

Synthesis of monoacylglycerol rich in polyunsaturated fatty acids from tuna oil with immobilized lipase AK

Ratchapol Pawongrat^a, Xuebing Xu^b, Aran H-Kittikun^{a,*}

^a Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112, Thailand

^b BioCentrum-DTU, Technical University of Denmark, Building 227, DK-2800 Kgs, Lyngby, Denmark

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Abstract

The aim of this study was to produce monoacylglycerols (MAG) rich in polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), by glycerolysis of tuna oil with lipase AK from *Pseudomonas fluorescens* immobilized on Accurel EP-100 (IM-AK). *tert*-Butyl methyl ether (MTBE) was the most suitable organic solvent after screening a list of different solvents and their mixtures. The optimum conditions for MAG production were: 10% w/v of tuna oil in MTBE; mole ratio of glycerol to tuna oil 3:1, water added 4 wt% in glycerol; the amount of IM-AK 30 wt%, based on tuna oil. The temperature was controlled at 45 °C. Under these conditions, with a 24 h reaction, the yield of MAG was 24.6%, but containing 56.0 wt% PUFA (EPA and DHA). Stability of the IM-AK was also studied. The hydrolytic activity of the enzyme remained at 88% and 80% of initial activity after incubating in MTBE for 24 h at 4 and 45 °C, respectively. The K_m and V_{max} values of the lipase-catalyzed glycerolysis of tuna oil in MTBE were found to be 19.5 mM and 2.71 mg MAG/min, respectively, for IM-AK.

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1. Introduction

Tuna oil is currently one of the major sources of polyunsaturated fatty acids (PUFA), especially, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The oil contains approximately 5.7% EPA and 18.8% DHA, which are distributed in mixed triacylglycerols (TAG) with ordinary fatty acids (Wongsakul, Prasertsan, Bornscheuer, & H-Kittikun, 2003). PUFA have received much attention in recent years because of their health benefits, including reduced risk of coronary disease, prevention of certain cancers, and improved immune functions (Narayan, Miyashita, & Hosakawa, 2006; Ruxton, Reed, Simpson, & Millington, 2004).

Monoacylglycerols (MAG), or mixtures with diacylglycerols (DAG), account for approximately 75% of the

emulsifier production and have various applications in different fields (Bornscheuer, 1995; Damstrup et al., 2005). MAG are nonionic emulsifiers, widely used in bakery products, margarines, dairy products and confectionery because of their emulsifying, stabilizing and conditioning properties. Moreover, MAG are also used in pharmaceuticals as binders in tablets and as emollients for transdermal, slow-release drugs. Due to the worldwide importance of MAG and their derivatives, as surface active additives in a wide range of foods, considerable attention has recently been paid to improving the synthesis of MAG. They are also of great interest in synthetic organic chemistry where they are utilized as synthetic intermediates and as chiral building blocks (Monteiro, Nascimento, & Ninow, 2003).

Chemically, MAG can be synthesized at high temperatures, using several metallic catalysts. Commercial MAG are widely manufactured by glycerolysis of fats and oils. The glycerolysis reaction is accelerated by the use of inorganic alkaline catalysts, such as NaOH or Ca(OH)₂ at

* Corresponding author. Tel.: +66 7428 6363; fax: +66 7421 2889.
E-mail address: aranxyz@yahoo.com (A. H-Kittikun).

220–260 °C. However, this leads to a number of unwanted side products and the reaction occurs in a random manner, so that extensive purification of products is required (Damstrup et al., 2005; McNeill & Yamane, 1991; Yang, Rebsdorf, Engelrud, & Xu, 2005). Furthermore, a high-temperature chemical process is not suitable for the production of heat-sensitive MAG containing PUFA, in particular from fish oils. Production of heat-sensitive MAG is, however, of great commercial interest owing to their nutritive value, which could be applied in foods and pharmaceuticals (Damstrup et al., 2005; Kaewthong & H-Kittikun, 2004; Yang et al., 2005).

Due to disadvantages of the conventional process, the use of enzymes as catalysts, thus, seems to be a potential as well as imperative alternative for practical considerations. The much lower temperature used (less than 80 °C) improves product quality and makes production of heat-sensitive MAG feasible. Lipases show good stability and activity in hydrophobic solvents for MAG synthesis by glycerolysis (Damstrup et al., 2005; Kristensen, Xu, & Mu, 2005).

Several studies have dealt with the synthesis of MAG enriched in PUFA, such as alcoholysis of triacylglycerols with ethanol using a 1,3-regiospecific lipase or glycerolysis of fish oil using an immobilized lipase (Sakiyama, Yoshimi, Tanaka, Ozaki, & Nakanishi, 2001; Wongsakul et al., 2003; Yang et al., 2005).

Glycerolysis of triacylglycerols (TAG) with lipase in the liquid phase typically yields only 30–50% of MAG (Bornscheuer, 1995; Kaewthong & H-Kittikun, 2004). Some authors have improved their systems by carrying out the reaction first in a liquid state, but reducing the temperature to crystallize the formed MAG. This led to a shift in the reaction equilibrium so that yields increased to 70–90%. However, continuous production of MAG by this method was impossible (Kaewthong & H-Kittikun, 2004). For the bioconversion of various lipophilic or water-insoluble compounds, it is essential to introduce organic solvents into reaction systems to improve the solubility of these reactants. Furthermore, use of a suitable solvent system will result in a more homogeneous system and enhance the conversion of substrate, the reaction rate, and the production distribution in favour of MAG formation, as well as PUFA content in MAG.

In this work, using enzymatic glycerolysis with tuna oil as substrate, a list of solvents were first evaluated for the synthesis of MAG. Optimal reaction parameters were investigated in order to obtain MAG in high yields as well as with high PUFA (defined as EPA and DHA) content. Major attention was paid to the enrichment of EPA and DHA in the MAG fraction.

2. Materials and methods

2.1. Materials

Crude tuna oil, with a water content of 4.7%, was provided by the Chotiwat Industrial Co. Ltd. (Hat Yai, Thai-

land). The crude oil was obtained from skipjack tuna heads by a conventional pressing method. The refined oil was achieved through degumming, neutralization, bleaching, and deodorizing. Lipase AK, from *Pseudomonas fluorescens*, with water content of 0.04%, was a gift from Amano (Nagoya, Japan). Microporous polypropylene powder, Accurel EP-100 (particle size <400 µm), was a gift from Akzo Nobel Membrana (Obernburg, Germany). All other chemicals and solvents used were of reagent grade or analytical grade.

2.2. Hydrolytic activity of lipases

Hydrolytic activity of the immobilized lipase was determined by the modified cupric acetate method of Lee and Rhee (1993). One unit of hydrolytic activity was defined as the amount of enzyme which liberates 1 µmol equivalent of palmitic acid from palm olein in 1 min at 30 °C.

2.3. Preparation of immobilized lipase

Accurel EP-100 (10 g) was added to 100 ml of 0.1 M phosphate buffer (pH 7) containing app. 100 U/ml of lipase AK and the reaction mixture was stirred with a magnetic bar at 100 rpm for 30 min. Afterwards, 100 ml of 0.1 M phosphate buffer (pH 7) were added and the suspension was filtered through a Buchner funnel by vacuum. The immobilized enzyme was washed with 100 ml of the buffer to remove soluble enzyme and dried in a vacuum desiccator. The immobilized lipase AK on Accurel EP-100 (IM-AK) was stored at 4 °C for further uses.

2.4. Glycerolysis reaction

The initial glycerolysis experiments were carried out in a batch system. The reaction mixture consisted of IM-AK (water content 3.0%) 0.6 g, glycerol (99.5%, water content 0.2%) 1.78 g, and 20 ml of 30% (w/v) of tuna oil in organic solvents. An extra 4 wt% of water, based on the glycerol, was added directly to the glycerol. The temperature was controlled at 45 °C. The reaction mixture was agitated on the shaker at 300 rpm. Samples of the reaction mixture were centrifuged to remove IM-AK before analysis.

2.5. Analysis of glycerides by TLC-FID

The components of the oil phase were analyzed by thin-layer chromatography and with a flame ionization detector (TLC/FID) (IATROSCAN MK5, Iatron Laboratories Inc. Tokyo, Japan) for the contents of TAG, 1,2(2,3)-DAG, 1,3-DAG, MAG and free fatty acids (FFA) (Kaewthong & H-Kittikun, 2004). The sample, diluted in chloroform/methanol (2:1 v/v), was spotted onto the chromatrod and developed for 35 min in a mixture of benzene/chloroform/acetic acid (50:20:0.7 v/v/v) as developing solvent. After development and drying, the rods were subjected to scanning with FID. Standards were used to identify the

peaks. The peak areas were normalized and used for evaluation of the reactions.

2.6. Analysis of fatty acid compositions

The fatty acid compositions of glyceride species were determined by converting into fatty acid methyl esters (FAME), followed by GC analysis. After evaporating excessive solvent of the sample, the mixture was applied to the normal TLC-plate with silica gel and developed in benzene/chloroform/acetic acid (50:20:0.7 v/v/v). After drying, the MAG band was scraped off and methylated with 0.5% NaOH in methanol (1000 μ l) for 10 min at 60 °C. The methyl esters were extracted with *n*-hexane (300 μ l) for 1 min. The *n*-hexane layer was washed with 200 μ l distilled water and dried over anhydrous sodium sulfate. Analysis was carried out with a Perkin–Elmer Autosystem XL-GC gas chromatograph (Perkin–Elmer, Norwalk, CT) on a FFFAP column (PERMABOND-FFFAP DF-0.25, 25 m \times 0.25 mm i.d., MACHEREY-NAGEL, Germany). The carrier gas used was helium at a flow rate of 0.5 ml/min (15 psi) and operated in a split mode with a split ratio of 50:1. The temperature began at 150 °C for 0.50 min and increased at the rate of 4 °C/min to 170 °C, followed by a rate of 5 °C/min to 195 °C, and further with a rate of 10 °C/min to 215 °C. The temperature was kept at 215 °C for 14 min. Injector and detector temperatures were 250 °C (Joseph & Ackman, 1992). Response factors were determined using a standard mixture of fatty acid methyl esters.

2.7. Regiospecific analysis

The regiospecific analysis of tuna oil was conducted by Grignard degradation with allylmagnesium bromide, followed by isolation, methylation, and GC analysis (Soumanou, Bornscheuer, & Schmid, 1998; Wongsakul et al., 2003).

2.8. Karl Fischer water content determination

The water content in the tuna oil, the immobilized lipase, and glycerol, as well as in the solvents, was determined by the Karl Fischer method (720 KFS Titrino, Switzerland, using HYDRANAL titrant and solvents) (Xu, Fomuso, & Akoh, 2000).

2.9. Statistical analysis

The SPSS programme was used for data analysis (SPSS, 1989–2001). Analysis of variance and *t*-test were used to evaluate the significance and difference of data. Values were considered significant at the $P < 0.05$ level.

3. Results and discussion

3.1. Screening of solvents for the enzymatic glycerolysis of tuna oil

To select the most suitable solvent for the glycerolysis reaction system, the effect of organic solvents on the catalytic activity of the lipase was examined. The glycerolysis of tuna oil, with IM-AK as biocatalyst, was carried out in acetone, hexane, isooctane, *tert*-butyl methyl ether (MTBE) and their combinations. The results are shown in Fig. 1. It was found that MTBE gave the highest yield of MAG at 20.4 wt%, with yield of PUFA (EPA and DHA) of about 14.8 wt%. Previously, Kaewthong and H-Kittikun (2004) used the combination of an acetone/isooctane mixture (3:1, v/v) as solvent for glycerolysis of palm olein. Wongsakul et al. (2003) used acetone as organic solvent for alcoholysis of tuna oil by lipase PS-C. Moreover, Chang and Rhee (1991) used isooctane as the organic solvent for continuous glycerolysis of olive oil in CSTR. Therefore, the selection of solvent seems to be affected by many different issues. From information collected so far,

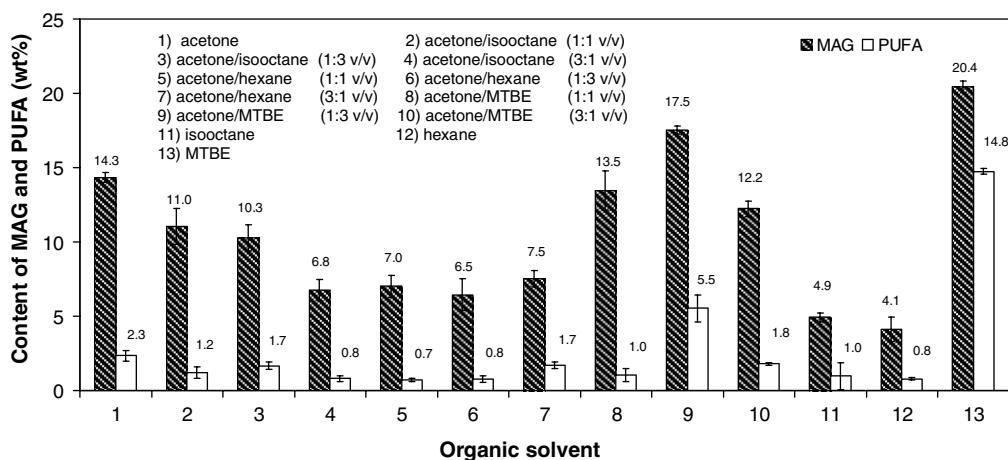


Fig. 1. Screening of organic solvents on MAG production with IM-AK. The reaction mixture contained 20 ml of 30% w/v tuna oil in organic solvents and 1.78 g glycerol with 4 wt% water. The amount of IM-AK used was 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h, PUFA-polyunsaturated fatty acids, here meaning EPA and DHA and MAG-monoacylglycerol. The Y-axis indicates the MAG weight content in the normalized lipid species profile analyzed by TLC/FID and PUFA weight content in the normalized fatty acid composition of the MAG fraction analyzed by GC.

the reaction is strongly dependent on the selection of lipases. Other issues, such as oil type, reactor selection, and reaction mechanism, might have effects as well. In this study, we decided to use MTBE for further study.

3.2. Effect of water content

Water content is recognized to be an important factor in transesterification reactions. A certain amount of water is necessary to preserve the catalytically-active conformation of the enzyme and to allow the formation of an acyl-enzyme complex. In contrast, excessive water causes acyl migration, leading to decrease in MAG yield (Wongsakul et al., 2003). Therefore, optimal water content is the foremost important factor that should be determined in the quite hydrophilic solvent. Initial water content of the glycerol in the range of 4–12 wt% was studied. The results are shown in Fig. 2. The highest yield of MAG of 20.7 wt%,

containing 15.7 wt% PUFA, was obtained when 4% water was added to the glycerol. When more than 4% water was added, the yield of MAG dropped gradually. This may be due to hydrolysis. Yamane, Kang, Kawahara, and Koizumi (1994) found that FFA content at equilibrium depended on the water concentration in the glycerol phase. This can eventually lead to decreases of MAG yields, as earlier mentioned.

3.3. Effect of substrate concentration in MTBE

In a solvent system, the concentration of substrate will eventually affect the reaction rate based on Michaelis–Menten kinetics, even though solvent can help create a homogeneous system. In order to select an efficient initial substrate (tuna oil) concentration for glycerolysis, the effect of tuna oil concentration was investigated. The results are shown in Fig. 3. The MAG yield increased with increasing

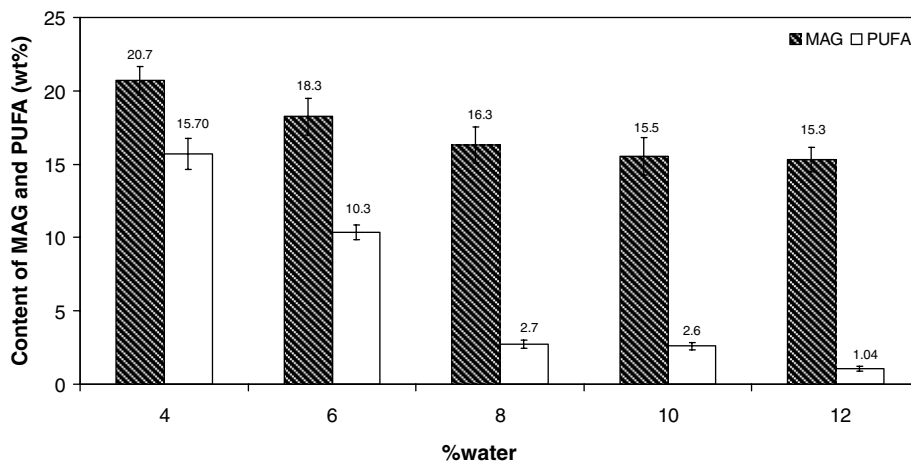


Fig. 2. Effect of water content in glycerol on MAG production by IM-AK. The reaction mixture contained 20 ml of 30% w/v tuna oil in MTBE and 1.78 g glycerol with various water contents. The amount of IM-AK used was 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h. For abbreviations and notes to Y-axis see Fig. 1.

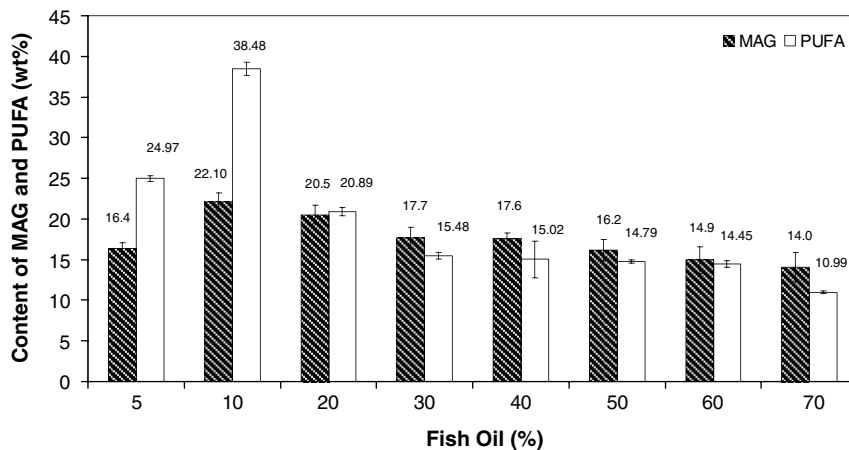


Fig. 3. Effect of fish oil concentration on MAG production by IM-AK. The reaction mixture contained various amounts of tuna oil in 20 ml of MTBE and 1.78 g glycerol with 4 wt% water. The amount of IM-AK used was 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h. For abbreviations and notes to Y-axis see Fig. 1.

concentration of tuna oil; also, PUFA content in MAG increased when tuna oil concentration was increased. At the concentration of 10% w/v of tuna oil in MTBE, with a mole ratio of glycerol to tuna oil of about 3:1, the best yield of MAG at 22.1 wt%, with a PUFA content of about 38.5 wt% was obtained after 24 h of incubation. When the concentration of tuna oil was below 10% w/v, the yield of MAG was decreased. Solvent plays multiple roles and has more than one function in the system. First, it makes a homogeneous system and increases mass transfer by reducing the viscosity of the system. However, solvent may increase inhibition of the lipase since it drives off water from the lipase structure. Certainly, addition of solvent decreases the amount of available substrate at the interface between the solvent and glycerol and hence decreases the MAG yield. The amount of glycerol also plays a role in reactions of the system. Glycerol in the mixture would create difficulty for the reaction system without solvent. On the other hand, Yang and Rhee (1991) suggested that glycerol could act as an effective stabilizer against thermal and solvent denaturation. However, Bornscheuer and Yamane (1994) showed that the optimum mole ratio of glycerol to palm olein for MAG production in the solid-phase system was 2.7:1, whereas, at a lower glycerol to TAG mole ratio (1:2), the main product of glycerolysis was diacylglycerols. Therefore, the amount of glycerol also affected the reaction equilibrium. In the present study, 10% w/v of tuna oil with ca. three-fold glycerol addition (mol) gave an optimal system for the glycerolysis reaction.

3.4. Effect of IM-AK loading

The effect of IM-AK loading on MAG production was determined. The results are shown in Fig. 4. Upon increasing the amount of IM-AK in the reaction mixture, the MAG production also increased. However, no benefit came from increasing IM-AK above 30 wt% of tuna oil.

Therefore, IM-AK at 30 wt% of tuna oil was used for further study.

3.5. Effect of temperature

Temperature plays two roles in the reaction system. First, higher temperature can reduce the viscosity as well as improve the substrate diffusion or its solubility. Second, enzymes usually have a temperature optimum. Therefore, an optimal temperature should be selected in terms of the overall performance of the reaction. The effect of temperature (30–50 °C) on MAG production from tuna oil was studied. When temperature was controlled at 30–45 °C, the MAG production increased with increasing temperature (data not shown). This result was a consequence of the increase in the reaction rate. In contrast, when increasing the temperature from 45 to 55 °C the yield of MAG was decreased. The temperature of 45 °C was considered an optimal temperature for the reaction system.

3.6. Stability of IM-AK in MTBE mixture

Stability of IM-AK in MTBE was studied at 4 and 45 °C. The results show that more than 88% and 80% of hydrolytic activity remained after incubation for 24 h, respectively (data not shown). However, Fukui, Kawamoto, Sonomoto, and Tanaka (1990) found that benzene was better for lipase stability, while it gave a moderate result for lipase activity. Kang and Rhee (1989) suggested that the immobilized lipase activity in a reverse-phase system decreased as the polarity of solvent increased. Kwon, Han, and Rhee (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. Stability is a very complicated issue for many lipases. It not only relates to the characteristics of a lipase

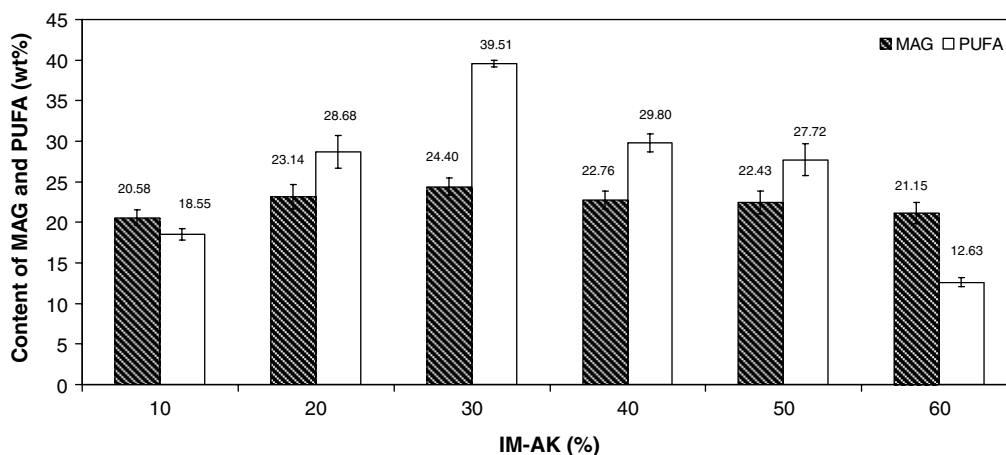


Fig. 4. Effect of IM-AK loading on MAG production. The reaction mixture contained 20 ml of 10% w/v tuna oil in MTBE and 1.78 g glycerol with 4 wt% water. The reaction was carried out at 300 rpm and 45 °C for 24 h. For abbreviations and notes to Y-axis see Fig. 1.

but also relates to the reaction system selected. More work is needed to improve the stability of the lipase used.

3.7. MAG production under optimal conditions

The optimal conditions for MAG production were decided as a tuna oil concentration of 10% w/v in MTBE, a mole ratio of glycerol to tuna oil of about 3:1, water content in glycerol of 4 wt% and using IM-AK at 30 wt% of tuna oil. The temperature was controlled at 45 °C. The reaction time course is given in Fig. 5. The yield of MAG was 24.5 wt% and PUFA (EPA and DHA) content was 56.0 wt% after 24 h of incubation. The reaction products were separated by TLC. The fatty acid profiles of each band (glyceride species) were determined by GC (Table 1). As shown, monodocosahexylglycerol and monooleylglycerol were the predominant MAG in the products.

3.8. Kinetics of the glycerolysis using both lipase AK and IM-AK

The kinetic constants (K_m and V_{max}) for glycerolysis of tuna oil with the non-immobilized lipase AK, as well as its immobilized form (IM-AK), were determined in MTBE by measuring initial reaction rates with varying amounts of tuna oil (50–500 mM). The results are shown in Fig. 6. The values of the kinetic constants were obtained from a Lineweaver–Burk plot. K_m and V_{max} of the original lipase AK were 39.3 mM and 11.4 mg MAG/min, respectively; while K_m and V_{max} of IM-AK were 19.5 mM and 2.7 mg MAG/min, respectively. IM-AK had smaller K_m and V_{max} values than had its original form, meaning that the catalytic capacity of the immobilized form was reduced. The potential allowed substrate concentration was also reduced. A similar result was obtained in hydrolysis of

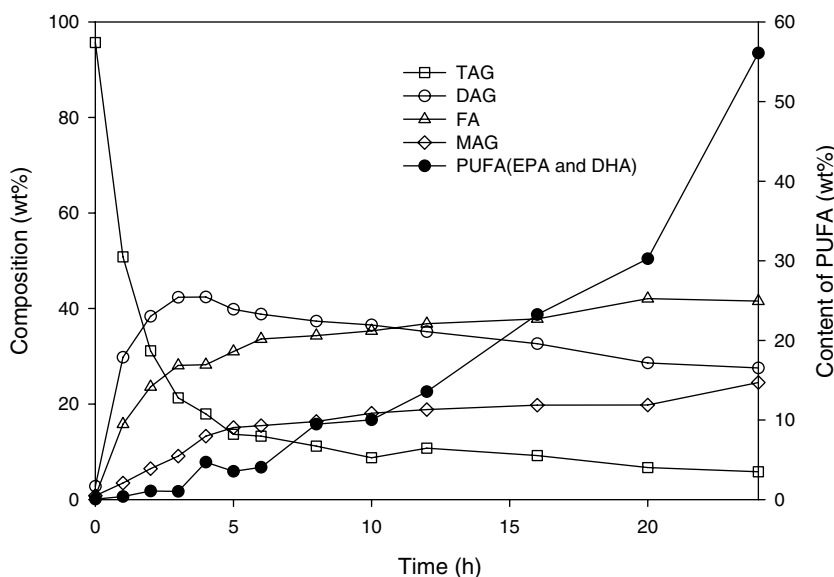


Fig. 5. Time course of glycerolysis by IM-AK in MTBE. The reaction mixture contained 20 ml of 10% w/v tuna oil in MTBE and 1.78 g glycerol with 4 wt% water. The amount of IM-AK was used 0.6 g (0.46 U/mg). The reaction was carried out at 300 rpm and 45 °C for 24 h. For abbreviations see Fig. 1.

Table 1
Fatty acid compositions of species of tuna oil and reaction product after TLC separation

Composition	wt%	Fatty acid composition (wt%) ^a							
		C14:0	C16:0	C16:1	C18:0	C18:1n-9	C18:2n-6	C20:5	C22:6
<i>Fish oil</i>									
TAG	99.3	4.2	30.2	4.7	9.3	16.3	2.6	4.2	27.9
MAG	0.4	14.6	58.9	20.3	–	5.9	–	–	–
FFA	0.3	14.1	75.3	9.9	–	–	–	–	–
<i>sn-2</i> ^b		0.7	–	16.5	5.7	8.0	13.9	21.2	33.4
<i>Product</i>									
TAG	5.8	10.8	6.1	43.4	–	0.7	20.4	1.4	17.0
1,2-DAG	18.9	3.1	26.5	5.1	5.8	6.2	18.4	9.2	25.3
1,3-DAG	9.2	7.9	5.8	41.6	4.1	12.4	13.1	2.2	12.6
MAG	24.5	1.7	12.7	2.5	5.4	6.6	14.8	7.7	48.2
FFA	41.6	3.4	3.9	27.8	5.2	15.4	7.4	7.9	28.5

^a Major fatty acids identified.

^b Fatty acid composition of tuna oil at the *sn-2* position before glycerolysis.

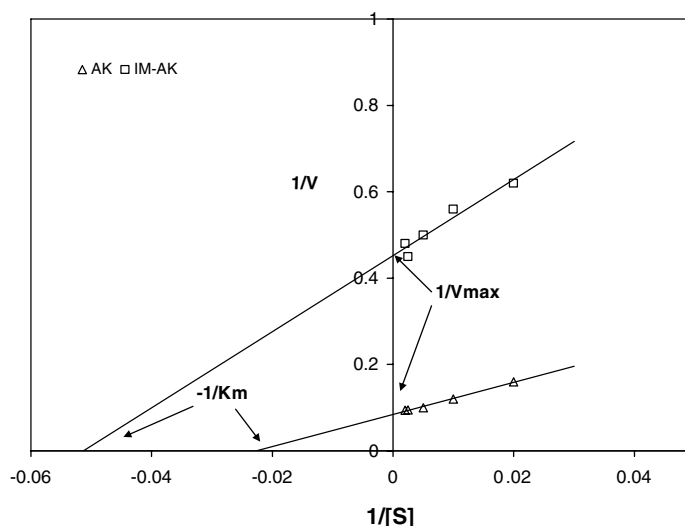


Fig. 6. Lineweaver–Burk plots for lipase AK and IM-AK-catalyzed glycerolysis of tuna oil.

olive oil by *Candida rugosa* lipase (Montero et al., 1993). In general, the immobilization of biocatalysts can lead to an activity reduction. It can also cause diffusional limitation of substrates in the immobilized biocatalyst system.

4. Conclusions

Glycerolysis of tuna oil was investigated to produce MAG rich in PUFA using immobilized lipase AK. The optimum conditions for MAG production were found to be 10% w/v of tuna oil in MTBE, glycerol to tuna oil ratio of ca. 3:1 mol/mol, water added to glycerol 4 wt% and the amount of IM-AK used 30 wt% of tuna oil. The temperature was controlled at 45 °C. Under these conditions, a yield of 24.6 wt%, containing of 56.0 wt% of PUFA (EPA and DHA), was obtained at 24 h. MAG were produced in good yield with high contents of PUFA, especially, EPA and DHA. Thus, a suitable product or starting material for synthesis of structured triglycerides can be obtained.

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