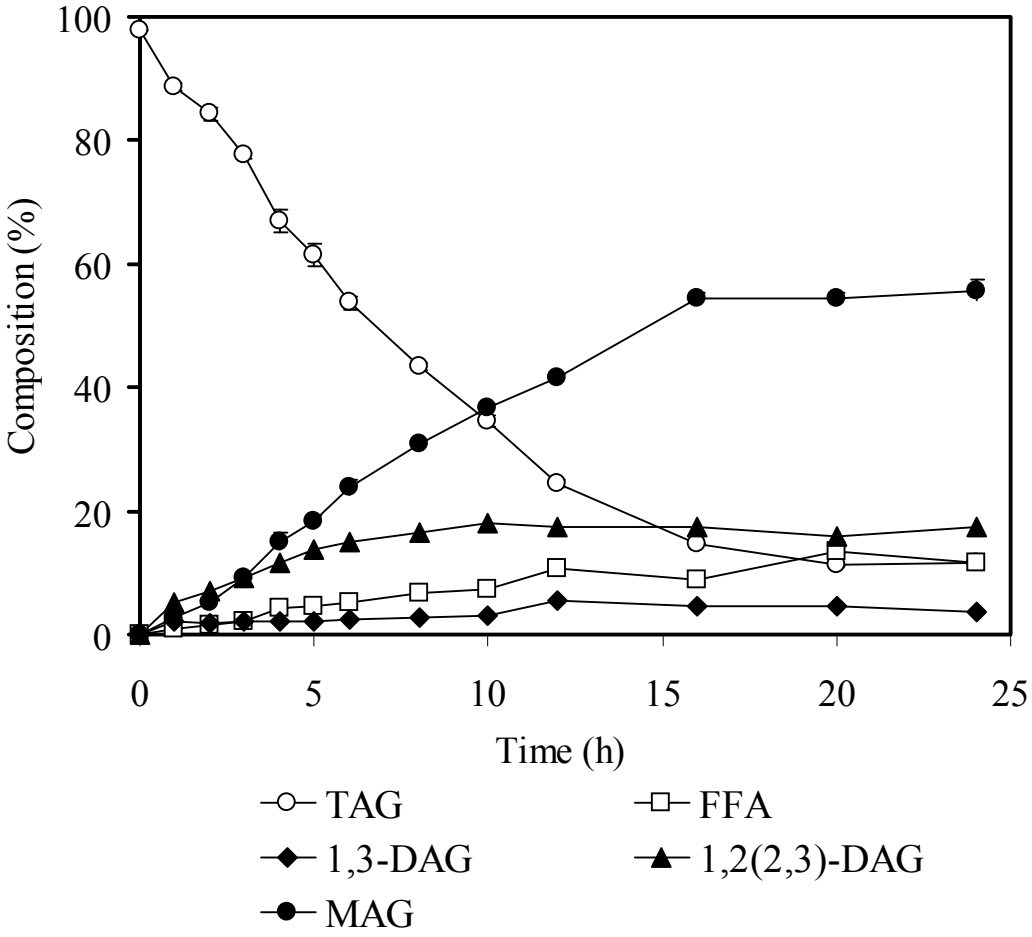


Figure 31 Time course of glycerolysis by IM-PS in acetone/isooctane mixture (3:1,v/v) under optimal conditions

The reaction mixture contained 20 mL of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of IM-PS used was 1 g (0.33 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

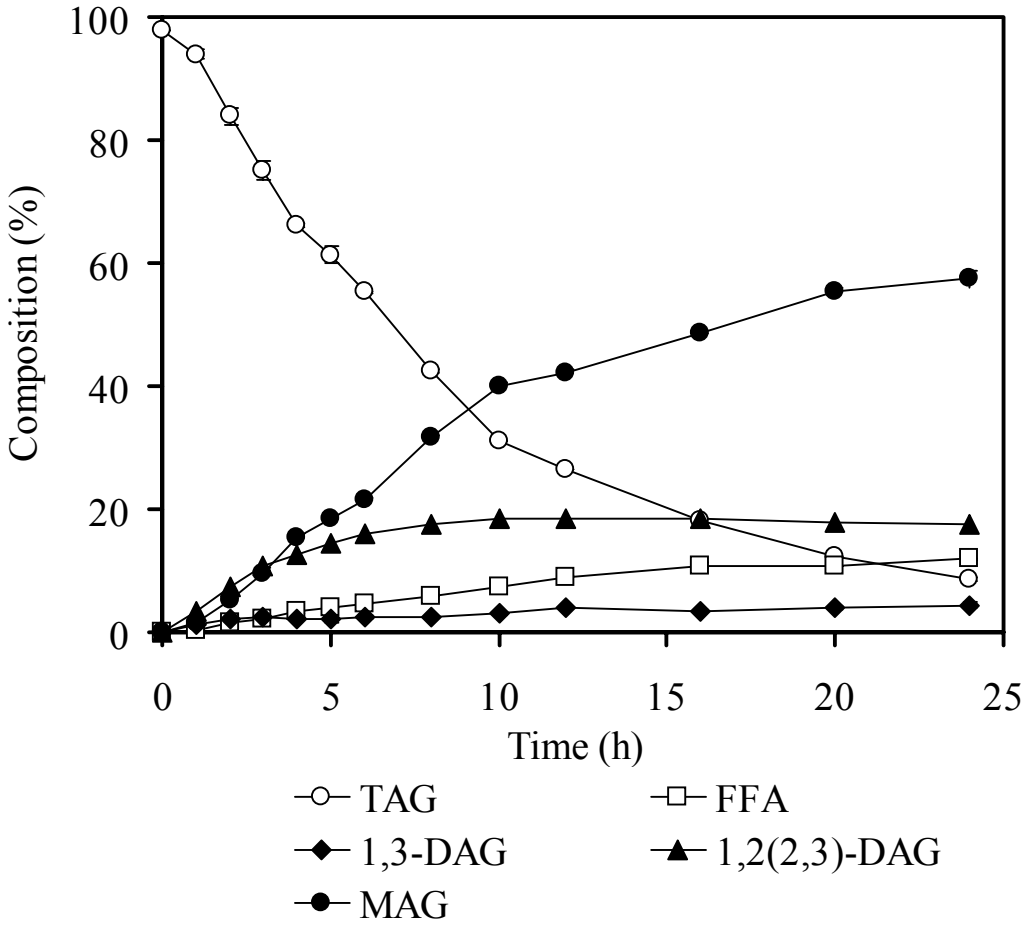


Figure 32 Time course of glycerolysis by lipase PS in acetone/isooctane mixture (3:1,v/v) under optimal conditions

The reaction mixture contained 20 mL of 10 %(w/v) of palm olein in acetone/isoocatne mixture (3:1,v/v) and 1.78 g glycerol with 10 %(w/w) water. The amount of soluble lipase PS used was 32 mg (10.31 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h.

10. Kinetics of glycerolysis of palm olein with glycerol by lipase PS and IM-PS

Kinetic constants (*Km* and *Vmax*) for glycerolysis of palm olein and glycerol by soluble lipase PS and IM-PS in organic solvents were determined by measuring initial reaction rate in organic solvent system containing varied amounts of palm olein (from 30 to 600 mM). The values of *Km* and *Vmax* were obtained from Linewever-Burk plot as given in Figure 33. For lipase PS, *Km* was 461.92 mM and *Vmax* was 1.68 mM/min. For IM-PS, *Km* was 229.05 mM and *Vmax* was 0.4 mM/min. The results showed that IM-PS had *Vmax* less than that half of *Vmax* of soluble lipase PS, but promoted higher affinity of the enzyme for this substrate. A similar result was obtained by Montero *et al.* (1993). They found that from hydrolysis of olive oil by *Candida rugosa* lipase, *Vmax* for soluble and immobilized lipase were 2810 and 89.5 μmol/min, respectively, *Km* for soluble and immobilized were 18.1 and 11.8 mg/mL, respectively. Padt *et al*. (1990) also reported esterification of decanoic acid with glycerol in a membrane reactor. When a lipase from *Candida rugosa* was immobilized by adsorption on cellulose, values of *Vmax* and *Km* changed from ca. 6.48 to 2.92 mol/min, and from ca. 3.88 to 0.54 mg/mL, respectively. Immobilization of biocatalyst can lead to an activity change not only caused by the immobilization itself, but also caused by diffusional limitation in immobilized biocatalyst system.

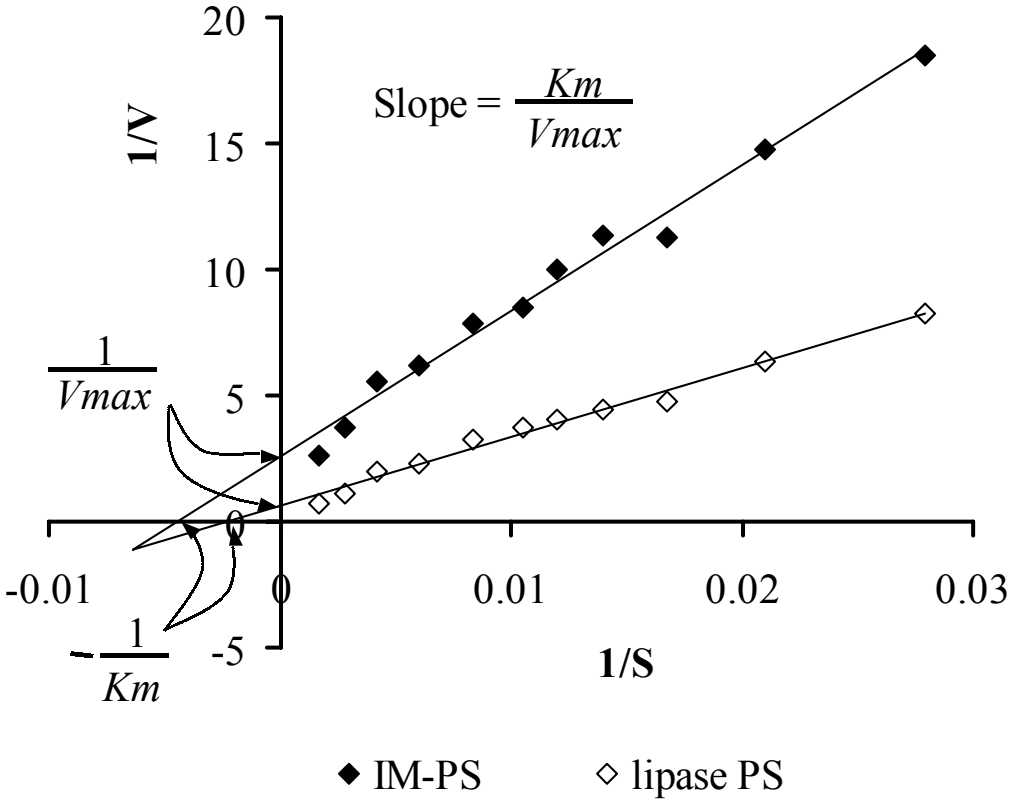


Figure 33 Lineweaver-Burk plot of glycerolysis by lipase PS and IM-PS with various palm olein concentrations in organic solvent system at 45 °C

11. Continuous MAG production in solvent system

11.1 Continuous MAG production in CSTR

The continuous glycerolysis of palm olein with glycerol was done in CSTR (4.5 cm ID, 6 cm height) (Figure 6). The substrate mixture was continuously pumped into the reactor. Agitation was supplied to the vessel by an overhead stirrer. The IM-PS powder mixed freely in the reactor and was retained in the reactor by a screen placed at the effluent take-off tube. This CSTR contained 1.5 g IM-PS (555 U) in a reactor volume of 50 mL. Average flow rates were 0.02 mL/min. The jacketed vessel was maintained at 45 °C. The reactor was in continuous operation for 72 h. Figure 34 showed a typical time

course of the continuous glycerolysis of palm olein in acetone/isooctane mixture (3:1, v/v). After 24 h of batch operation, continuous operation was started. The amount of MAG which is the major product of glycerolysis was slightly decreased at the initial continuous operation and nearly unchanged at a level about 24 % MAG for 72 h. The amount of TAG in the reaction was increasing with increasing the operation time. It may be caused by the substrates flow rate was faster than reaction velocity. A productivity of 1.32×10^{-3} g MAG/U.day was obtained while a theoretical productivity was 5.69×10^{-3} g MAG/U.day.

11.2 Continuous MAG production in PBR

The continuous glycerolysis of palm olein with glycerol was done in PBR (Figure 7). The immobilized enzyme was placed into the jacketed column, forming a bed of 0.68 cm diameter by 25 cm length. The substrate mixture was fed to the top of the column at the flow rate of 0.02 mL/min. The column temperature was maintained at 45 °C. The reactor was in continuous operation for 72 h. The results are shown in Figure 35. After 18 h of a transient state from the start of the continuous operation, a steady state was achieved and the yield of MAG was higher than 54 % during operation. A productivity of 2.93×10^{-3} g MAG/U.day was obtained while a theoretical productivity was 5.69×10^{-3} g MAG/U.day. Padt *et al.* (1992) produced MAG in a membrane bioreactor by esterification. The MAG production was about 18 % when immobilized *Candida rugosa* lipase on membrane and 50 % decanoic acid in hexadecane was used for production.

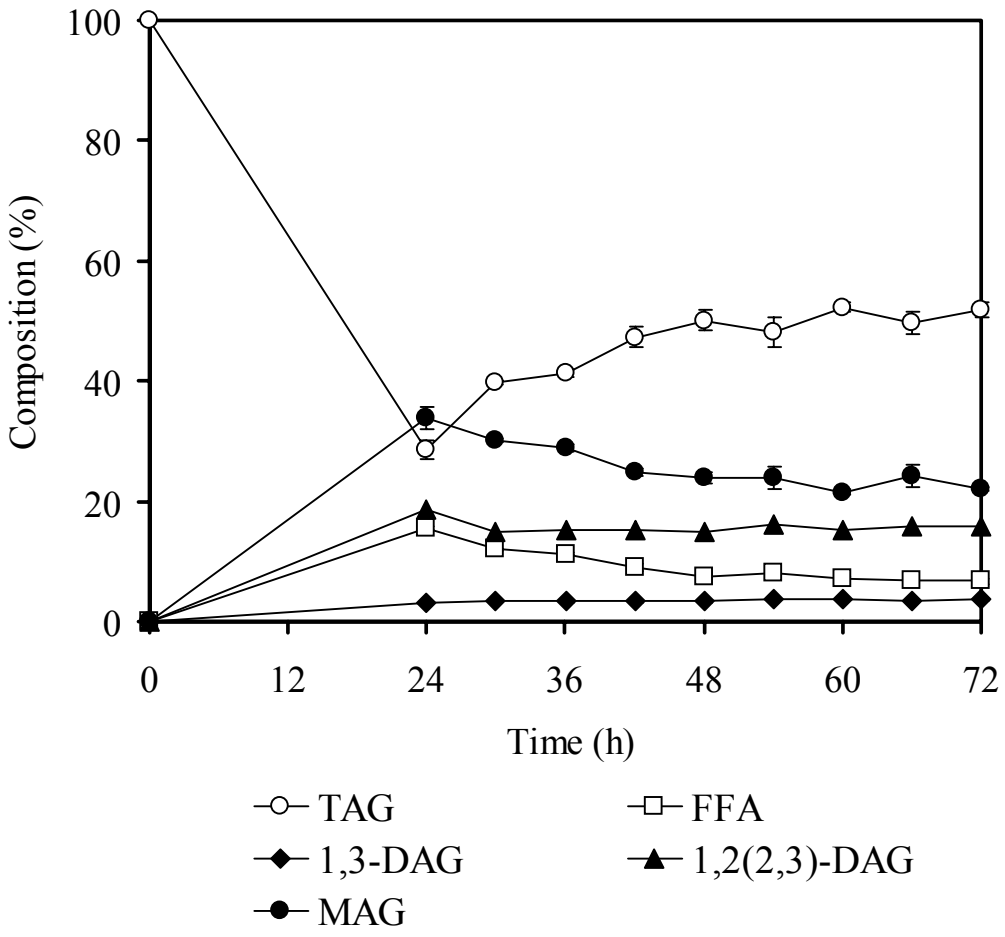


Figure 34 Continuous MAG production in solvent system by IM-PS in CSTR

The amount of IM-PS was 1.5 g (0.37 U/mg). Substrates consisted of 10 % (w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio was 8:1, 10 %(w/w) water in glycerol and acetone/isooctane mixture (3:1,v/v). The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

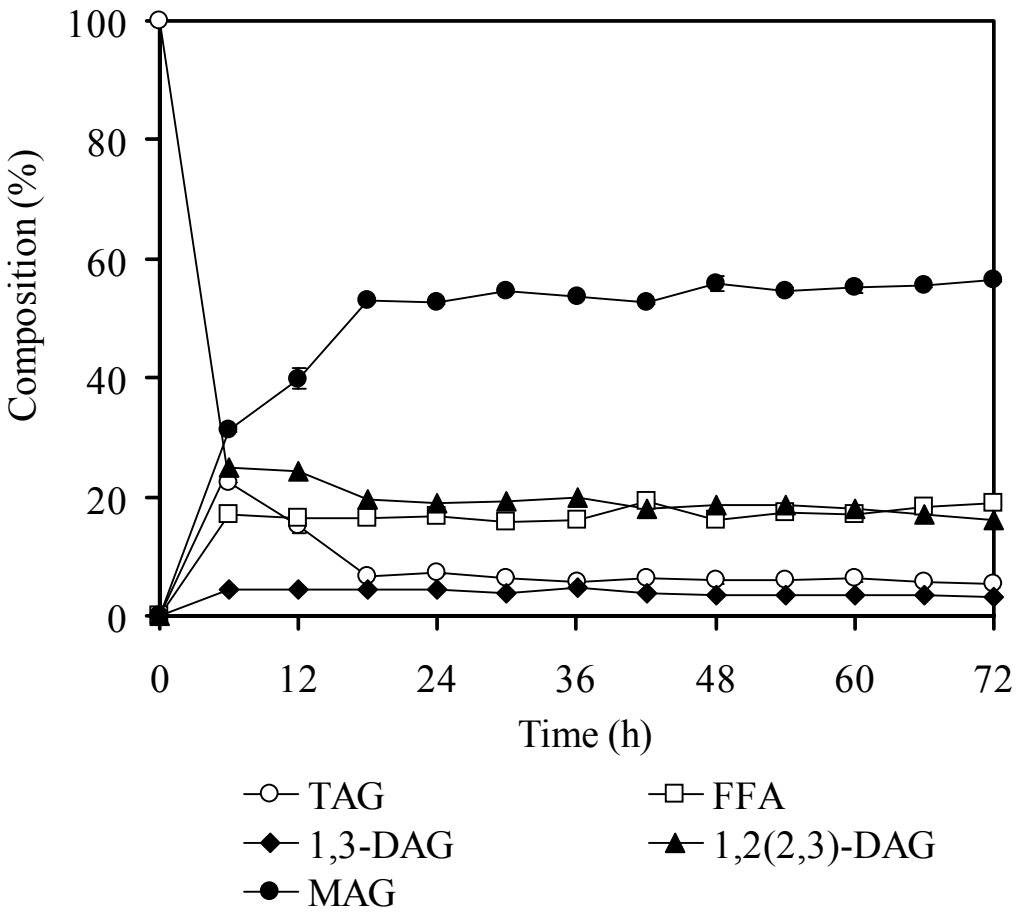


Figure 35 Continuous MAG production in solvent system by IM-PS in PBR

The amount of IM-PS was 1.5 g (0.37 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio was 8:1, 10 %(w/w) water in glycerol and acetone/isooctane mixture (3:1,v/v). The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

12. Optimization MAG production in PBR

The conditions that explained before were used as initial condition for following studies

12.1 Effect of the molar ratio of glycerol to palm olein on MAG production

The molar ratio of glycerol to palm olein between 0:1 and 16:1 were studied. The results are shown in Figure 36 and 37. It was found that when increasing glycerol concentrations, the yields of MAG production were increased. The molar ratios of glycerol to palm olein at 12:1 and 16:1 gave the highest yields of MAG. At a fixed water content in glycerol, increasing glycerol also increased water content in the reaction. Hence, hydrolysis might be occured. Consequently, the molar ratios of glycerol to palm olein at 12:1 was suitable for continuous MAG production in PBR. Yamane *et al.* (1994) found that when glycerol to triglyceride molar ratio was 1:2, the main product of glycerolysis was DAG. Brady *et al.* (1988) reported that glycerol stabilized the lipase dramatically as glycerol concentration was increased, however, it was difficult to use too high glycerol concentration because of its viscosity, which resulted in pressure-drop.

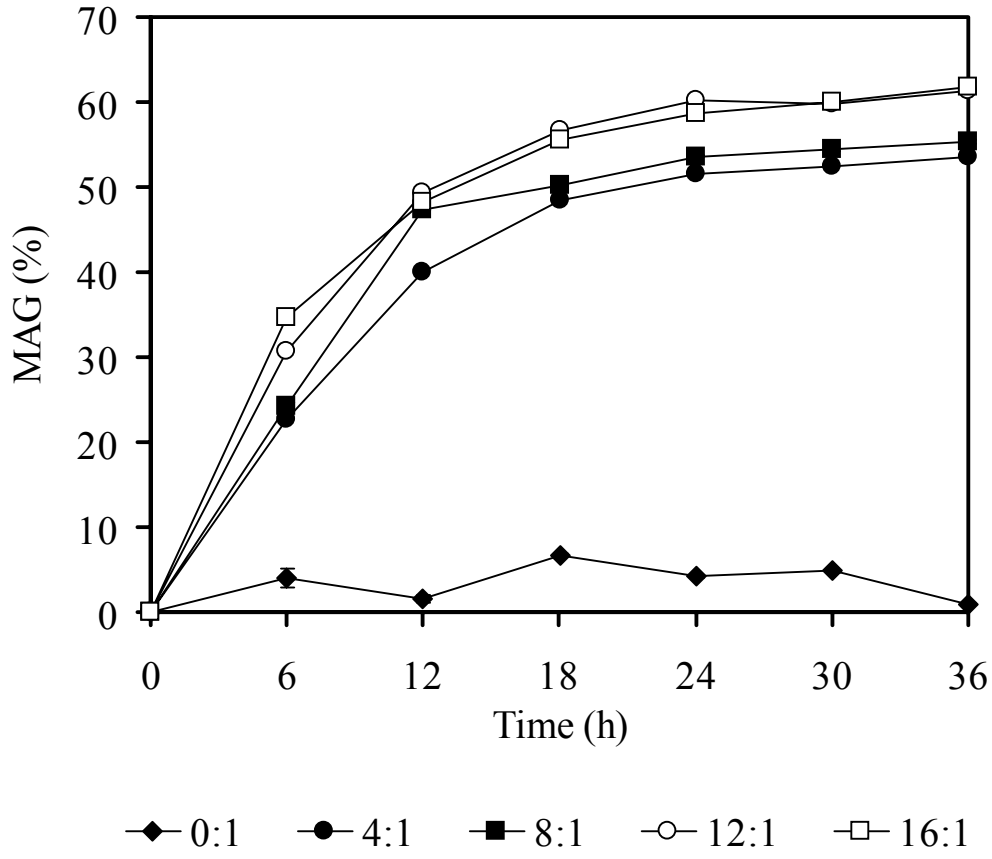


Figure 36 Effect of molar ratio of glycerol to palm olein on continuous MAG production by IM-PS in PBR

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), 10 %(w/w) water in glycerol and varied glycerol to palm olein molar ratios. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

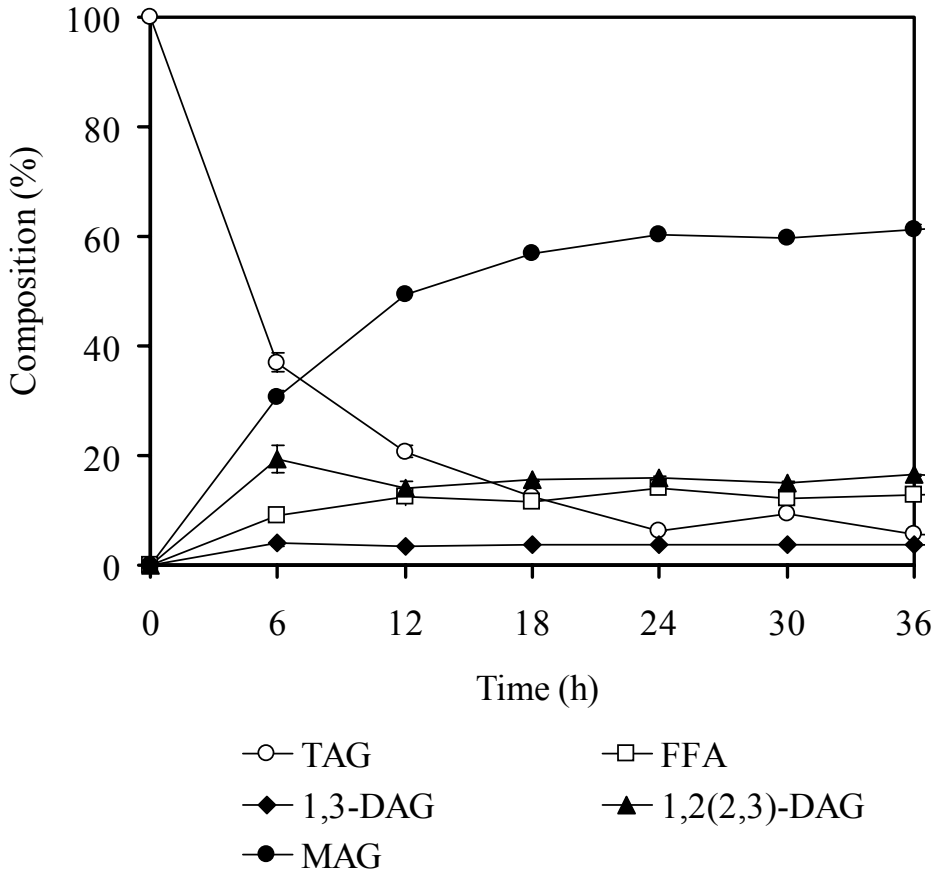


Figure 37 Time course of continuous MAG production by IM-PS in PBR using the molar ratio of glycerol to palm olein of 12:1

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), 10 %(w/w) water in glycerol and glycerol to palm olein molar ratio of 12:1. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

12.2 Effect of water content in glycerol on MAG production

Lipase-catalyzed glycerolysis involves a hydrolysis and esterification. In the first step, water is a reactant. In the second step, it is a product. Thus a suitable water content is necessary to maximize the reaction of both steps. In addition, water is crucial to maintain enzyme structure and stability. A trace amount of water is necessary to maintain the hydration layer around lipase molecules and maintains the enzyme activity, but it also promotes hydrolysis when the amount of water reaches a critical level. An effect of water content on glycerolysis in PBR in the range of 5-20 % (w/w) in glycerol was therefore studied. The results are shown in Figure 38 and 39. The water content of 10 %(w/w) in glycerol gave the best yield of MAG. When water content in glycerol was increased to 15 and 20 %(w/w) the yields of MAG were not increased, but the yields of FFA were increased. Therefore, the water content in glycerol at the value 10 %(w/w) was optimal to obtain the highest MAG production with FFA at a minimum level. The result obtained in present study was similar to that of continuous glycerolysis of olive oil in CSTR reported by Chang *et al*. (1991).

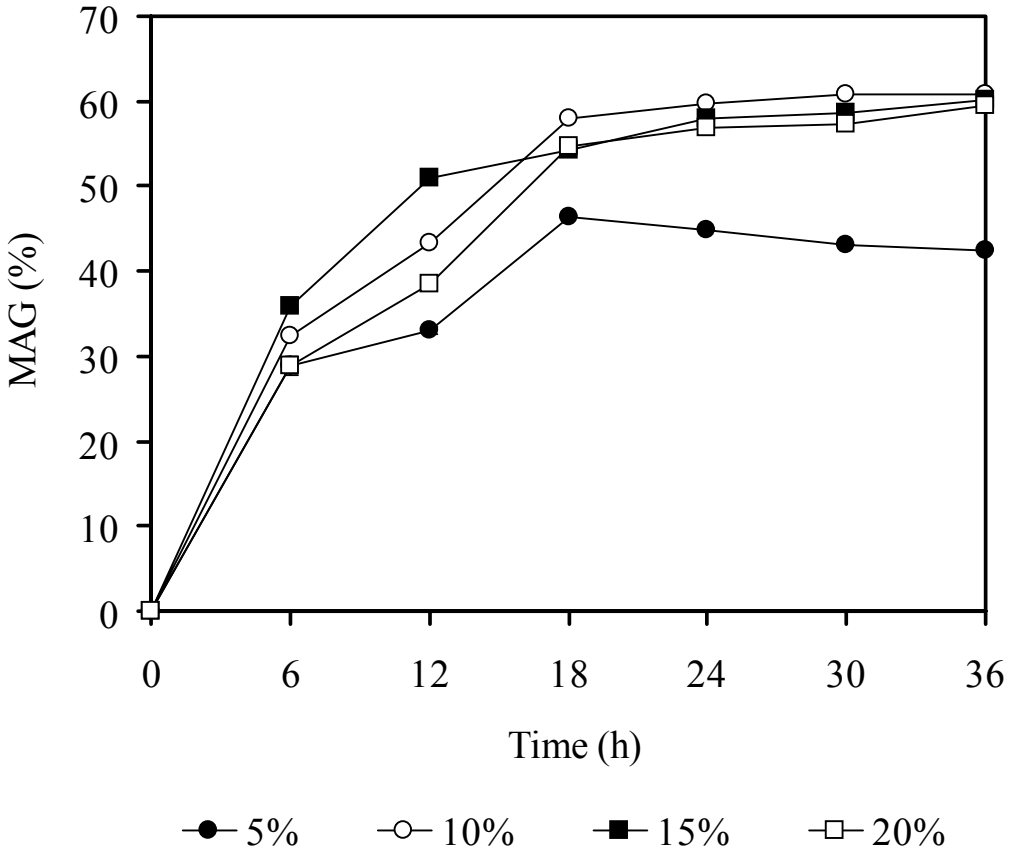


Figure 38 Effect of water content in glycerol on continuous MAG production by IM-PS in PBR

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and varied water contents in glycerol. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

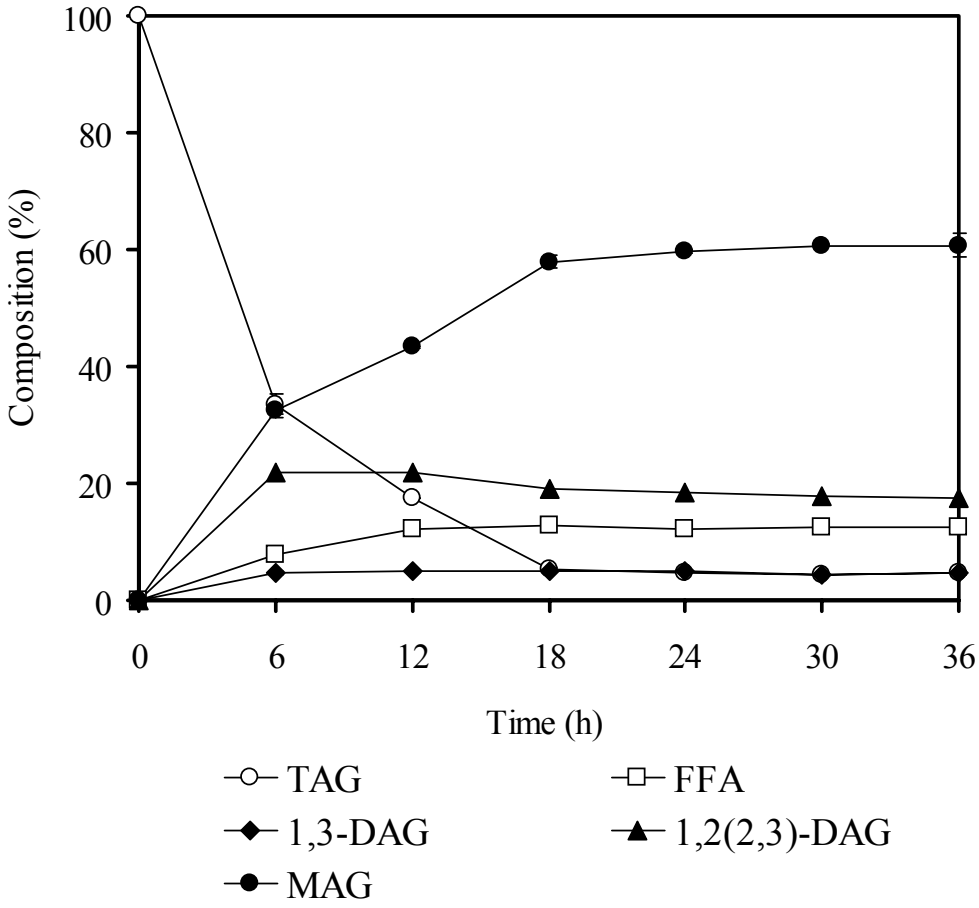


Figure 39 Time course of continuous MAG production by IM-PS in PBR using 10 %(w/w) water in glycerol

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

12.3 Effect of substrate flow rate on MAG production

An effect of substrate flow rate on MAG production in PBR was investigated. The results are shown in Figure 40 and 41. The MAG production decreased with increasing flow rate because of the shorter residence time of oil in the bioreactor. At low flow rates, the MAG production approached their maximal values. However, substrate flow rate decreased from 0.02 to 0.01 mL/min, the yield of MAG increased only 9.62 %. MAG production was drastically decreased at flow rate higher than 0.02 mL/min. Therefore, in order to obtain a high MAG production in PBR, the reaction should be carried out at a flow rate of 0.02 mL/min. Chang *et al*. (1991) also found that yield of MAG increased with decreasing in substrate flow rate for continuous glycerolysis in CSTR and suggested that the length of transient state was depended on the flow rate of substrate. In other word, the higher the flow rate, the shorter the transient state. According to the results of continuous glycerolysis of soybean oil in supercritical carbon dioxide reported by Jackson and King (1997), increased flow resulted in a decreased percentage of conversion of soybean oil. This may be expected that the amount of triglyceride exceeds the catalytic activity of lipase at high substrate flow rate.

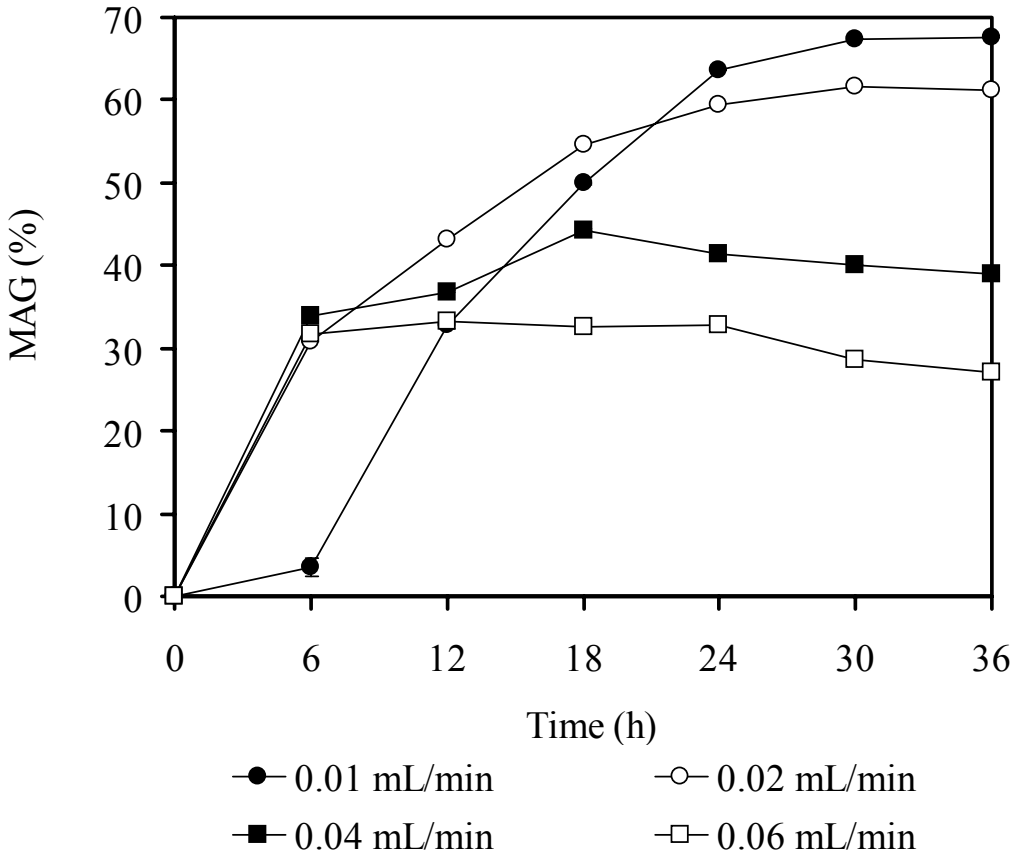


Figure 40 Effect of substrate flow rate on continuous MAG production by IM-PS in PBR

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was varying. The temperature was controlled at 45 °C.

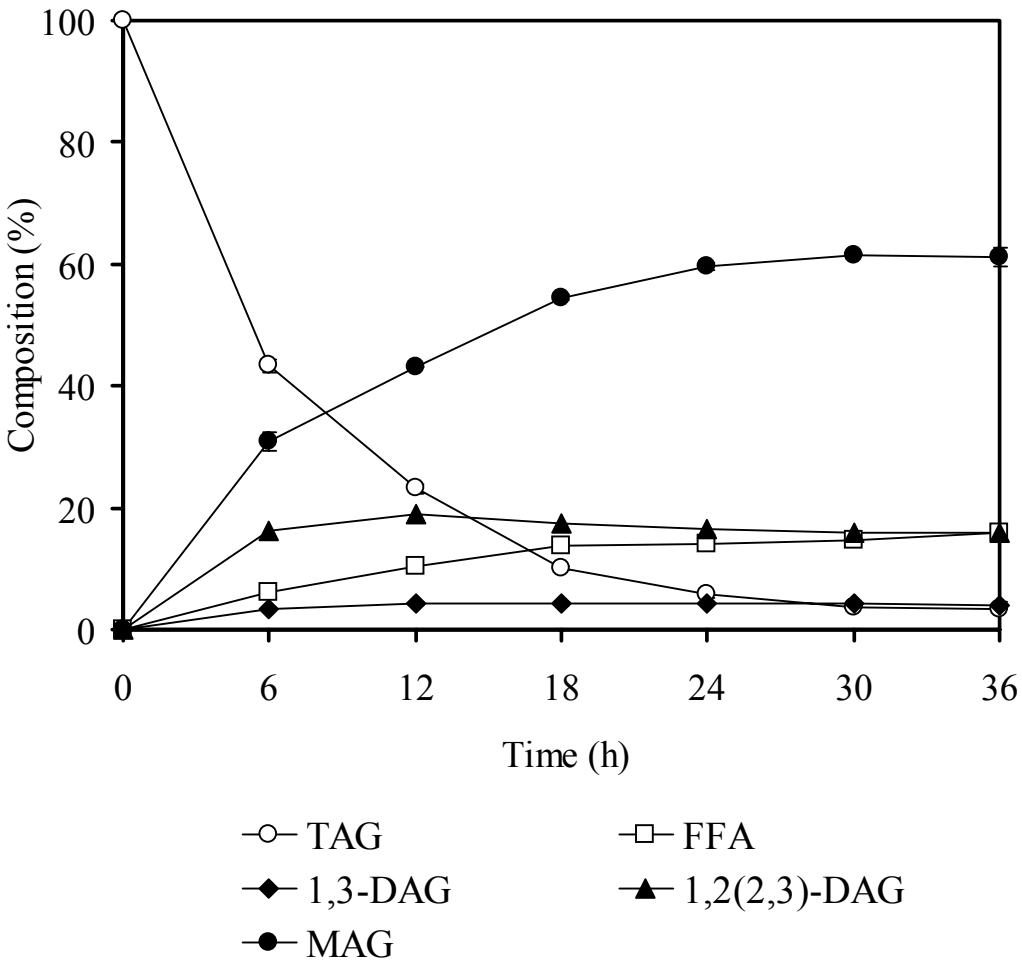


Figure 41 Time course of continuous MAG production by IM-PS in PBR using substrate flow rate of 0.02 mL/min

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

12.4 Effect of temperature on MAG production in PBR

The temperature of the reactor was maintained at 35-55 °C. The results are shown in Figure 42 and 43. It was found that yield of MAG increased as temperature increased from 35 to 45 °C. An increased incorporation at high temperature may result from a higher activity of enzyme and improve contact between reaction substrate and enzyme as the viscosity of the substrate decreases. When the temperature of the reactor was maintained at 45 °C the highest yield of MAG was obtained with 60 %. For reaction at 55 °C, according to the result of batch reactor studies, the final mixture contained low yield of MAG. It might be caused by more rapid deactivation of IM-PS at this temperature. Therefore, the temperature at 45 °C was the best condition for continuous MAG production in PBR by IM-PS.

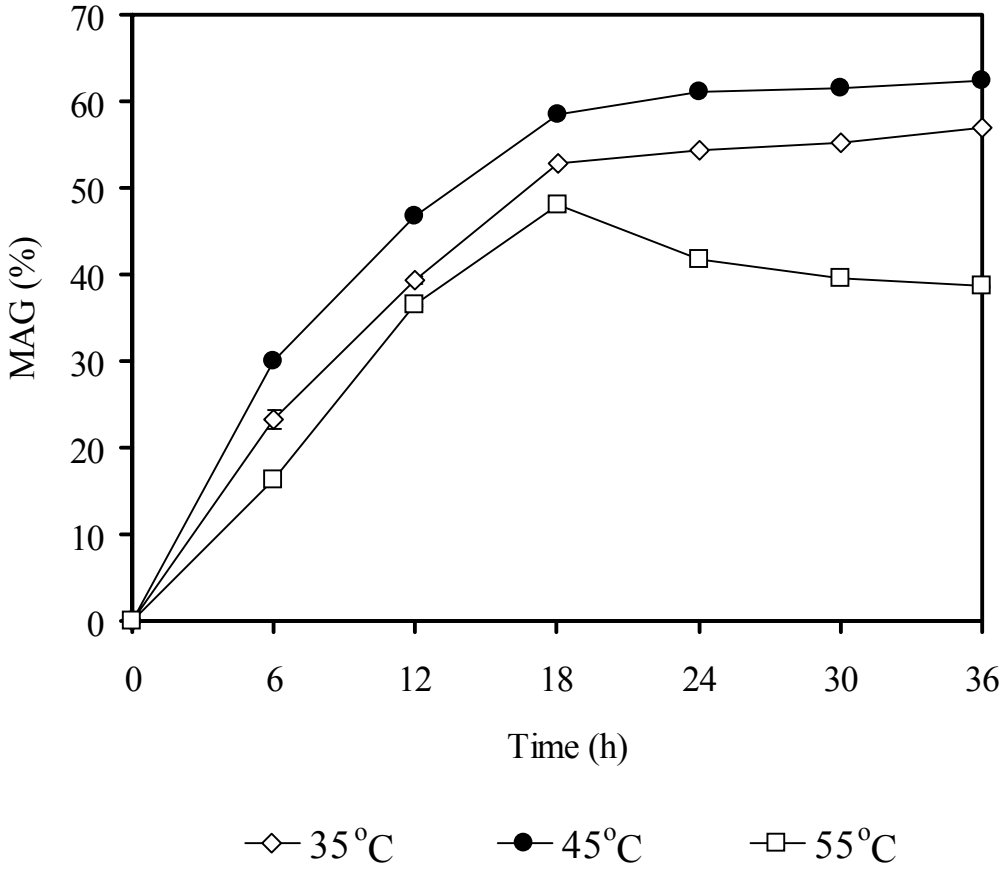


Figure 42 Effect of temperature on continuous MAG production by IM-PS in PBR

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min. The temperature was varied.

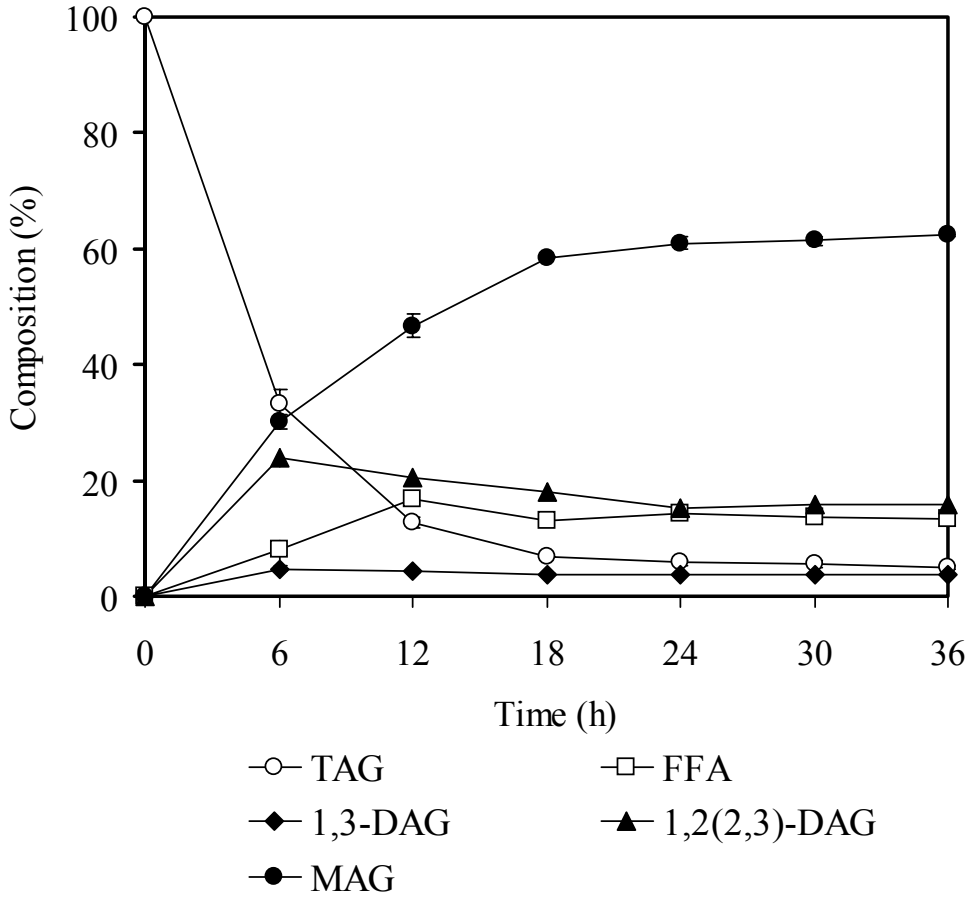


Figure 43 Time course of continuous MAG production by IM-PS in PBR at 45 °C

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

12.5 Continuous MAG production in PBR under optimal conditions

Glycerolysis of palm olein in PBR was dependent on several important operational parameters. The optimal molar ratio of glycerol to palm olein, water content in the glycerol phase, substrate flow rate and operation temperature for MAG production were 12:1, 10 %(w/w), 0.02 mL/min and 45 °C, respectively. These optimum conditions were used for continuous MAG production in PBR. The results are shown in Figure 44. It was found that after 24 h of a transient state from the start of the continuous operation, a steady state was achieved and the yield of MAG was slightly decreased with increasing the operation time. The average MAG yield about 55.84 % was obtained after 216 h operation. TAG was remained less than 10 %. The other products such as 1,2(2,3)-DAG, 1,3-DAG and FFA were less than 20 %. A productivity of 3.0×10^{-3} g MAG/U.day was obtained while a theoretical productivity was 5.66×10^{-3} g MAG/U.day. The compositions of product mixture are shown in Figure 45.

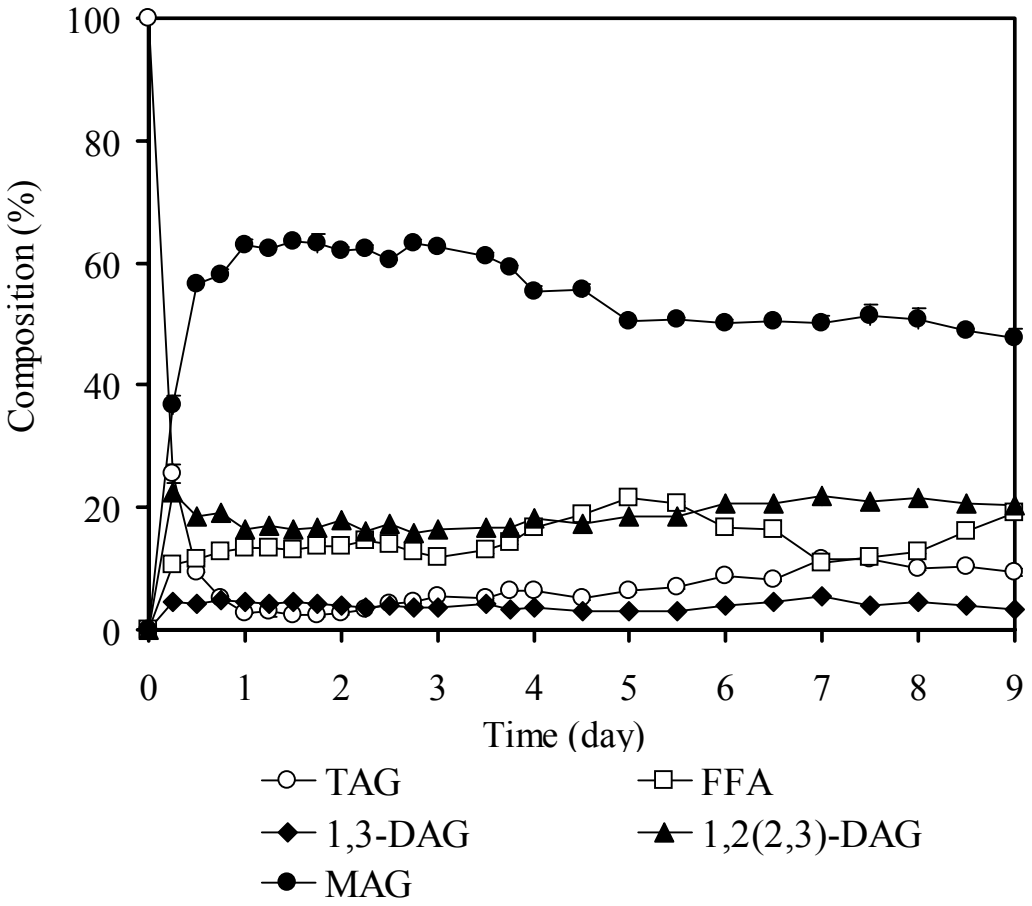
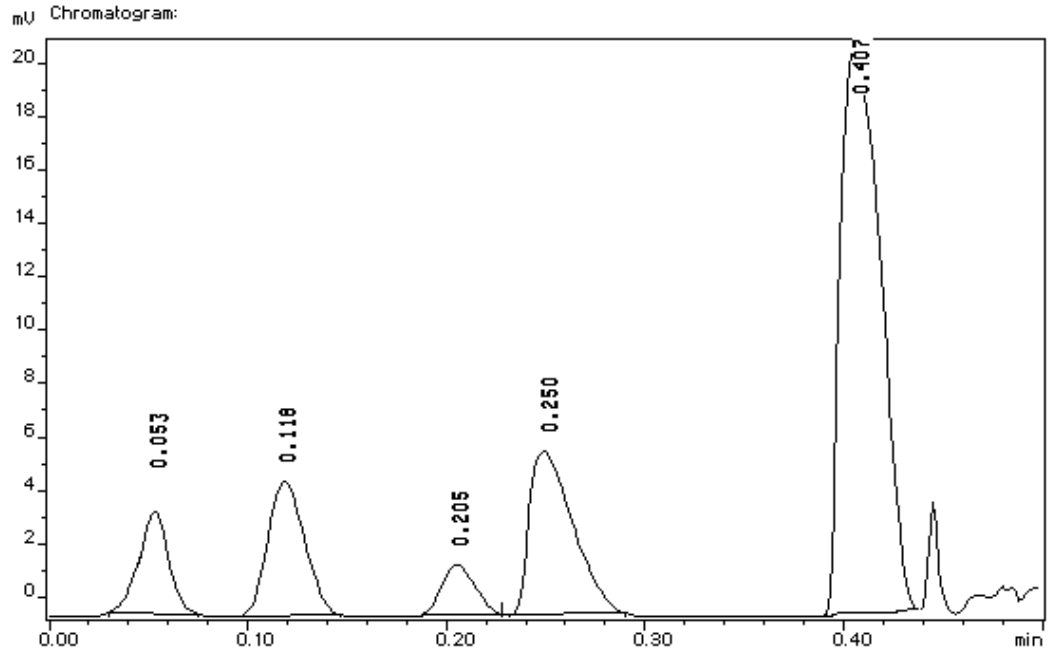


Figure 44 Continuous MAG production in solvent system by IM-PS in PBR

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.



Peak No	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area %
1	TAG	0.053	0.030	0.075	1968	4.02	7.084
2	FFA	0.118	0.097	0.147	4042	5.02	14.549
3	1,3-DAG	0.205	0.188	0.228	1113	1.88	4.007
4	1,2(2,3)-DAG	0.250	0.228	0.290	5146	6.11	18.524
5	MAG	0.407	0.390	0.438	15512	21.34	55.836
					27781	38.37	100.000

Condition:

Stationary phase : CHROMAROD-SIII

Mobile phase : benzene : chloroform : acetic acid (50:20:0.7)

Gas flow : H_2 160 mL/min, Air 2.0 L/min

Scanning speed : 30 s/scan

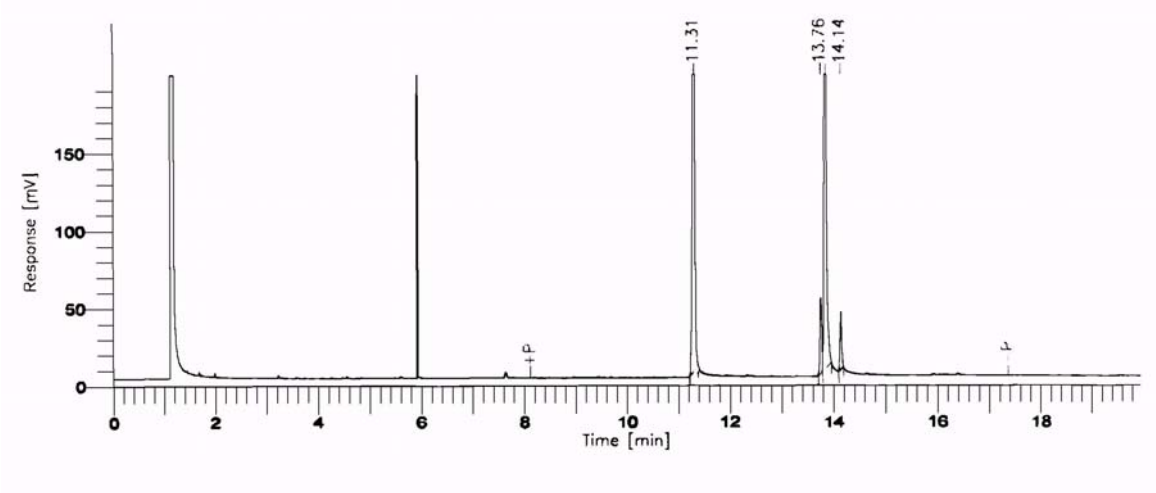
Figure 45 TLC/FID chromatogram of product mixture from continuous MAG production in solvent system by IM-PS in PBR

Fatty acid profile of product mixtures

The components of product mixtures were separated and recovered by thin-layer chromatography (TLC). Approximately 20 mg of the reaction mixture of glycerolysis was dissolved in 200 μL chloroform. Development solvent was chloroform/acetone in the ratio of 96:4. The dissolved sample was applied to TLC plate of silica gel and visualized with iodine. Each fraction was scraped off the plate and extracted with chloroform. Each fraction was identified by converting the recovered bands to methyl ester followed by GC analysis. The results are tabulated in Table 13. The fatty acid components of MAG are shown in Figure 46. The fatty acid components of MAG in product mixture were similar to fatty acid components of palm olein, indicating that formation of MAG was random. It agrees with the results of continuous glycerolysis of soybean oil in supercritical carbon dioxide reported by Jackson and King (1997).

Table 13 Fatty acid profile of product mixtures

Product mixtures		Fatty acid composition (%,w/w)			
Composition	% (w/w)	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$
TAG	7.08	51.82	3.95	44.23	-
FFA	14.55	36.12	5.14	52.80	5.93
1,2(2,3)-DAG	18.52	44.21	3.23	47.08	5.49
1,3-DAG	4.01	51.09	3.58	41.19	4.14
MAG	55.84	44.76	4.03	45.19	6.02



Peak #	Name	Time (min)	Area (μV.s)	Height (μV)	Norm Area (%)	BL	Area (%)
1	Palmitic acid	11.311	884283.51	308514.58	0.00	BB	44.76
2	Linoleic acid	13.758	118832.88	49047.10	0.00	BV	6.02
3	Oleic acid	13.856	892712.95	321065.48	0.00	VB	45.19
4	Stearic acid	14.143	79698.68	37258.35	0.00	BB	4.03
			1975528.01	715885.51	0.00		100.00

Condition:

Column : Optima-5-0.25 μm 25 m x 0.25 mm

Carrier gas : Helium at 1.65 mL/min

Temp : 150 °C (40 °C/min, 0.5 min), 170 °C (5°C/min), 195°C (10°C/min) and 215°C (9.5 min)

Injector temp : 250 °C

Detector : FID at 250 °C

Figure 46 GC chromatogram of MAG in product mixture from continuous MAG production in solvent system by IM-PS in PBR

13. Time course of continuous MAG production in PBR

Based on the results obtained, the long-term, continuous MAG production using glycerolysis of palm olein with glycrol by IM-PS in PBR was investigated. Under optimal conditions obtained, the reactor could be successfully operated by maintaining about 31 % yield of MAG (a half of the highest MAG yield) for 780 h without pressure drops problem (Figure 47). The highest MAG yield (61.5 %) was obtained at 24 h and nearly stable for 30 h. Subsequently, the MAG yield was decreased with increasing the operation time. Both 1,2(2,3)-DAG and 1,3-DAG yields were slightly stable through operation. On the other hand, FFA yield was fluctuated near 10 % through operation. A productivity of 2.23 x 10^{-3} g MAG/U.day was obtained at 780 h. The prolonged high activity of the enzyme may be caused by the combination of both protecting effect of substrates (glycerol and palm olein) and stabilizing effect of immobilization of enzyme. Polyhydric alcohols, e.g., glycerol protect enzymes from their denaturation (Hoq *et al*., 1984). Chang *et al*. (1991) found that the half-life of the immobilized lipase during continuous glycerolysis of olive oil in stirred vessel reactor was about 7 weeks. Fukui *et al*. (1990) could produce highly optically active 2-(4-chlorophenoxy) propionic acid continuously for 63 days by celite-adsorbed lipase OF 360 in water-saturated carbon tetrachloride-isooctane (8:2, v/v) as reaction solvent. Yang and Rhee (1992) found that operational half life was 220 h at 30 °C for continuous hydrolysis of olive oil in isooctane by immobilized lipase from *Candida rugosa* in PBR. Moreover, Kang and Rhee (1989a) suggested that substrate binding to lipase can reduce the conformational changes of the lipase and preserve the activity of immobilized lipase.

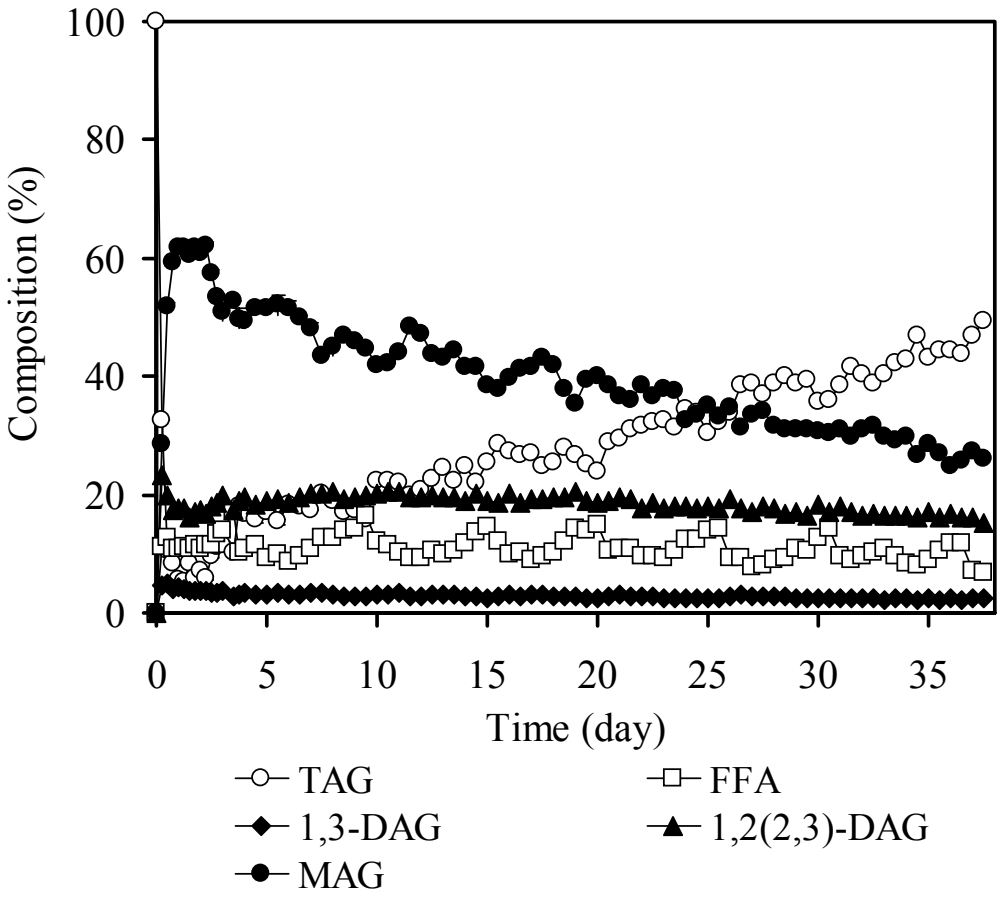


Figure 47 Time course of continuous MAG production in solvent system by IM-PS in PBR

The amount of IM-PS was 1.5 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was 0.02 mL/min. The temperature was controlled at 45 °C.

14. Large scale MAG production in PBR

The semi-fundamental method in combination with different methods (e.g. dimensional analysis/regime analysis, rules of thumb, scale-down approach/regime analysis, trial and error) were used to scale up reactor and MAG production processes.

The PBR was scaled up 10 times for MAG production. The IM-PS (15 g) was packed in a jacketed column (1.5 cm ID, 50 cm long). The temperature was controlled at 45 °C. The substrate was introduced at the top of the column with the flow rate of 0.2 mL/min. This system was operated for 96 h. The results are shown in Figure 48. Firstly, the MAG yield was rapidly increased with increasing the operation time and the highest yield of MAG (70.11 %) was obtained at 24 h. Subsequently, the yield of MAG was slightly decreased with increasing the operation time. The FFA yield was nearly constant at 10 % through continuous operation. The average yield of MAG with 61.3 % was obtained. A productivity of 3.32×10^{-3} g MAG/U.day was obtained while a theoretical productivity was 5.66×10^{-3} g MAG/U.day. It was similar to the yield of MAG (59.31 %) derived from small PBR (0.68 cm ID, 25 cm long). Consequently, large scale of PBR could be used to produce MAG without changing the yield of MAG in 96 h of operation time.

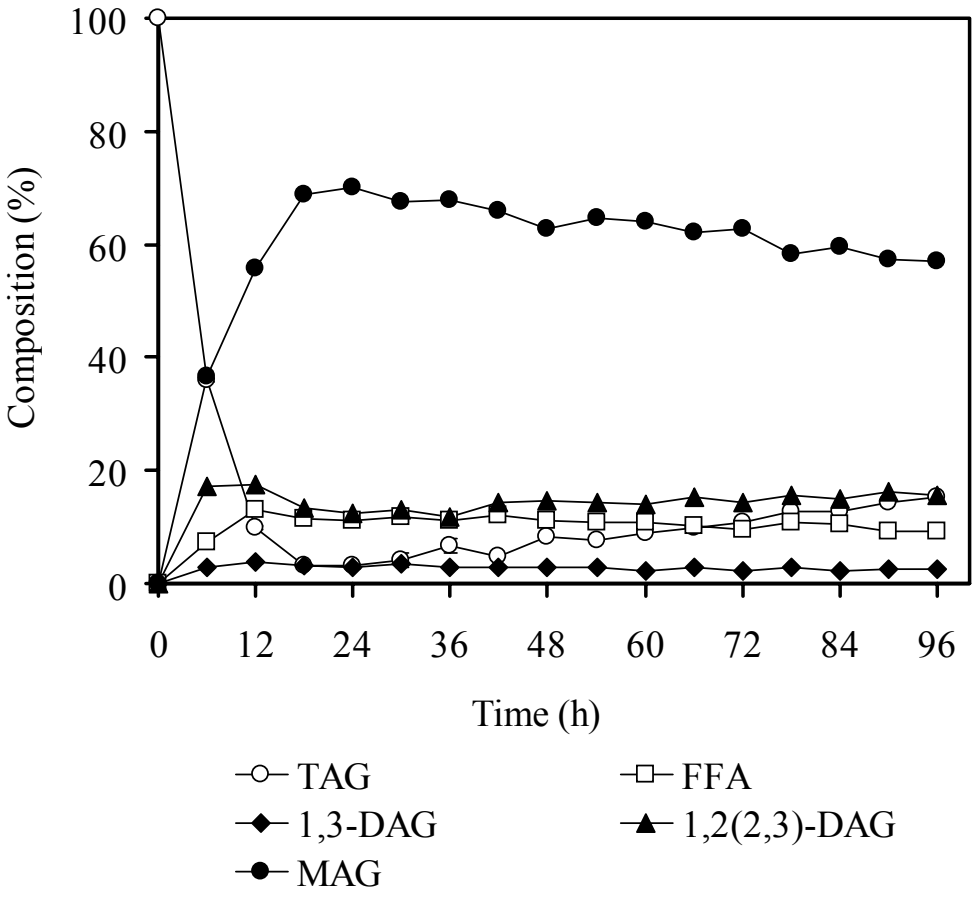


Figure 48 Large scale of continuous MAG production by IM-PS in PBR

The amount of IM-PS was 15 g (0.36 U/mg). Substrates consisted of 10 %(w/v) palm olein in acetone/isooctane mixture (3:1,v/v), glycerol to palm olein molar ratio of 12:1 and 10 %(w/w) water in glycerol. The substrate flow rate was 0.2 mL/min. The temperature was controlled at 45 °C.

15. Recovery of MAG

After continuous MAG production, purification was needed not only to remove impurities but also to concentrate MAG. Several methods to purify MAG were investigated.

15.1 Crystallization using temperature control

Residual glycerol was removed from the product mixture by separation with separating funnel and used for further study.

15.1.1 Effect of temperature on MAG crystallization from product mixture

Effect of temperature on MAG crystallization from product mixture was studied at temperatures between 10 and –10 °C. The product mixture was incubated at various temperatures for 8 h. The results are shown in Table 14. It was found that MAG did not crystallized at 10 °C, but crystallized at temperature between 5 and –10 °C. No different in purity of MAG was obtained when using temperature for crystallization between 5 and –5 °C. MAG crystallized at –5 °C gave more purity than at –10 °C. The result might be caused by increasing of non-MAG crystals at –10 °C. However, MAG crystallization at –5 and –10 °C gave higher yields of MAG. Therefore, MAG crystallization at –5 °C for 8 h was chosen for further study.

Table 14 Effect of temperature on MAG crystallization from product mixture

Temperature (°C)	Purity (%)	Yield (%)
5	87.95±0.20[a] a[b]	22.10±1.09 c
0	88.55±0.62 a	23.66±0.75 b
-5	87.64±0.52 a	25.45±0.29 a
-10	85.99±0.21 b	25.54±0.39 a

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

15.1.2 Effect of time on MAG crystallization from product mixture

Effect of time on MAG crystallization from product mixture was studied at –5 °C. The MAG crystallization was performed at various incubation times between 2 and 24 h. The results are shown in Table 15. It was found that the purity of MAG was slightly different when using incubation times between 2 and 24 h. However, yields of MAG were increased with increasing the incubation times from 2 to 8 h and nearly stable when using incubation times between 8 and 24 h. Consequently, 8 h for incubation time was enough for MAG crystallization. When crystallizing at –5 °C for 8 h, the purity and yield of MAG of 87.76 and 25.58 % were obtained, respectively.

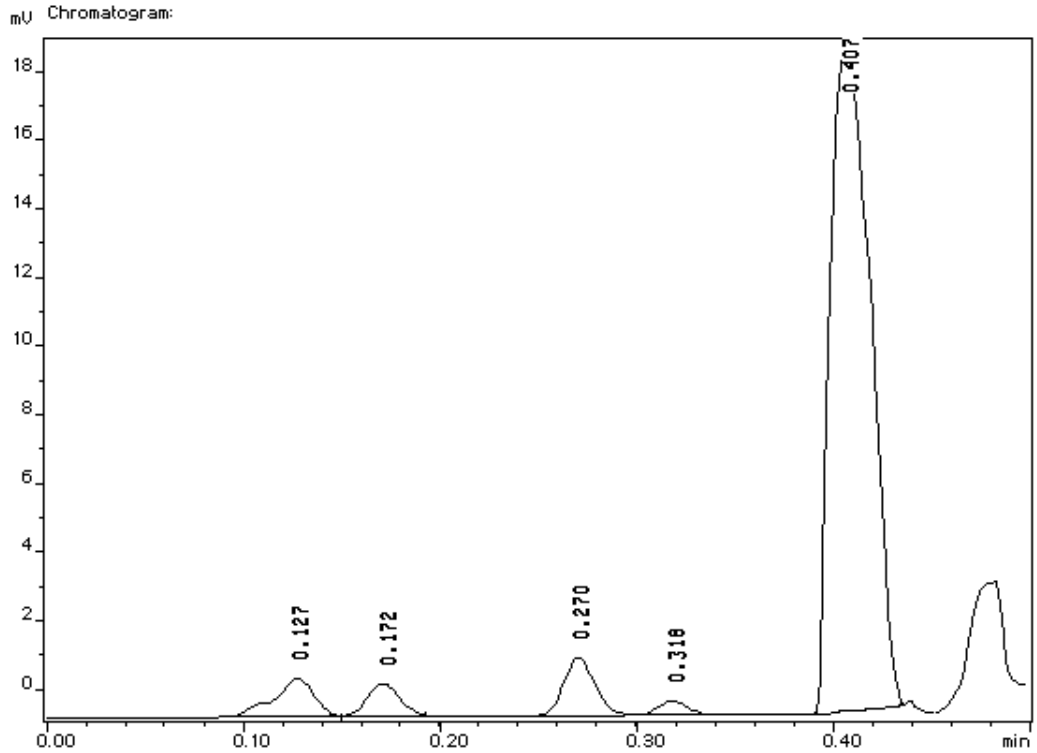
Table 15 Effect of time on MAG crystallization from product mixture

Time (h)	Purity (%)	Yield (%)
2	86.49±0.31[a] b[b]	21.11±0.46 c
4	87.13±0.51 b	24.08±0.35 b
8	87.76±0.12 ab	25.58±0.18 a
12	87.88±1.45 ab	25.88±0.55 a
18	86.86±0.67 b	25.57±0.48 a
24	88.65±0.74 a	25.69±0.27 a

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

MAG product derived from crystallization in acetone/isooctane mixture (3:1, v/v) at -5 °C for 8 h was determined for purity by TLC/FID (Figure 49) and fatty acid components by gas chromatography (Figure 50). It was found that MAG contained palmitic acid, oleic acid and stearic acid of 94.45, 3.14 and 2.41 %, respectively. The major MAG in MAG product was monopalmitin.



Peak No	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area %
1	TAG	0.127	0.095	0.150	687	1.08	4.016
2	FFA	0.172	0.152	0.193	417	0.91	2.435
3	1,3-DAG	0.270	0.250	0.293	754	1.66	4.407
4	1,2(2,3)-DAG	0.318	0.303	0.340	237	0.39	1.385
5	MAG	0.407	0.388	0.452	15023	19.51	87.757
					17119	23.56	100.000

Condition:

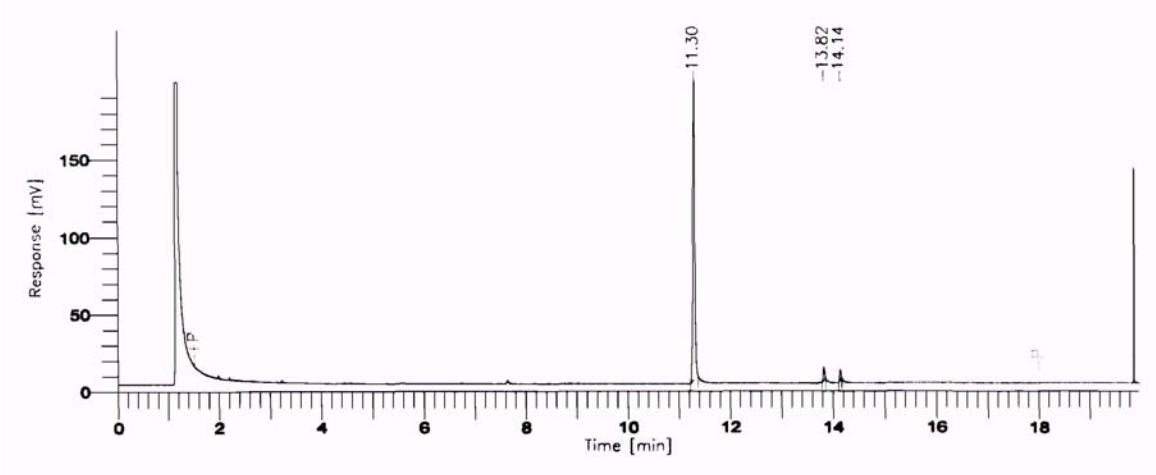
Stationary phase : CHROMAROD-SIII

Mobile phase : benzene : chloroform : acetic acid (50:20:0.7)

Gas flow : H_2 160 mL/min, Air 2.0 L/min

Scanning speed : 30 s/scan

Figure 49 TLC/FID chromatogram of MAG product after crystallization in acetone/isooctane mixture (3:1,v/v) at –5 °C for 8 h



Peak #	Name	Time (min)	Area (μV.s)	Height (μV)	Norm Area (%)	BL	Area (%)
1	Palmitic acid	11.298	501650.10	199969.33	0.00	BB	94.45
2	Linoleic acid	13.821	16665.82	8632.84	0.00	BB	3.14
3	Oleic acid	14.141	12798.26	7001.92	0.00	BB	2.41
			531114.19	215604.08	0.00		100.00

Condition:

Column : Optima-5-0.25 μm 25 m x 0.25 mm

Carrier gas : Helium at 1.65 mL/min

Temp : 150 °C (40 °C/min, 0.5 min), 170 °C (5°C/min), 195°C (10°C/min) and 215°C (9.5 min)

Injector temp : 250 °C

Detector : FID at 250 °C

Figure 50 GC chromatogram of MAG after crystallization in acetone/isooctane (3:1,v/v) at –5 °C for 8 h

15.2 Crystallization using extraction by organic solvent with temperature control

After continuous MAG production in PBR, residual glycerol was removed from the product mixture by separation with separating funnel. The removal of organic solvents was achieved by evaporation using rotary evaporator. Crude MAG product was used for further study.

15.2.1 Effect of organic solvents on MAG crystallization

Effect of various organic solvents on MAG crystallization were studied. MAG crystallization was carried out by dissolving crude MAG product in several organic solvents: hexane, isooctane and acetone with 10% w/v concentration and kept at 0 °C for 8 h. The results are shown in Table 16. Isooctane was the best organic solvent for MAG crystallization. Forty percentage yield of MAG and 84.39 % purity of MAG were obtained when MAG was crystallized in isooctane at 0 °C for 8 h. Acetone/isooctane mixture (3:1,v/v) and single solvent gave same % purity of MAG but acetone/isooctane mixture (3:1,v/v) gave lower % yield of MAG than isooctane alone. When crystallizing in isooctane at 0 °C for 8 h, the purity and yield of MAG of 84.39 and 40.98 % were obtained, respectively. Therefore, isooctane was chosen for MAG crystallization in further study.

15.2.2 Effect of crude MAG product concentration in isooctane on MAG crystallization

Effect of crude MAG product concentration in isooctane on MAG crystallization was determined. MAG crystallization from crude MAG product was carried out by dissolving crude MAG product in isooctane with several concentration and kept at 0 °C for 8 h. The results are shown in Table 17. It was found that % yield of MAG product was increased with increasing crude MAG product concentration in isooctane. Besides, concentration of crude MAG product in isooctane had not effect on purity of MAG. Twenty percentage crude

MAG product concentration in isooctane was suitable for MAG crystallization at 0 °C for 8 h. However, solution was solidified when crude MAG product concentration in isooctane was 25 %(w/v)

Table 16 Effect of organic solvents on MAG crystallization

Solvent	Purity (%)	Yield (%)
Hexane	84.65±0.17[a] ab[b]	37.20±1.56 b
Isooctane	84.39±0.16 ab	40.98±0.64 a
Acetone	83.20±0.64 b	25.07±1.34 cd
Hexane/acetone (1:3,v/v)	76.51±2.02 d	18.43±1.64 e
Hexane/acetone (1:1,v/v)	85.26±0.66 a	12.11±0.82 f
Hexane/acetone (3:1,v/v)	78.45±0.69 c	18.37±0.87 e
Isooctane/acetone (1:3,v/v)	85.00±0.42 a	23.73±0.85 d
Isooctane/acetone (1:1,v/v)	79.85±0.45 c	16.92±1.14 e
Isooctane/acetone (3:1,v/v)	74.80±0.69 e	25.81±0.80 c

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

Table 17 Effect of crude MAG product concentration in isooctane on MAG crystallization

Crude MAG product (%)	Purity (%)	Yield (%)
2.5	85.96±0.18[a] a[b]	16.52±2.35 d
5	85.10±0.69 a	30.25±1.65 c
10	85.01±0.20 a	39.75±1.10 b
15	85.92±0.15 a	47.17±1.24 a
20	85.15±0.78 a	49.19±0.84 a

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

15.2.3 Effect of temperature on MAG crystallization in isooctane

Effect of temperature on MAG crystallization in isooctane was determined between 10 and –10 °C. MAG crystallization from crude MAG product was carried out by dissolving crude MAG product in isooctane with 20 %(w/v) concentration and kept at several temperature for 8 h. The results are shown in Table 18. It was found that temperature at 5 and 0 °C gave reasonably high purity of MAG but temperature at 0 °C gave higher % yield of MAG than at 5 °C. At –5 and –10 °C the whole solution was solidified and the MAG product could not be separated. Therefore, temperature at 0 °C was suitable for crystallized of MAG. The purity and yield of MAG of 84.25 and 49.26 % were obtained, respectively when 20 % of crude MAG product in isooctane was crystallized at 0 °C for 8 h.

Table 18 Effect of temperature on MAG crystallization in isooctane

Temperature (°C)	Purity (%)	Yield (%)
10	82.14±0.81[a] b[b]	36.93±1.67 b
5	85.76±0.18 a	41.57±2.89 b
0	84.25±0.26 a	49.26±2.48 a

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)

15.2.4 Effect of time on MAG crystallization in isooctane

Effect of time on MAG crystallization in isooctane was determined between 2 and 18 h. MAG crystallization from crude MAG product was carried out by dissolving crude MAG product in isooctane with 20 %(w/v) concentration and kept at 0 °C. The results are shown in Table 19. It was found that both purity and yield of MAG were increased with increasing crystallization time from 2 to 8 h. However, the crystallization time more than 8 h has not effect on both purity and yield of MAG. Consequently, incubation for 8 h was enough for MAG crystallization in isooctane at 0 °C. Therefore, the suitable condition for MAG crystallization included 20 %(w/v) of crude MAG product in isooctane and incubated at 0 °C for 8 h.

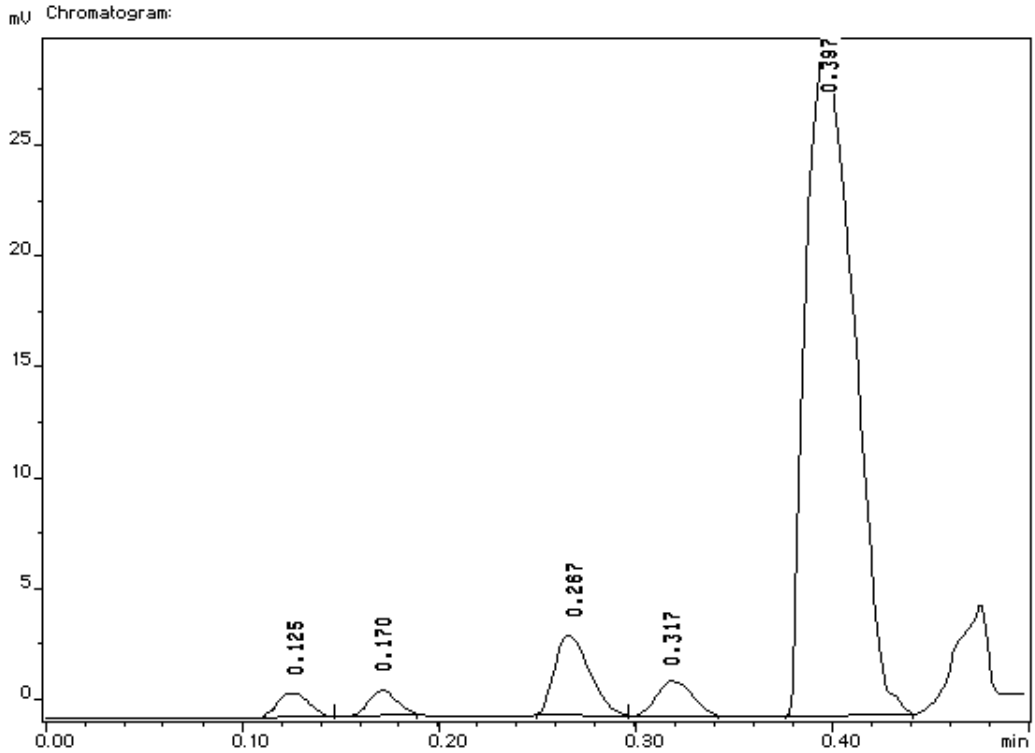
Under these conditions, the yield of MAG with 49.55 % and purity of 85.35 % were obtained (Figure 51). The MAG contained palmitic acid, oleic acid, stearic acid and linoleic acid with 72.20, 22.88, 3.55 and 1.37 %, respectively (Figure 52).

Table 19 Effect of time on MAG crystallization in isooctane

Time (h)	Purity (%)	Yield (%)
2	78.98±0.17[a] ab[b]	45.20±1.35 b
4	80.92±0.54 b	46.79±0.44 b
8	85.35±0.54 a	49.55±0.32 a
12	86.17±0.20 a	49.02±0.11 a
18	86.25±0.05 a	49.61±2.02 a
24	86.45±0.25 a	49.21±0.85 a

[a] Mean ± standard deviation from triplicate determination

[b] Different letters in the same column indicate significant differences ($p < 0.05$)



Peak No	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area %
1	TAG	0.125	0.118	0.147	578	1.03	1.835
2	FFA	0.170	0.147	0.193	695	1.11	2.207
3	1,3-DAG	0.267	0.250	0.297	2427	3.59	7.706
4	1,2(2,3)-DAG	0.317	0.297	0.333	915	1.57	2.906
5	MAG	0.397	0.377	0.442	26885	30.24	85.346
					31501	37.55	100.000

Condition:

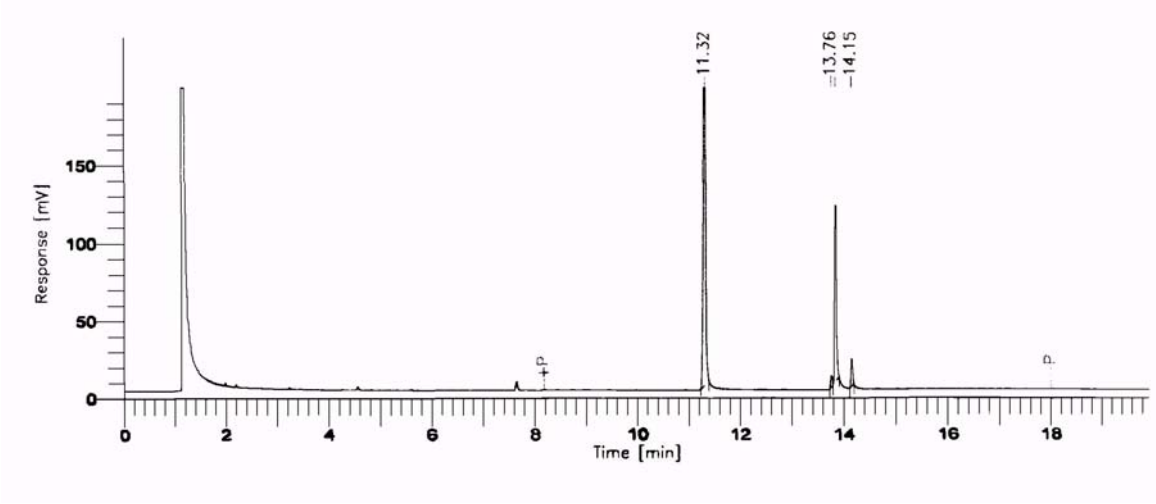
Stationary phase : CHROMAROD-SIII

Mobile phase : benzene : chloroform : acetic acid (50:20:0.7)

Gas flow : H_2 160 mL/min, Air 2.0 L/min

Scanning speed : 30 s/scan

Figure 51 TLC/FID chromatogram of MAG product after crystallization in isooctane at 0 °C for 8 h



Peak #	Name	Time (min)	Area (μV.s)	Height (μV)	Norm Area (%)	BL	Area (%)
1	Palmitic acid	11.316	785860.09	287783.50	0.00	BB	72.20
2	Linoleic acid	13.761	14964.07	7363.86	0.00	BV	1.37
3	Oleic acid	13.842	249023.87	114218.48	0.00	VB	22.88
4	Stearic acid	14.152	38670.19	18195.08	0.00	BB	3.55
			1088518.22	427560.92	0.00		100.00

Condition:

Column : Optima-5-0.25 μm 25 m x 0.25 mm

Carrier gas : Helium at 1.65 mL/min

Temp : 150 °C (40 °C/min, 0.5 min), 170 °C (5°C/min), 195°C (10°C/min) and 215°C (9.5 min)

Injector temp : 250 °C

Detector : FID at 250 °C

Figure 52 GC chromatogram of MAG after crystallization in isooctane at 0 °C for 8 h

15.3 Fractionation by silica gel 60

After evaporation of the solvents, crude MAG product was dissolved in hexane (50 %,w/v). One mL crude MAG product in hexane was applied on the column (0.68 cm ID, 25 cm long contained 5 g silica gel 60).and eluted with 5 mL hexane at a flow rate of 0.5 mL/min following 5 mL 5 %(v/v) ethanol in hexane with the same flow rate. The eluting solution was collected 0.5 mL/fraction. The composition of each fraction was analyzed by TLC/FID. By this method, most of TAG and FFA were eluted with hexane. DAG [1,2(2,3)-DAG and 1,3-DAG] were eluted with 5 % ethanol in hexane at early fractions and then MAG was eluted. It may be caused by the higher non-polarity of TAG and FFA than DAG and MAG. A similar observation was made by Padt *et al*. (1992) during separation of MAG from membrane bioreactor. The yield of MAG after filtration was 87.66 % (Figure 53) and the purity of MAG was 95.36 % (Figure 54). When the fatty acid components of MAG was determined by gas chromatography, the MAG contained palmitic acid, oleic acid and stearic acid with 71.85, 24.88 and 3.28 %, respectively (Figure 55).

The comparison of three methods for recovery of MAG; crystallization in acetone/isooctane mixture (3:1,v/v), crystallization in isooctane and fractionation by silica gel column, are shown in Table 20. It was found that fractionation by silica gel column gave the highest both purity and yield of MAG with 95.36 and 87.6 %, respectively. Crystallization of MAG in isooctane gave higher yield than crystallization of MAG in acetone/isooctane mixture (3:1,v/v) while the purity of MAG in both methods was similar. Monopalmitin was the major MAG in crystals when crystallization of MAG in acetone/isooctane mixture (3:1,v/v) was used. Moreover, crystallization of MAG in isooctane and fractionation by silica gel column gave the same fatty acid composition in MAG.

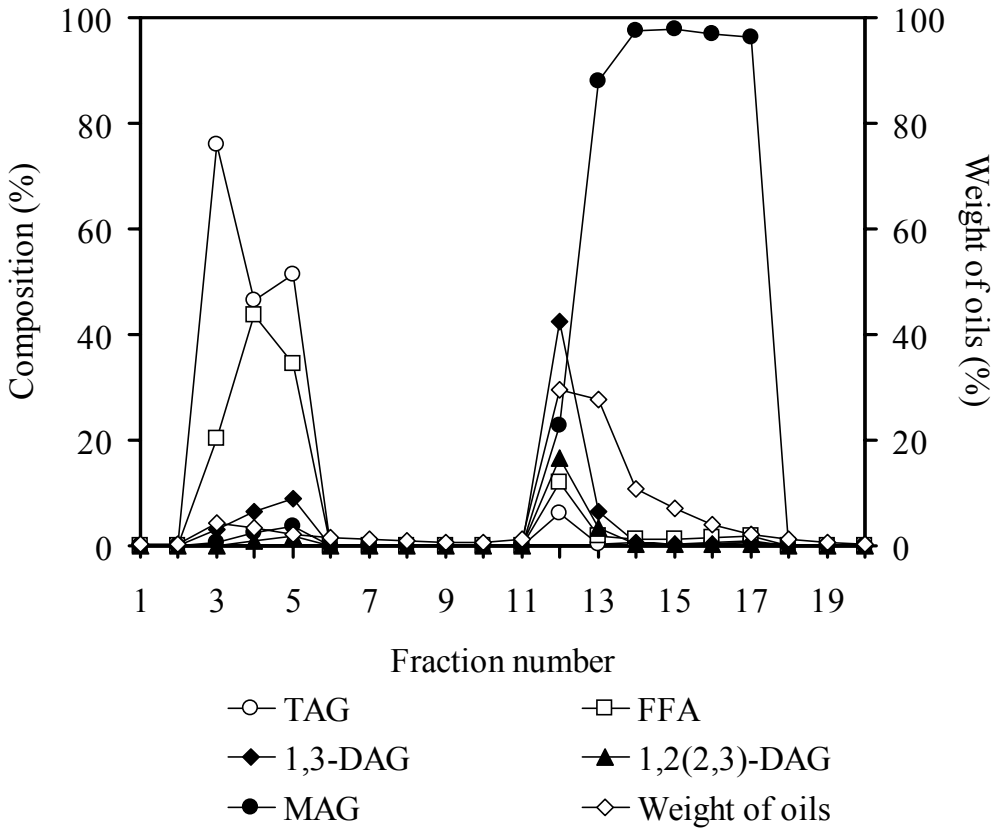
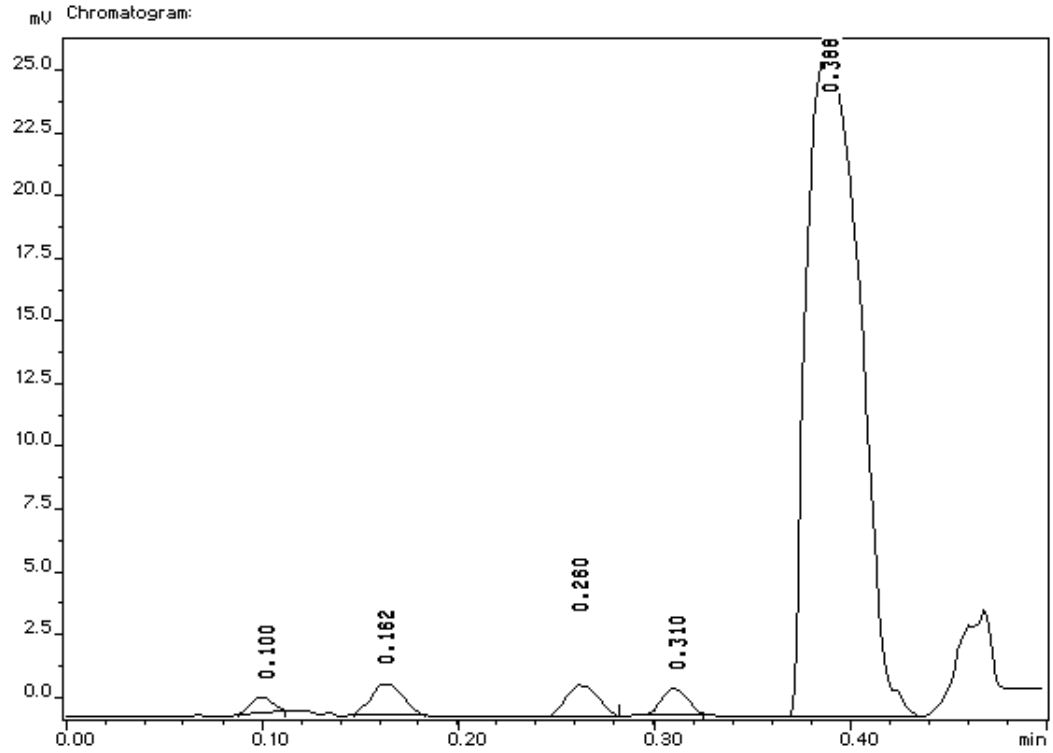


Figure 53 Fractionation of crude MAG product by silica gel column chromatography

The sample applied on the column (0.68 cm ID, 25 cm long) was eluted with hexane fraction in 5 mL at a flow rate of 0.5 mL/min for 10 fractions following 5 % (v/v) ethanol in hexane with the same flow rate for 10 fractions. The eluting solution was collected 5 mL/fraction.



Peak No	Name	Ret. Time (min)	Pk. Start (min)	Pk. End (min)	Area	Height (mV)	Area %
1	TAG	0.100	0.088	0.112	170	0.60	0.573
2	FFA	0.162	0.147	0.183	477	1.23	1.604
3	1,3-DAG	0.260	0.243	0.282	491	1.24	1.653
4	1,2(2,3)-DAG	0.310	0.290	0.328	242	1.02	0.814
5	MAG	0.388	0.368	0.435	28346	26.80	95.356
					29726	30.89	100.000

Condition:

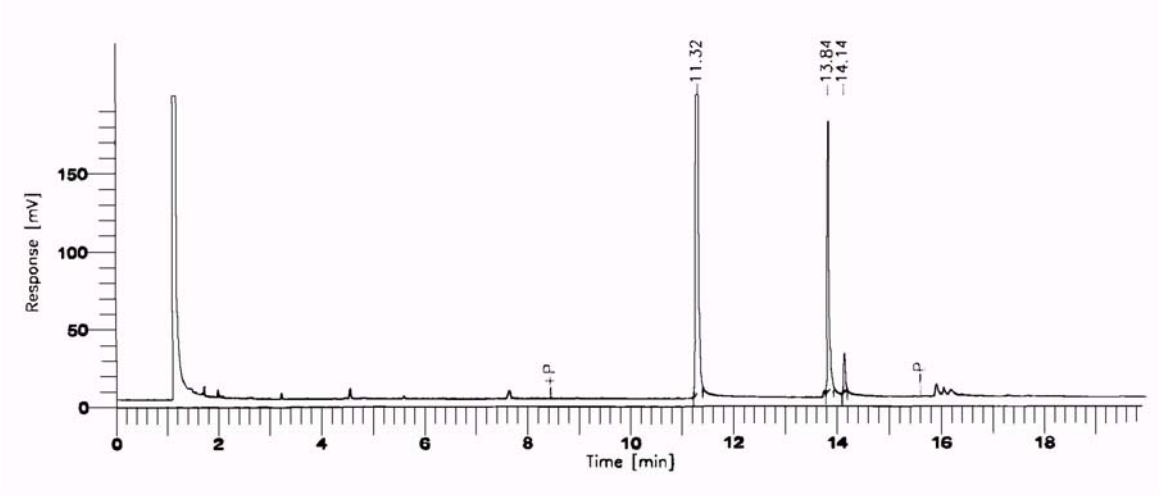
Stationary phase : CHROMAROD-SIII

Mobile phase : benzene : chloroform : acetic acid (50:20:0.7)

Gas flow : H_2 160 mL/min, Air 2.0 L/min

Scanning speed : 30 s/scan

Figure 54 TLC/FID chromatogram of MAG product after fractionation by silica gel column



Peak #	Name	Time (min)	Area (μV.s)	Height (μV)	Norm Area (%)	BL	Area (%)
1	Palmitic acid	11.320	1178269.57	382967.94	0.00	BB	71.85
2	Linoleic acid	13.840	407971.77	173060.35	0.00	BB	24.88
3	Oleic acid	14.140	53769.44	24987.79	0.00	BB	3.28
			1640010.77	581016.08	0.00		100.00

Condition:

Column : Optima-5-0.25 μm 25 m x 0.25 mm

Carrier gas : Helium at 1.65 mL/min

Temp : 150 °C (40 °C/min, 0.5 min), 170 °C (5°C/min), 195°C (10°C/min) and 215°C (9.5 min)

Injector temp : 250 °C

Detector : FID at 250 °C

Figure 55 GC chromatogram of MAG after fractionation by silica gel column

Table 20 Comparison of various methods for MAG recovery

	Palm olein	Crude MAG product	Method of recovery		
			Crystallization in acetone/isooctane mixture	Crystallization in isooctane	Fractionation by Siliga gel Column
Purity of MAG (%)		55.84	87.76	85.35	95.36
Yield of MAG (%)			25.58	49.55	87.6
Composition (%)					
TAG	96.07	7.08	4.02	1.84	0.57
FFA	-[a]	14.55	2.44	2.21	1.60
1,3-DAG	2.84	18.52	4.41	7.71	1.65
1,2(2,3)-DAG	1.09	4.01	1.39	2.91	0.81
MAG	-[a]	55.84	87.76	85.35	95.36
Fatty acid composition in MAG (%)					
Palmitic acid ($C_{16:0}$)		44.76	94.45	72.20	71.85
Stearic acid ($C_{18:0}$)		4.03	2.41	3.55	3.28
Oleic acid ($C_{18:1}$)		45.19	3.14	22.88	24.88
Linoleic acid ($C_{18:2}$)		6.02	-[a]	1.37	-[a]

[a]- could not detect