CHAPTER 4

PHYSICOENZYMATIC PRODUCTION OF MONOACYLGLYCEROLS ENRICHED WITH VERY LONG CHAIN POLYUNSATURATED FATTY ACIDS

4.1 Abstract

The aim of this study was to develop an efficient enzymatic glycerolysis system together with physical fractionation operation for the production of monoacylglycerols (MAG) containing polyunsaturated fatty acids (PUFA), especially, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) from tuna oil. The enzymatic glycerolysis system at 40-70 °C is unfortunately a multiple-phase system, which leads to lower reaction efficiency. A solvent system targeting on highly efficient reactions was therefore the focus. Based on previous progress, tertiary alcohols were selected. Therefore, a further screening of solvent mixtures involving tertiary alcohols was made and a number of mixtures gave a good performance. Novozym 435 was employed as a catalyst in glycerolysis following enzyme screening. Basic reaction parameters were thoroughly studied. In the batch reaction system with *tert*-Butanol (TB) as solvent, the following conditions for MAG production was recommended as: mole ratio of glycerol to tuna oil 4.0:1.0, the weight ratio of TB to tuna oil 2.0:1.0, 15 wt% Novozym 435 (based on glycerol and tuna oil), and no additional water. The temperature was controlled at 40 °C. Under these conditions, the yield of MAG was up to 90% after 3 h incubation. Crude MAG from glycerolysis of fish oil was fractionated with acetone and hexane as solvent to produce MAG with higher EPA and DHA content. Using acetone at 0 °C was the suitable condition for the fractionation. Under these conditions, the yield of MAG was about 50% but contained EPA and DHA up to 71% in comparison with around 30% in tuna oil.

4.2 Introduction

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; Ernst and Patrick, 1997). In food industry, MAG are widely used in bakery products, margarines, diary products, and confectionary because of their emulsifying, stabilizing, and conditioning properties. There are also important in cosmetic and pharmaceutical industries as drug carrier and for consistency improvement in creams and lotions. MAG for food industries are manufactured by chemical glycerolysis of fats and oils. High temperature (220-250 $^{\circ}$C) and inorganic alkaline catalysts are used to accelerate the reactions. Due to this, chemical processes are not quite suitable to those heat-sensitive oils and fats concerning nutritional or biological properties.

Lipase-catalyzed glycerolysis of fats and oils at atmospheric pressure and low temperature has attracted interest in both academia and industry, which is believed to be a practical alternative method for chemical methods in the production of commercial MAG. Several glycerolysis systems have been investigated with or without organic solvents, with immobilized or non-immobilized enzymes, and in microemulsion or other media.

Glycerolysis system with an immobilized lipase as catalyst is a three-phase system: a hydrophobic oil phase, a hydrophilic glycerol phase, and a solid enzyme phase. Since the more hydrophilic characteristic of the enzyme, glycerol often binds to enzyme particles so that the access of oil molecules to the enzyme is difficult. The mass transfer of glycerol is also limited. Because of this reason, the reaction efficiency is usually low even though the efficiency can be improved through optimization in a low range. It is reported that glycerol can be immobilized on silica gel so as to overcome the problem (Bornscheuer, 1995; Peng *et al*., 2000). The improvement is only minor, however, not to say difficulties in practical operations. Therefore, a solvent medium is actually an important solution to improve the homogeneity of the system.

The single solvent that could hold oil and glycerol in homogeneous system is actually very difficult to find. The hydrocarbon solvents were generally impossible for this

purpose. After evaluation, a few alcohols more than five carbons can be considered since they contain a polar-OH group and a nonpolar carbon chain. However, alcohols are naturally reaction competitors to glycerol, especially those primary alcohols. From the study of Damstrup *et al.* (2005) and Yang *et al.* (1992) the use of tertiary alcohols has no problem since the tertiary structure will have strong steric hindrance of the enzyme activity. Therefore, *tert*-butanol or *tert*-pentanol is promising for the glycerolysis system. Higher yield of MAG has been achieved with *tert*-alcohols in the glycerolysis system.

Polyunsaturated fatty acids (PUFA) containing MAG is interesting for many potential uses or applications in food, drug, or cosmetic production. Omega-3 PUFA have received much attention in recent year because of the health benefits they offer, including reduced risk of coronary disease, prevention of certain cancers, and improved immune function (Narayan *et al*., 2006; Ruxton *et al*., 2004). Their MAG forms may offer new possibilities in different applications. We have intended to synthesize MAG from fish oil with higher content of PUFA through alcoholysis of fish oil with 1,3-specific lipases (Klinkesorn *et al*., 2004; Wongsakul *et al*., 2003; Pawongrat *et al.*, 2007). The reaction strategy was mainly to produce 2-MAG since usually more omega-3 PUFA is located at 2-position of fish oil (Klinkesorn *et al*., 2004; Wongsakul *et al*., 2003).

One possible approach to obtain a dedicated fraction with different melting points is the application of fractionation. There are several approaches available to fractionate fats and oils including dry fractionation (without solvent), solvent fractionation (wet fractionation), and super-critical fluid fractionation (Lee and Foglia, 2000; Lee *et al*., 2001). Using these fractionation processes, lipid fractions with different nutritive properties can be produced since the melting behavior of lipids is strongly relates to the number of double bonds, meaning PUFA fractions and their derivatives can have very different melting properties from the rest fractions in the mixture.

Therefore, in this study, we designed the production of omega-3 MAG rich in PUFA (EPA and DHA) into a two step operation. In the first step, we intended to build an efficient glycerolysis system for the enzymatic production of MAG from fish oil using tertiary

alcohols or their mixtures with other solvents. As previously studied using other than fish oil, the high yield of MAG can be expected after optimization (Damstrup *et al*., 2005). With such a condition, the second step was targeted to fractionate the MAG containing PUFA. Normally, such MAG have much lower melting points than MAG containing saturated or monounsaturated fatty acids. Therefore, a physical fractionation system was also studied to isolate the omega-3 PUFA MAG from other MAG.

4.3 Materials and Methods

4.3.1 Materials

Crude tuna oil from Skipjack tuna head, with water content of 4.4% and free fatty acid contents of 0.36%, was provided from Chotiwat Industrial Co. Ltd. (Hat Yai, Thailand). The oil was prepared from crude tuna oil obtained from skipjack tuna heads by a conventional pressing method. The refined oil was achieved through degumming, neutralization, bleaching, and deodorizing. The major fatty acid compositions of the refined oil (wt%) was the following: C14:0, C16:0, C18:0, C18:1, C18:2, C20:5 and C22:6 (4.2, 30.6, 9.3, 17.3, 2.6, 6.7 and 29.0, respectively). The glycerol was analytical grade with 0.2% water. The properties of *tert*-butanol are boiling point 83 °C, melting point 25 °C , relative density (water=1) 0.8, octanol/water partition coefficient (log $P_{o/w}$) 0.4, and with colorless appearance. Commercially immobilized lipase, Novozym 435, from *Candida antarctica* lipase B, was obtained from Novozymes (Bagsvaerd, Denmark) and *Pseudomonas fluorescens* lipase (Lipase AK) was a gift from Amano Pharmaceutical Co. Ltd (Nagoya, Japan). Accurel EP-100, a microporous polypropylene powder (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.

4.3.2 Preparation of the immobilized lipase

Accurel EP-100 (10 g) was added to 100 mL of 0.1M phosphate buffer (pH 7) containing approximately 100 U/mL Lipase AK and the mixture was stirred with a magnetic bar at 100 rpm for 30 min. Afterward, 100 ml of 0.1 mol/L phosphate buffer (pH 7) was added and the suspension was filtered through a Buchner funnel by vacuum. The immobilized enzyme (IM-AK) was washed with 100 mL of buffer to remove the soluble enzyme.

According to Lee and Rhee (1993), for the water activity, the immobilized enzyme were adjusted by different method (dried in vacuum (a_w=0.389), acetone washing (a_w=0.019) and equilibrated over saturated salt solution of LiCl (a_w=0.113)) and incubated separately in desiccator to obtained a defined initial water activity. Equilibration was performed for at least 16 h at 25 °C.

4.3.3 Enzymatic glycerolysis of tuna oil

The mixture of 10 g of tuna oil with required amounts of glycerol and amount of *tert*-Butanol (TB) were incubated in a capped 25-mL flask at the designed conditions under 400 rpm shaker. The reaction was initiated by the addition of lipases. At selected intervals, 0.25 mL of reaction mixture was withdrawn and the lipase was removed by filtration and the solvent was removed by vacuum. All samples were stored at -20 °C before analysis. Experimental repeatability for batch reactions was conducted through three experiments under the following condition: temperature 45 °C, glycerol/tuna oil molar ratio 4.5:1.0, TB/tuna oil 2.2:1.0 (w/w), 15 wt% Novozym 435 (based on oil and glycerol), and no additional water.

4.3.4 Fractionation of PUFA-MAG from reaction mixture of glycerolysis of tuna oil

The product collected after reaction under the optimal conditions is subjected to solvent removal under vacuum. The product in this stage is named as crude MAG. The crude MAG in 0.1 g was dissolved in different solvents or mixtures with 30 mL. Acetone and hexane were commonly used solvents in literature and industrial uses. Therefore, these two solvents were selected together with one mixture between the two solvent in 50/50 (v/v). The fractionation was

conducted under different temperatures. Based on melting points of different MAG fractions, the following three temperatures were selected for evaluation including 10, 4, and 0 °C. The fractionation was conducted in a selected solvent and temperature for 3 h. Afterwards the samples were then centrifuged at the same temperature for 30 min under 10,000 rpm. The supernatant was removed and the solids were washed several times with the same solvent cooled to the same temperature. The liquid parts were collected together and the solvents were removed by a vacuum evaporator for both solids and liquids. The two fractions were then weighted and used for further analysis.

4.3.5 Analysis of acylglycerols by TLC-FID

The components of oil phase were analyzed with a thin-layer chromatography with flame ionization detector (TLC/FID)(IATROSCAN MK5, Iatron Laboratories Inc., Tokyo, Japan) for the content of TAG, 1,2(2,3)-DAG, 1,3-DAG, MAG and free fatty acids (FFA) (Kaewthong and H-Kittikun, 2004). The samples diluted in chloroform/methanol (2.0:1.0 v/v) was spotted onto the chromarod and developed for 35 min in a mixture of benzene/chloroform/acetic acid (50:20:0.7, v/v/v) as developing solvent. After developing and drying, the rods were subjected to scanning with FID. Standards were used to identify the peaks. The peaks areas were normalized and used for evaluation of reactions. Triplicate analysis was conducted and the average was used to be reported in this paper.

4.3.6 Analysis of fatty acid compositions

The fatty acid compositions of acylglycerol 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 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 μL), for 10 min at 60 °C. The methyl esters were extracted with *n*-hexane (300 μL) for 1 min. The *n*-hexane layer was washed with 200 μL distilled water and dried over anhydrous sodium sulfate. Analysis was carried out

with a Perkin-Elmer Autosystem XL-GC gas chromatograph (Perkin-Elmer Corporation, Norwalk, CT) on a FFFAP column (PERMABOND-FFFAP DF-0.25, 25m×0.25mm *i.d.*, MACHEREY-NAGEL, Germany). The carrier gas used was helium set at a flow rate of 0.5 mL/min (15 psi) and operated in a spit ratio of 50:1. The temperature was started from 150 °C for 0.50 min and increased at the rate of 4 °C/min to 170 °C, followed with the rate of 5 °C/min to 195°C, and further with the rate of 10 °C/min to and 215 °C the temperature was kept at 215 °C for 14 min. Injector and detector temperatures were 250 °C (Joseph and Ackman, 1992). Response factors were determined using a standard mixture of FAME. Duplicate analysis was carried out for all the analysis and the average was used in the paper.

4.3.7 Statistical analysis

The SPSS program analysis 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 $P < 0.05$ level.

4.4 Results and Discussion

4.4.1 Screening of lipases

Enzyme characteristics can have determinant functions for the product development and process development. In recent progress of enzymatic production of MAG under solvent systems, Novozym 435 was recommended under the tertiary solvent system (Damstrup *et al*., 2005; Yang *et al*., 2005). Kaewthong and H-Kittikun (2004) was concluded in a list of solvent screening that the immobilized Lipase AK showed good activity as well. To find an appropriate catalyst for aimed MAG, two immobilized lipases were used in experiments: Novozym 435 from *Candida antarctica* lipase (nonspecific) and Lipase AK from *Pseudomonas fluorescens* lipase (1,3-specific lipase). Under TB and hexane media, experiments were conducted at the ratio of organic solvent to tuna oil 2.2/1.0 (w/w), 15 wt% lipase load, 4.5:1.0 (mol/mol)

glycerol/tuna oil, 45 °C, and reaction time 8 h. The results showed that glycerolysis of tuna oil of both enzymes was slow under hexane medium with very low TAG conversion (less than 20%), while in TB much more MAG were formed (Figure 16). Under TB, Novozym 435 showed higher activity with 90% yield of MAG, while Lipase AK gave only 70% with higher amount of FFA (21%) than Novozym 435 (Figure 17). The study was further conducted concerning the pretreatment of the IM-AK to see if the performance can be further improved in comparison with Novozym 435. The reason is that it will be very interesting to have a cheaper alternative to Novozym 435 but with similar performances, especially with a lower FFA content as well. Therefore, the IM-AK was pre-dried in three different ways, vacuum drying, acetone precipitation, and salt conditioning (Figure 18). As seen from the figure, once water content is down, the enzyme activity was decreased as well for the immobilized enzyme, meaning the enzyme was water dependent. This implies that the lipase needs higher amount of water to maintain the activity, but such a high amount of water will lead to the stronger hydrolysis reaction so as to form higher amount of FFA. In general, some minimal water content (critical) is needed to activate enzymes, but different lipases can have very different minimal water requirement. Novozym 435 was demonstrated to have less water dependence (Piyatheerawong *et al*., 2004). As a general conclusion, Novozym 435 was selected for further process studies. Since its low water requirement, the process can have a big benefit, that is, a lower free fatty acid content in the products. This is a very important issue for industrial applications since higher FFA content will lead to the loss of oils as well as the difficulty of the process.

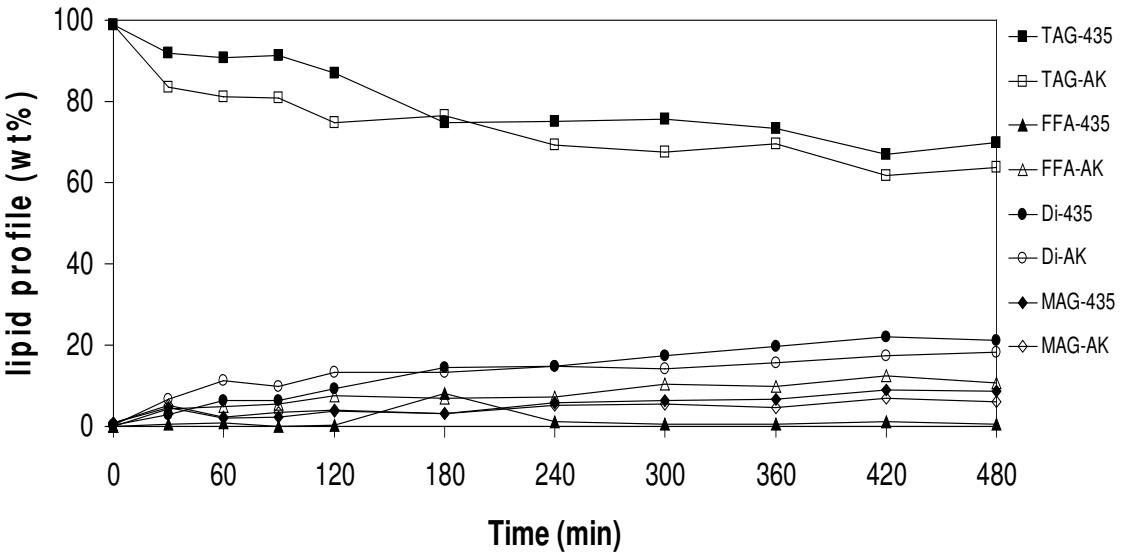

Figure 16. Glycerolysis of tuna oil in hexane with different immobilized lipases. Reaction conditions: temperature 45 °C, glycerol/tuna oil molar ratio 4.5:1.0, tert-butanol/tuna oil 2.2:1.0 (wt/wt), 15 wt% lipase (based on total substrates), and no additional water. *Abbreviations:* TAG (triacylglycerols), DAG (diacylglycerols), MAG (monoacylglycerols), FFA (free fatty acids)

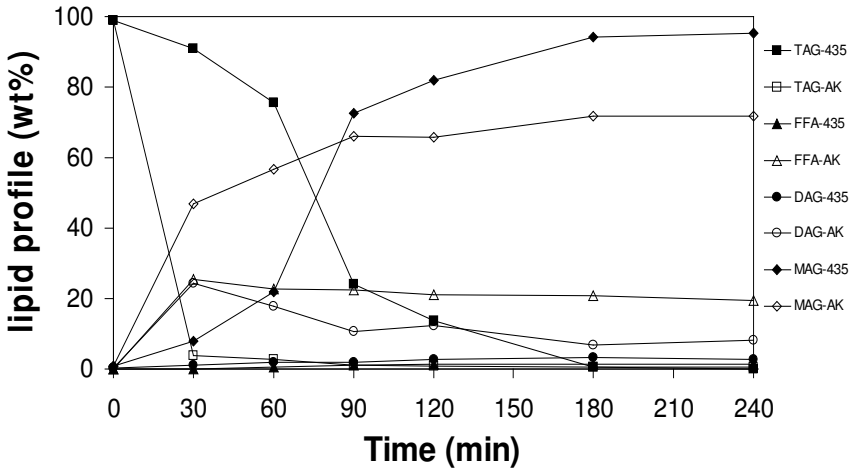

Figure 17. Glycerolysis time courses of tuna oil in *tert*-butanol. Reaction conditions: temperature 45 °C, glycerol/tuna oil molar ratio 4.5:1.0, tert-butanol/tuna oil 2.2:1.0 (wt/wt), 15 wt% lipase (based on total substrates) and no additional water.

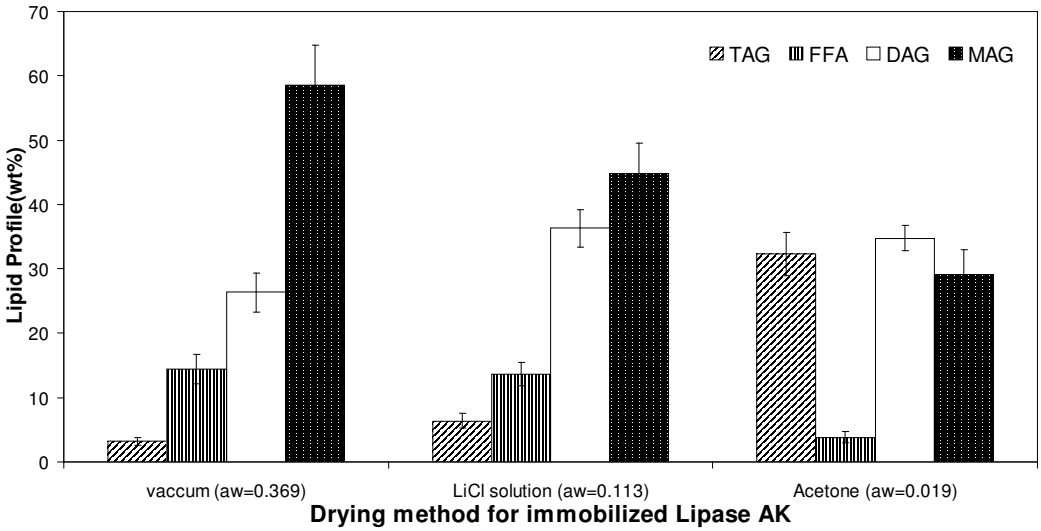

Figure 18. Effect of drying method for immobilized lipase AK on the glycerolysis of tuna oil in *tert*-butanol. (a). dry with acetone (b) adjusted water content with a_W = 0.113 by saturated salt (LiCl) (c). dry in vacuum. Reaction conditions: temperature 45 °C, glycerol/oil molar ratio 4.5:1.0, *tert*-butanol/oil 2.2:1.0 (w/w), 15 wt% IM-AK (based total substrates) and no addition water. See Fig. 15 for abbreviations.

4.4.2 Optimization of glycerolysis of tuna oil with Novozym 435

4.4.2.1 Effect of solvent mixtures for glycerolysis of tuna oil

As demonstrated in a few recent publications (Damstrup *et al.,* 2005; Yang *et al.,* 2005) tertiary alcohols are suitable solvents for the efficient glycerolysis system with very short reaction time but high MAG yields. However, *tert*-butanol (TB) has a high melting point (25-26 °C) while *tert*-pentanol (TP) is much more expensive (2-3 fold higher than TB). Therefore, a mixture is a choice for practical and cost-effective processes. For that thinking, the mixtures from the two solvents as well as mixtures with hexane were evaluated for the reaction system. The glycerolysis reaction was carried out in such solvent mixtures as shown in Figure 19. Tertiary alcohols and their mixtures gave higher yields of MAG in general, even though there were slight differences between each other. Low amount of hexane (20%) also gave reasonably

good results, but higher amount of hexane led to a lower yields of MAG. Yields of 90-95% were occurred in mixtures of TB/hexane (down to 20% v/v hexane) and TB/TP in vary ratios (20:80, 50:50, and 80:20 v/v). This offers a variety of possibilities of solvent selection in practical uses. To simplify the study, TB was selected for the following experiments.

4.4.2.2 Effect of enzyme loading

The amount of enzyme used is a crucial economical factor for successful industrial applications. The effect of enzyme loading on glycerolysis of tuna oil with glycerol was examined with various amount of enzyme rage from 5 to 25 wt% based on glycerol and tuna oil. The results are shown in Figure 20. Enzyme load more than 10% resulted in little increase of MAG yield and no increase of MAG yield was obtained after 15% Novozym 435. For the economic point of view, 10-15% enzyme load is enough for the maximum reaction performance and used for further reaction.

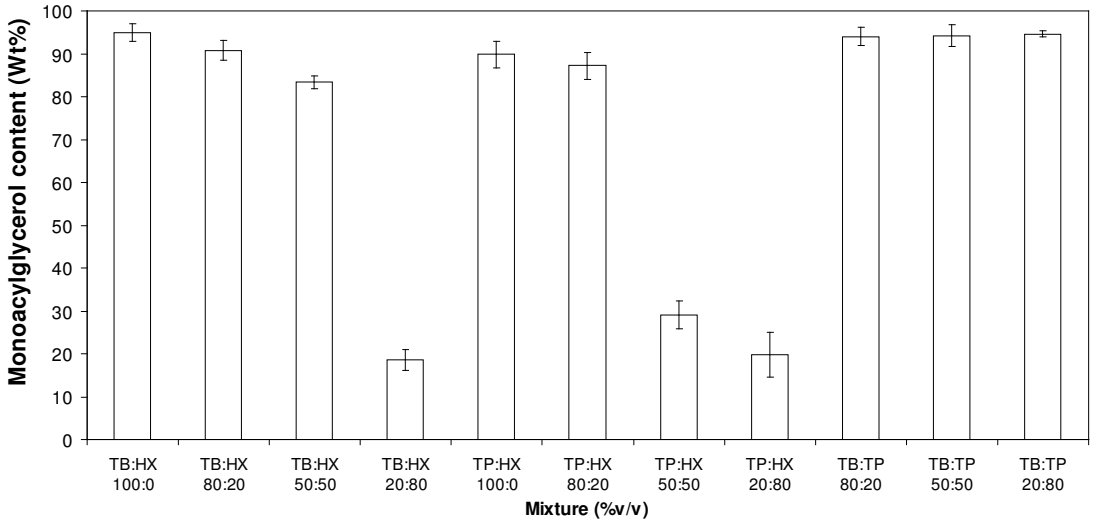

Figure 19. Effect of solvent mixtures on glycerolysis of tuna oil by Novozym 435. Reaction conditions: temperature 45 °C, glycerol/tuna oil molar ratio 4.5:1.0, *tert*-butanol/tuna oil 2.0:1.0 (w/w), reaction time 3 h, 15 wt% Novozym 435 (based on total substrates) and no additional water.

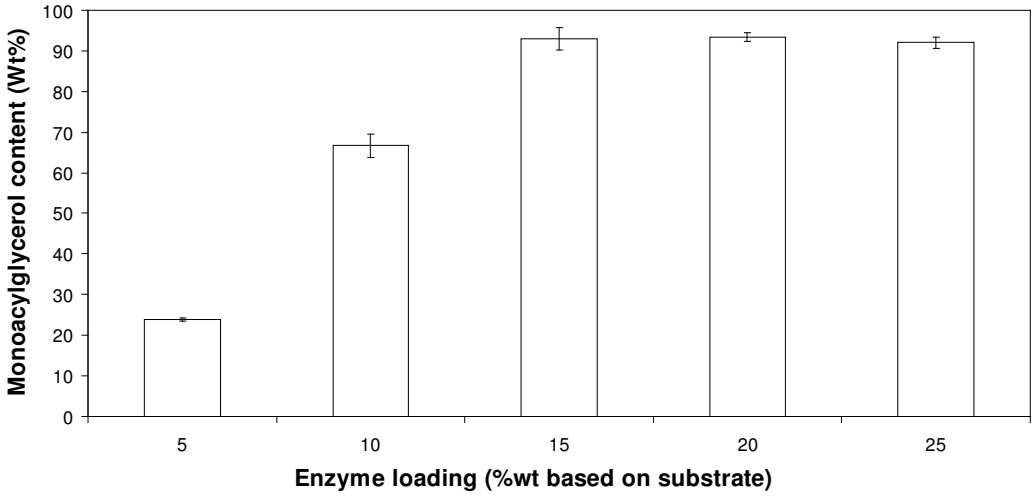

Figure 20. Effect of enzyme loading on glycerolysis of tuna oil in *tert*-butanol by Novozym 435. Reaction conditions: temperature 45 °C, glycerol/tuna oil molar ratio 4.5:1.0, *tert*-butanol/tuna oil 2.0:1.0 (w/w), reaction time 3 h and no additional water.

4.4.2.3 Effects of solvent amount and substrate ratio

The effect of substrate ratio can be in two ways. The increase of TB can improve the system homogeneity and stability as well as reduce the viscosity and mass transfer limitations. On the other hand, the medium will reduce concentration of substrates so as to reduce reaction rates as indicated by Michaelis-Menten equation. The effect was evaluated in the weight ratio of TB to tuna oil of 1.0:1.0, 1.5:1.0, 2.0:1.0, 2.2:1.0 and 2.5:1.0. The results are given in Figure 21. In general, the change from 2.0:1.0 to 2.5:1.0 had little difference in MAG yields (90-93%), especially after 2.0:1.0. FFA was increased, however, when high amount of solvent was used,

probably because of the water brought in by solvent. As conclusion, the ratio 2.0:1.0 was selected for the next studies.

Higher amount of glycerol can shift the reaction equilibrium to the MAG formation. However, the removal of glycerol after reaction may increase the process cost dramatically. Furthermore, the glycerol can affect the system polarity so as to influence the system stability and homogeneity. The experiments were conducted in the following molar ratios between glycerol and oil: 2.0:1.0, 3.0:1.0, 4.0:1.0, 4.5:1.0 and 5.0:1.0. The results are indicated in Figure 22. Less glycerol certainly produced less MAG since the maximum yield was also reduced. However, more glycerol did not produce more MAG since 4.0:1.0 to 4.5:1.0 (mol/mol) glycerol/tuna oil had little difference in MAG yield. This is not due to equilibrium problem. An optimal ratio must exist considering both effects of glycerol on reaction equilibrium and the system homogeneity. In this study, 4.0:1.0 (mol/mol) glycerol/tuna oil was taken for next studies.

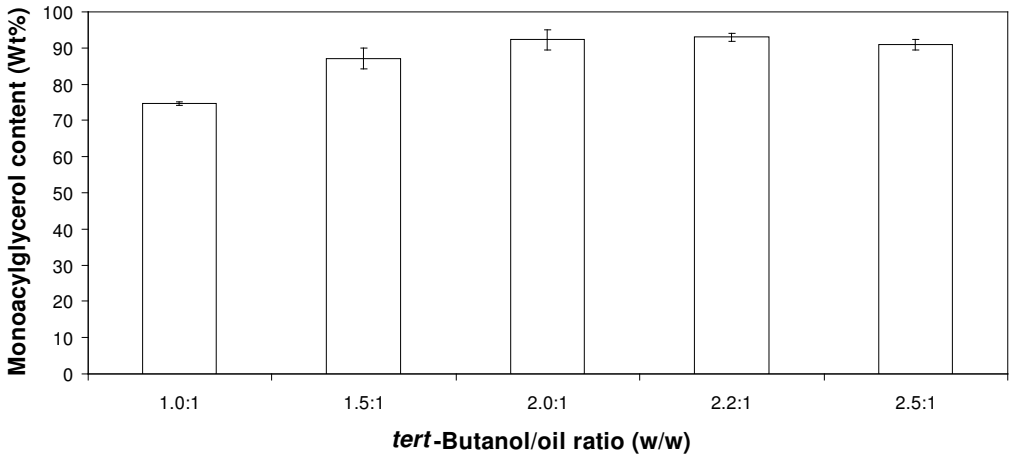

Figure 21. Effect of *tert*-butanol/tuna oil ratio (w/w) on glycerolysis of tuna oil in *tert*-butanol by Novozym 435. Reaction conditions: temperature 45°C, glycerol/tuna oil molar ratio 4.5:1.0, reaction time 3 h, 15 wt% Novozym 435 (based on total substrates) and no additional water.

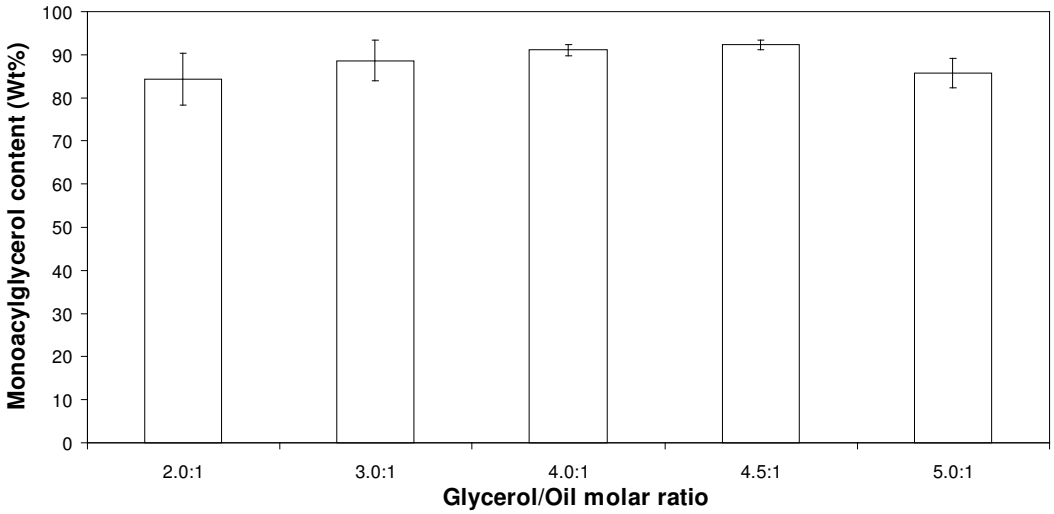

Figure 22. Effect of glycerol/tuna oil ratio on glycerolysis of tuna oil in *tert*-butanol by Novozym 435. Reaction conditions: temperature 45 °C, *tert*-butanol/tuna oil 2.0:1.0 (w/w), reaction time 3 h, 15 wt% Novozym 435 (based on total substrates) and no additional water.

4.4.2.4 Effect of temperature

Adequate temperature control is important for reproducible assay of enzymatic catalyzed reactions. Temperature also has multiple functions. In one way, higher temperature can improve mass transfer through viscosity reduction as well as molecular activity. It also can improve enzyme activity according to Arrhenius law. On the other hand, too high temperature may reduce the enzyme activity and stability. The temperature effect on the glycerolysis of tuna oil was carried out at 30, 35, 40, 45 and 50 °C. The result is shown in Figure 23. As the reaction temperature was increased, there was a trend in higher formation of MAG. However, the MAG yield was almost constant after 40 °C. In general, the differences from different temperatures were not highly significant. This might indicate that temperature is not crucial for such a system, but this could be also because the reaction equilibrium has reached. In general, 40 °C was used in the following studies since lower temperature was recommended with respect to the product quality.

4.4.2.5 MAG production under optimal conditions

The optimal conditions for MAG production were finalized as using TB as the medium, the mole ratio of glycerol to tuna oil with 4.0:1.0, the weight ratio of TB to tuna oil with 2.0:1.0, using 15 wt% Novozym 435 (based on glycerol and tuna oil), and no additional water. The temperature was controlled at 40 °C. Under these conditions, the yield of MAG of 90.8 wt% was obtained after 3 h incubation and the remained TAG was only 5.5 wt% (Figure 24).

The major fatty acid compositions of the MAG fraction after separating by thin layer chromatography were determined by gas chromatography as follows: C14:0, C16:0, C18:1, C18:2, C20:5 and C22:6 (3.5, 29.7, 8.6, 17.1, 3.6, 6.3 and 30.5 wt%, respectively). In general, the fatty acid composition had no significant difference from the original tuna oil, meaning such a reaction gave little fatty acid selectivity for the formation of MAG. This is a reasonable conclusion since the reaction was an interesterification process where positional and fatty acid

selectivity of the lipase will place no difference for the product formation. Furthermore, the lipase is commonly regarded as non-specific. Selective reactions may not exist.

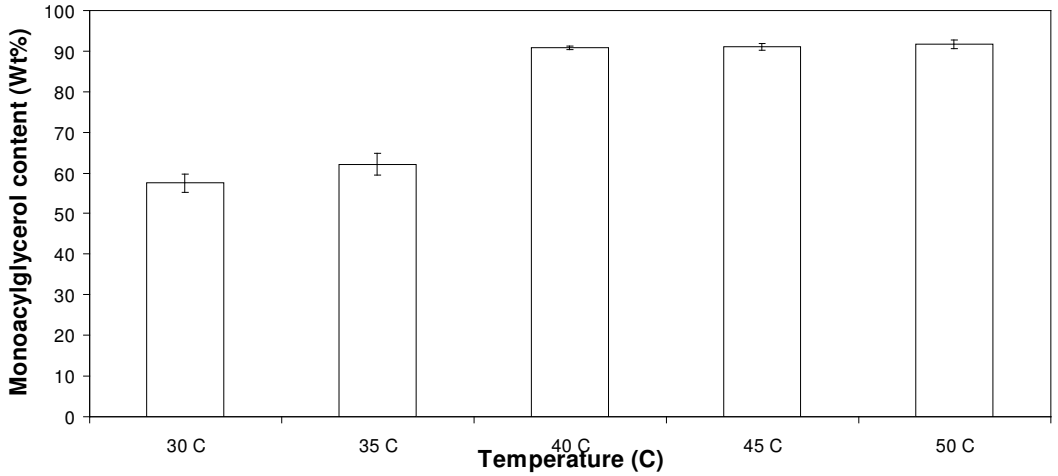

Figure 23. Effect of temperature on glycerolysis of tuna oil in *tert*-butanol by Novozym 435. Reaction conditions: glycerol/tuna oil 4:1.0 (mole/mole), tert-butanol/tuna oil 2.0:1.0 (w/w), reaction time 3 h, 15 wt% Novozym 435 (based on total substrates) and no additional water.

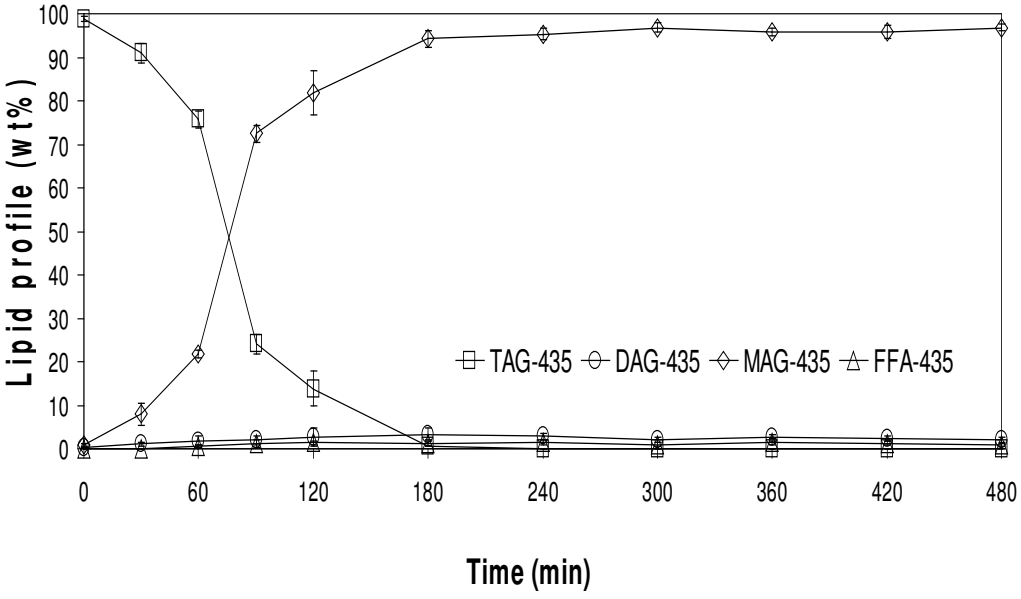

Figure 24. Time course of glycerolysis by Novozym 435 in TB. The reaction mixture contained the mole ratio of glycerol to tuna oil with 4.0:1.0, the weight ratio of TB to tuna oil with 2.0:1.0, reaction time 3 h, 15 wt% Novozym 435 (based on glycerol and tuna oil), and no additional water.

4.5 Fractionation of the reaction mixture from glycerolysis of tuna oil

Temperature fractionation of fats or oils or their derivatives can be regarded as a thermo-mechanical separation process and has been widely used in industry. Individual species (for example TAG or MAG) for a given material are selectively crystallized from the melt or liquid phase. During cooling of the liquid oil or melted material, the species with the highest melting point preferentially crystallized, resulting in solid phase within the system. For natural fats and oils, they are mostly complex mixtures of individual TAG that can contain from one to three different fatty acyl residues on their glycerol backbone. Because of this there is the large variation in the melting points of the TAG species, which complicates the fractionation process (Lee and Foglia, 2000; Lee and Rhee, 1993). For MAG product, single fatty acid residue is attached to glycerol backbone, the melting point profile is largely dependent on the fatty acids attached. Therefore, a simple separation of MAG with different fatty acids having different unsaturation is theoretically possible. In particular, EPA and DHA have 5-6 double bond, the melting points of their MAG will be largely different from the rest of fatty acid in tuna oil. For this reason, a fractionation system with solvent used (so-called wet fractionation) was studied.

Temperature is a critical issue for fractionation. Theoretically, MAG from C16:0 to C18:0 have a melting point (mp) in the range between 69-75°C under pure lipid phase and stable crystal structures. The C18:1 based MAG has a mp around 24°C and C18:2 based MAG around 9°C (Lee and Foglia, 2000) no information can be collected for EPA and DHA based MAG, but a melting point much lower can be expected. Once solvent applied, the melting behavior is completely different from pure lipid phase. Both solvent and concentration in the solvent can have effect on the crystallization temperatures. Based on applied wet fractionation in industry as well as literature (Lee and Rhee, 1993), three temperatures (0, 4 and 10°C) were selected for this study.

Type of solvents is another issue. Acetone and hexane have been commonly applied in industry and many previous studies (Lee and Foglia, 2000; Lee and Rhee, 1993). Considering the higher polarity of the material, the polarity of solvent may have effects on the fractionation process (Xu *et al.,* 2000). Therefore, both two solvents were selected for further evaluation including their mixtures.

The effects of solvent and temperature on yield and fatty acid composition were evaluated. Table 16 shows that percentage of EPA and DHA were higher in liquid fraction than solid fraction. The yield of liquid fraction was decreasing in general with the decreasing of temperature. Consequently, the EPA and DHA content in the MAG of the liquid fraction was increasing. The effect of solvent mixing was not very significant. There was a tendency that better fractionation was made in the acetone system than in the hexane system. Yang *et al*. (1992) found that the percentage of saturated fatty acid of stearin decreased with increasing solvent polarity, and percentage of EPA and DHA increased with solvent polarity and fractionation temperature. Lee *et al.* (2001) also reported that fractionation with acetone at low temperature was effective for enriching the monounsaturated fatty acid of chicken fat in the liquid fraction. Yokochi *et al*. (1990) reported that the winterization process with acetone at -20°C showed higher separation efficiency for tri-unsaturated TAG into liquid fraction than the other solvents.

In general, C16:0 and C18:0 were dramatically reduced in the liquid fraction and increased in the solid fraction. The oleic acid was also changing but not highly consistent. The crystallization of oleic acid based MAG may need further lower temperature. This needs further study. As commonly known, the yield of the liquid fraction will be decreasing with the decreasing of the temperature. The loss of the liquid fraction which is trapped by the solid, will be also increasing. With the present set-up, the liquid fraction with around 50% yield and containing around 70% EPA and DHA was obtained at 0 °C using acetone.

4.6 CONCLUSION

Physicoenzymatic production of MAG containing PUFA especially EPA and DHA was investigated. A few solvent mixtures were suitable for production of MAG by using

Novozym 435 as a catalyst in glycerolysis of tuna oil with glycerol. A few reaction parameters have been evaluated including solvent amount, substrate ratio, enzyme load, and temperature. The yield of MAG up to 90.8% could be achieved with suitable conditions. The temperature fractionation under different solvents was evaluated in order to produce a fraction with higher content of EPA and DHA. Temperature was a critical parameter for effective fractionation. A fraction under 0°C fractionation could be obtained with around 70% EPA and DHA and in a yield of around 50%. A possibility of enriching the EPA and DHA into MAG has been built.

Table 16. Major fatty acid compositions of crude MAG fraction after solvent fractionation at low temperature from the mixture of hexane and acetone*

Temperature (°C)	**Solvent mixture (hexane /acetone, v/v)**	**Yield of liquid and solid fractions (wt%)****		**Major fatty acid content (wt%)**						
				C14:0	**C16:0**	**C18:0**	**C18:1**	**C18:2**	**C20:5**	**C22:6**
10	100/0	L	84.9	2.9	30.3	8.3	17.1	3.7	4.9	30.9
		S	15.1	6.6	38.6	16.6	14.6	0.7	10.1	10.3
	50/50	L	69.8	3.7	23.3	5.0	20.2	3.0	7.0	37.4
		S	30.2	2.7	45.1	15.3	24.8	3.7	2.7	5.5
	0/100	L	63.1	3.9	15.2	3.2	16.9	4.2	7.3	47.5
		S	36.9	1.6	49.2	15.6	20.3	3.3	3.9	5.5
4	100/0	L	67.9	2.5	13.2	4.0	21.1	4.0	5.6	48.6
		S	32.1	6.7	61.5	21.2	4.5	0.5	2.2	1.8
	50/50	L	71.6	2.2	15.4	4.6	19.8	4.1	5.9	46.3
		S	28.4	6.7	66.4	16.6	4.7	1.1	1.4	2.2
	0/100	L	65.3	3	4.9	3.5	22.4	6.2	8.5	50.3
		S	44.7	3.9	72.3	13.8	3.2	0.4	3.1	1.2
0	100/0	L	52.8	1.7	4.2	0.3	23.7	5.1	8.0	56.0
		S	47.2	4.2	66.3	17.2	7.7	0.1	2.0	1.8
	50/50	L	50.6	1.9	0.3	0.4	20.8	4.9	8.6	60.5