CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

Thailand has become the world's largest exporter of canned tuna and largest importer of fresh and frozen tuna. Canned tuna exports accounted for 77% of total canned seafood exports of Thailand in 2006, which was about 456,000 metric tons of tuna and valued about $1,000 million (Nidhiprabha *et al.,* 2006). Tuna processing produces 30-35% product yield. 20-35% of solid waste or by-product is in the form of low values applications, such as fishmeal or pet food (Yamprayoon and Virulhakul, 1999). One possible way to use the tuna wastes is extraction of oil for human consumption.

There are a numbers of processes that can be used to convert raw tuna or tuna waste into oil. These consist of wet rendering, dry rendering, hydrolysis, and solvent extraction. The wet rendering process widely used in the factories producing fish oil. The major steps of this process are cooking, pressing and separating (Bimbo, 1990). Crude oil from precooked and non-precooked tuna waste (Skipjack's heads) could be separated by a wet rendering method, yielding 2.8 and 4.8%, respectively (Chantachum *et al*., 2000). Another processing technique is solvent extraction. Oil from tuna wastes could be obtained by using solvent extraction. The results showed that eyes and heads of tuna contained 14-18% oil while that form the gut contained only 2.8-3.9% oil (Kungsuwan *et al*., 1996).

Tuna oil and other marine oils such as squid oil are rich in *n*-3 polyunsaturated fatty acids (*n*-3 PUFA), especially eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acids (DHA, C22:6) that have been shown to be important for prevention a range of human diseases and disorder (Uauy and Valenzuela, 2000; Harris, 2001; 2004). Due to there beneficial health properties, *n*-3 PUFA have great potential as functional food ingredients. Generally, tuna oil has complex mixtures of fatty acids with varying chain lengths and degree of unsaturated. Over consumption of tuna oil to obtain *n*-3 PUFA may increase the intake of cholesterol and

other saturated fatty acids by consumers (Haagsma *et al.,* 1982; Shahidi and Wanasundara, 1998). Therefore, concentration or enrichment of tuna oil in *n*-3 PUFA could help avoid the consumption of saturated fatty acids.

Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) are found widely in animals, plants, and microorganisms. Microbial lipases have increasing important in industrial applications (Wong, 1995). In addition to hydrolysis of fats and oils, lipases are also capable in catalyzing the reverse reaction of hydrolysis (esterification), and tranesterification reactions in the organic solvent or near anhydrous condition. Lipases are currently used as biocatalyst for the modification of existing lipids, to synthesize new lipid, such as structured triacylglycerides. The applications of lipases have increased impressively in recent years. The main applications are in the enantioselective synthesis of precursors of pharmaceutically active compounds and the conversion of natural fats and oils into high value products such as cocoa butter equivalent, oils enriched omega three fatty acids (Keawthong and H-Kittikun, 2000).

The family of oil enriched in omega three fatty acids also know as *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) has attracted the interest of experts in nutrition because of their beneficial effects on human health (Garcia *et al.,* 2001). For example, Siscovick *et al.* (1995) found that the risk of primary cardiac arrest was inversely correlated with dietary intake of *n*-3 fatty acid. Moreover, dietary intake of *n*-3 fatty acids, such as EPA and DHA, is effective in reducing in an incidence of coronary heart disease, arteriosclerosis, cancer, diabetes, high blood pressure, and depression (Thude *et al.,* 1997). DHA has been shown to be essential in the proper functioning of eyes and the nervous system, whereas EPA serves as precursor of a large group of lipid mediators that have linked to the prevention and/or treatment of disease processes. The extensive clinical results of Siscovick *et al.* (1995), demonstrate that dietary intake of approximately 5.5 g of *n*-3 fatty acids (EPA and DHA) per week from seafood is associated with a 50% reduction in risk of primary cardiac arrest. Consequently, oils containing *n*-3 PUFA have been in high demand for pharmaceutical and dictator purposes. Many investigations have been actively undertaken in search of methods for concentrating *n*-3 polyunsaturated fatty acids from marine oils.

However, the PUFA are highly labile and sensitive to heat and oxidation. The all *cis n*-3 structure is prone to partial destructure by oxidation, double bond migration, *cis-trans*

isomerization, or polymerization (Jonzo *et al.,* 2000). Modification of lipid from marine oil normally involves lipase-catalyzed process. First, the application of lipase as biocatalysis in processes such as hydolysis of lipids (Knezevic *et al.,* 1998), triacylglycerol modification (Jonzo *et al.,* 2000), and ester synthesis (Ward *et al.,* 1997) is attractive because lipase efficiency is high and only a low amount of catalyst is required especially when immobilized. From marketing view, immobilization technology offer many advantages such as ability to reuse the catalyst, an easy separation of the product from the enzyme by filtration and a great potential to run continuous processes on large scale. Secondly, lipase-catalyzed reactions are carried out at mild conditions and lead to the formation of specific compounds, which are easily isolated with out molecular distillation in comparison to equivalent chemical processes, which usually occur at higher temperature. Therefore, the use of lipases to enrich PUFA in tuna oil will be investigated.

Literature Review

1. Lipases

Lipases (triacylglycerol ester hydrolase, E.C. 3.1.1.3) are ubiquitous enzymes whose biological function is to catalyze the hydrolysis of triacylglycerols (TAG) into monoacylglycerols (MAG), diacylglycerols (DAG), free fatty acids (FFA) and glycerol. In addition, lipases are capable of catalyzing a variety of alternative enzymatic reactions, many of which have considerable commercial potential (Montero *et al*., 1993). The combination of broad substrate range and high selectivity make lipases an ideal catalyst for organic synthesis (Bornscheuer and Kazlauskas, 1999).

1.1 Sources of lipases

There are a variety of lipases that could be used for the production of structured lipids because of their regiospecificity or stereospecificity. Although widespread in nature, lipases have only recently become available in large quantities for industrial purposes. Some of the major application possibilities have only been established within the last 15-20 years, due to the new discovery that lipases can be used in microaqueous systems and at high temperatures up to 70-80°C (Zaks and Klibanov, 1984).

Microbial lipases have been the most attractive enzymes as they are thermostable, without co-lipase requirements, and of different specifications, which have been extensively described (Eigtved *et al*., 1987). Using genetic engineering, recombinant or mutant lipases may be produced in a suitable microorganism such as *Escherichia coli* with a more directed approach towards desired properties and efficient expression. A list of regiospecific lipases is given in Table 1.

Table 1. Specific lipases for the production of specific structured lipids

Lipase source	**Fatty acid specificity**	**Regiospecificity (*sn*)**
Aspergilus niger	S, M, L	1, 3>>2

Candida lipolytica	S, M, L	1, 3>2	
Thermomyces lanuginosa	S, M, L	1, 3>>2	
Mucur javanicus	M, L>>S	1, 3,>2	

Table 1. (Continued)

Rhizomucor meihei	S>M, L	1>3>>2	
Pancreatic	S>M, L	1, 3	
Pre-gastric	S>M>>L	1, 3	
Penicillium camembettii	MAG, DAG>TAG	1, 3	
Penicillium roquefortii	S, M>>L	1, 3	
Rhizopus delemar	M, L>>S	1, 3>>2	
Rhizopus javanicus	M, L>S	1, 3>2	
Rhizopus japonicus	S, M, L	1, 3>2	
Rhizopus niveus	M, L>S	1, 3>2	
Rhizopus oryzea	M, L>S	1, 3>>>2	
Pseudomonas fluorescens	M, L>S	1, 3>2	
Pseudomonas sp.	S, M, L	1, 3>2	
Rhizopus arrhizus	S, M>L	1, 3	

Source: Xu *et al*. (2000)

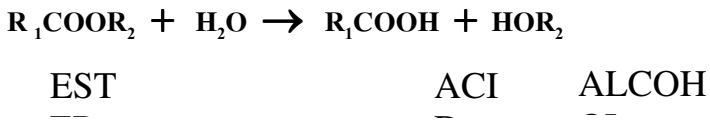
S – short-chain free fatty acids; M – medium-chain free fatty acids; L – long-chain free fatty acids

1.2 Lipase-catalyzed reactions

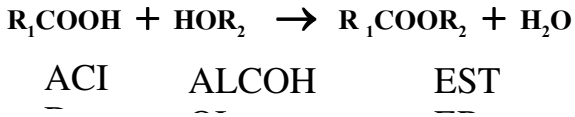
Lipases catalyze a series of different reactions. In fact, although they were designed by nature to cleave ester bonds of TAG with the concomitant consumption of water molecules (hydrolysis), lipases are also able to catalyze the reverse reaction under microaqueous condition, viz. information of ester bonds between alcohol and carboxylic acid moieties (ester synthesis). These two basic processes can then be combined in a sequential fashion to give rise to a set reaction generally termed interesterifications. Depending on the particular starting point in terms of substrates, one may have acidolysis (where an acyl moiety is displaced between an acylglycerol and a carboxylic acid), alcoholysis (where an acyl moiety is displaced between an

acylglycerol and an alcohol), transesterification (where two acyl moieties are exchanged between two acylglycerols) and aminolysis (where an amino group is displaced between an acylglycerol and an alcohol). All these processes are depicted in Figure 1 (Yamane, 1987; Balcao *et al*., 1996).

(1) Hydrolysis of Ester

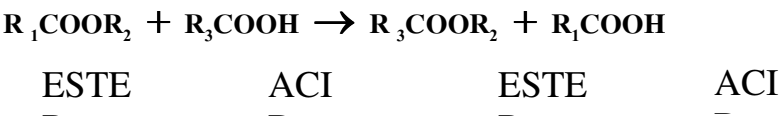


(2) Synthesis of Ester

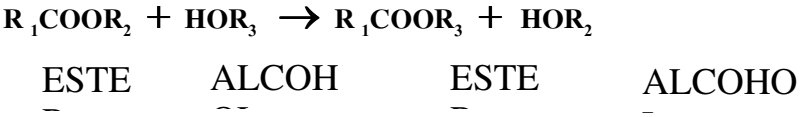


(3) Transesterification

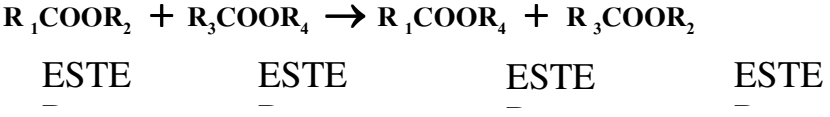
(3.1) Acidolysis



(3.2) Alcoholysis



(3.3) Ester Exchange (Interesterification)



(3.4) Aminolysis

Figure 1. Lipase-catalyzed reactions

Source: Yamane (1987)

1.3 Specificity of lipases

Because enzymes are specific to their substrates and operated at mild reaction conditions, they are preferred over chemical catalyst for the modification of lipids in trended for the modern consumers who demand more nature and less synthetic food products and additives (Akoh, 1996).

Based on specificity of lipases, Figure 2, the main group of lipases can be defined;

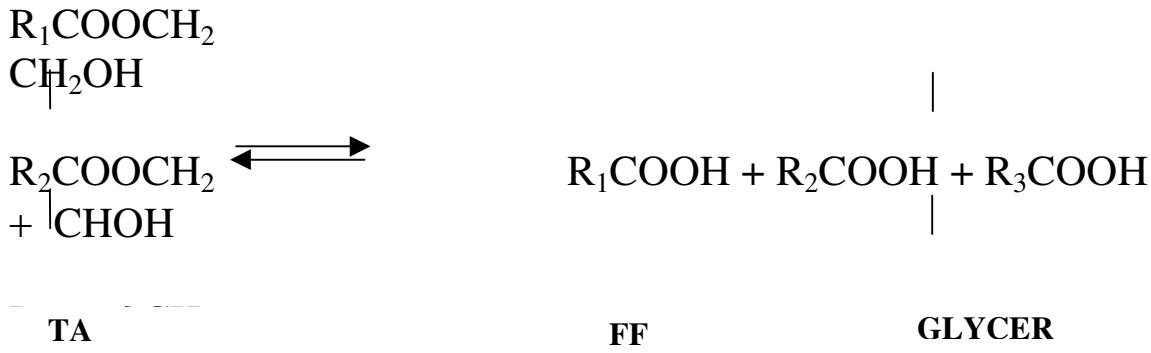
(i) random or **non-specific lipases**, which catalyze reactions at all positions on the glycerol molecule e.g. lipase from *Pseudomonas fluorescens* (Yamane *et al*., 1987), *Pencilium cyclopium* (Okumura *et al*., 1980), and *Candida cylindracea* (Seong *et al*., 1996).

(ii) *sn* 1,3-specific or **positional specific lipases**, which act preferentially at the *sn* 1- and 3-positions of glycerol molecule e.g., lipase from *Aspergillus niger*, *Rhizopus delemar* (Okumura *et al*., 1980) and *Mucor miehei* (Ergan *et al*., 1990; Pecnik and Knez, 1992).

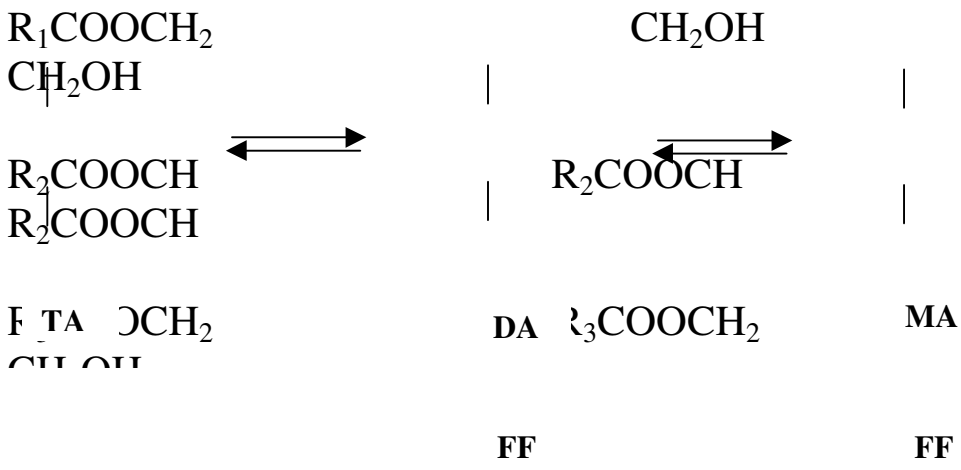
(iii) substrate specific lipases, which show specificity toward specific types of fatty acids e.g. lipase from *Yarrowia lipolytica* hydrolyzed triacylglycerides containing long-chain fatty acid (oleic acid, C 18:1*n*-9) better than triacylglycerides containing medium-chain fatty acid (capric acid, C 10)(Yahya *et al*., 1989). In addition, lipase from *Rhizopus japonicus* NR 40 not hydrolyzes triacylglycerides containing saturated fatty acids such as tristearin or tripalmitin (Suzuki *et al*., 1988).

The position specificity of lipases is usually retained when they are placed in organic solvents (Fomuso and Akoh, 1998; Wongsakul *et al*., 2004).

(1) Nonspecific lipases



(2) 1,3-specific lipases



(3) Fatty acid specific lipases

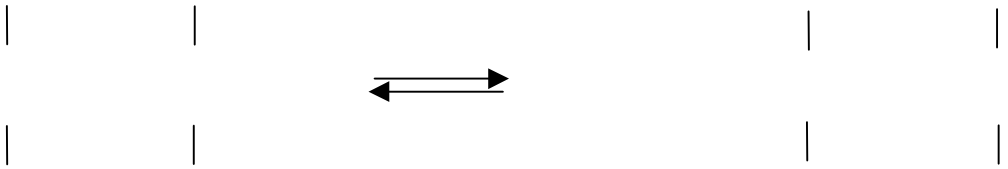


Figure 2. Specificity of lipases

Source: Macrae (1983)

2. Immobilized lipases

Lipases are soluble in aqueous solution (as a result of their globular protein nature), but their natural substrates (i.e. lipids) are not. Although the use of a proper organic solvent or an emulsifier helps overcoming the problem of intimate contact between substrate and enzyme, the practical use of lipases in such pseudo homogeneous reaction systems poses technological and economic difficulties. The former leads to constraints on the product level, because the final characteristics of the product depend on such post processing conditions as storage time and temperature. The latter leads to constraints on the process level, because the useful life of enzyme is restricted to the life-time of reactor. In both cases, part of the overall potential enzymatic activity is lost. If the lipase is immobilized, then it becomes an independent phase within the reaction system, which may be easily retained in the reactor via mechanical means with concomitant advantages in preventing contamination of the products and extending its useful active life (Balcao *et al*., 1996).

Immobilization of lipases from several microbial and animal sources has been performed by different methods, including covalent attachment to activated supports, entrapment with photocrosslinkable resins and poly (vinyl) chloride or colloid ion membranes, and adsorption on different materials such as ionic resins (Montero *et al*., 1993). Immobilizations by adsorption are usually carried out by incubation of the support and the enzyme in buffer or by precipitating the lipase from a buffer with acetone into a surface of the support (Mustranta *et al*., 1993). The activity yields of the immobilized lipases so far reported are considerably lower than those

reported for many other enzymes. The activity yield in immobilization of lipase is often only a few percent (Sarabok, 2000; Kaewthong and H-Kittikun, 2004).

2.1 Effect of support matrix

The catalytic activity of immobilized lipase is influenced by the properties of the support matrix and the method of immobilization (Mustranta *et al*., 1993). The support can affect the partitioning of substrates, product and water in the reaction mixture and, thereby, indirectly influence the catalytic properties of enzyme (Castro *et al*., 1999). When the lipases are immobilized on porous solid support, the highest retention of lipolytic activity is usually observed when the carrier possesses hydrophobic characteristics (Malcata *et al*., 1992).

The use of porous support material is recommended so that a suitable amount of lipase can be spread on a surface area without conformational changes. Comparative studies indicate that dramatic differences in activity are observed among lipases on supports of different materials. A good correlation was found between the water-attracting capacity (hydrophilicity) of the support and the reaction rate obtained with enzymes deposited on the support; low hydrophobicity resulted in high catalytic activity (Adlercreutz, 1991). It has been suggested that both ionic and hydrophobic interactions between the lipase and the surface are important for the non-covalent immobilization of lipase. A number of bacterial lipases can be immobilized in a rapid and strong fashion on octyl-agarose gels. It has been shown that adsorption rates in absence of ammonium sulfate are higher than in its presence, opposite to the observation for the typical hydrophobic adsorption of proteins, and these immobilized lipase molecules show a dramatic hyperactivation. It is suggested, from the results, that lipases recognize theses well-defined hydrophobic supports as solid interfaces and they become adsorbed through the external areas of the large hydrophobic active centers of their open and hyperactivated structure (Bastida *et al*., 1998). It was found that interesterification activity was better using resin and celite as carrier compared to polypropylene (EP 100) and Hyflo Super Cel was the least suitable support for interesterification of triolein and caprylic acid (Soumanou *et al*., 1998).

2.2 Effects of solvents

The solvents used in immobilization have been shown to have the great effect on the applicability of a covalently immobilized *Rhizopus* species (Mustranta *et al*., 1993). In the study of Castro *et al*. (1999), porcine pancreatic lipase was absorbed on celite, either by direct binding from aqueous solution or deposition from aqueous solution by the addition of organic solvent. Better activities of lipase were displayed when the immobilized lipase was prepared in an aprotic solvent such as hexane. It appeared that the use of solvent, which has lower polarity (higher log P value), was capable of creating a specific microenvironment around the enzyme that may enhance its stability and activity.

Non-aqueous solvents can have a variety of effect on enzymes. A layer of bound water (or water hydration shell) plays a key role in maintaining the structural integrity and catalytically active conformation of lipases since it affect intramolecular salt bridges and hydrophobic interactions. Therefore, the existence of trace amount of water in the immediate vicinity of lipase is a prerequisite for successful functioning of a lipase in a microaqueous system, in which lipase-catalyzed interesterification or ester synthesis reactions are occurring (Malcata *et al.,* 1992).

Mustranta *et al*. (1993) studied the immobilization of lipases from *Candida cylindacea*, *Aspergillus niger*, and *Psuedomonas fluorescens* by adsorption on anion exchange resin and diatomaceous earth in buffer or hexane. It was found that immobilized *C. cylindracea* was more active in n-hexane. For *A. niger* lipase, hexane was also a better reaction medium and anion exchange resin was a better support than diatomaceous earth.

2.3 Effect of water on enzyme activity in organic media

In particular, the effects of water associated with the enzyme have been studied in some detail. It appears that different enzymes display vastly different requirements with regard to how much bound water they need to maintain an appreciable level of catalytic activity in non-aqueous media. It has also been shown that the stability of enzyme markedly increased under low water condition, making it possible to perform biotransformations at temperatures higher than those used in conventional aqueous solutions (Turner and Vulfson, 2000). A layer of bound water

plays a crucial role in maintaining the structural integrity and catalytically active conformation of lipases. Thus, the existence of trace amounts of water in the immediate vicinity of lipase is a perquisite for successful functioning of a lipase in a microaqueous system (Malcata *et al*., 1992). The work of Turner and Vulfson (2000) demonstrated that the temperature at which a protein undergoes thermal denaturation (T_d) was strongly dependent on the amount of water that associated with the protein and could be correlated with the thermodynamic water activity (a_W) of enzymes. It was found that esterases pre-equilibrated over saturated salt hydrates to a fixed a_W had higher T_d at low a_W. These observations suggested that at low water activity, enzymes should be catalytically active at temperatures of around 9-120 °C.

3. Application of immobilized lipases

Lipases have high potential for hydrolysis, glycerolysis, and alcoholysis of a bulk fats and oils because of their high specificity and the relative purity of the products derived there from (Balcao *et al*., 1996). Immobilized lipase is use for hydrolysis and partial hydrolysis of fats and oils for the industrial production of fatty acids (FA), diacylglycerols (DAG), and monoacylglycerols (MAG), which are widely used as emulsifiers in food and pharmaceutical industrials. Examples of product obtained via lipase-catalyzed esterification include partial acylglycerol, TAG, shot chain esters, and terpene esters, which are used as flavor or fragrance. Sugar esters, which can be used as emulsifiers or fat substitutes, can be also synthesized by this reaction. Acidolysis and interesterification reaction have been extensively used for the production of partial acylglycerols, sugar esters, designer fats such as cocoa butter and other structure triacylglycerols (ST). Lipases are also used for modifying the FA composition of vegetable oils according to several functional, health and nutritional reasons. Lipase is currently used for production of optically active compounds for the fine chemicals and pharmaceutical industry, such as hydroxymethyl glutaryl coenzyme A reductase inhibitor, which is a potential anticholesterol drug (Akoh, 1996).

The immobilized lipase from *Rhizopus delemar* on a ceramic carrier could be used for a continuous tranesterification of borage oil with caprylic acid for more than 100 days

without being destroyed in the packed-bed reactor. Thus, the reaction system was effective for the industrial production of ST (Shimada *et al*., 1999).

Rhizopus arrhizus lipase immobilized on Celite was used to prepare isomerically pure 2-MAG by alcoholysis of TAG in organic media. The enzyme preparation showed high stability in repeated-batch reaction (Millqvist *et al*., 1994).

4. Fish oils: The essential nutrients

There is considerable evidence that fish oils are beneficial to heart health, reduce the risk of cancer, and benefit mental health. The active components of fish oils are ecosapentaenoic acid (EPA), a polyunsaturated fatty acid with 20 carbon atoms in its backbone, and docosahexaenoic acid (DHA), a polyunsaturated fatty acid with 22 carbon atoms. Both are the members of *n*-3 PUFA group of essential fatty acids. EPA and DHA are found exclusively in marine animals; fatty fishes such as herring, sardines, salmon, and tuna (Powlosky, 2001).

There are many researches showing the difference in contents of DHA and EPA of extracted marine fish oil (Sarabok, 2000; Wongsakul *et al.,* 2004). Table 2 indicates that tuna oil has high contents of DHA and EPA compared with other marine fish oils (Table 3). Tuna oil contained 99.32 % of triglyceride and the main fatty acids contents are palmitic acid (20.78%), stearic acid (6.58%), oleic acid (10.73%), EPA (6.42%), and DHA (27.18%) (Table 4).

5. *n*-3 Polyunsaturated Fatty Acids (*n*-3 PUFA)

FA are monobasic carboxylic acids consisting of a single carboxylic group attached to the end of a straight hydrocarbon chain. With some exceptions, most FA that occur in nature are straight chain acids which contain an even number of carbon atom (Sonntag, 1979). The fatty acids that contain double bond are termed unsaturated fatty acids. The term PUFA, consisting more than one double bond, cover a wide range of acids of 18, 20 and 22 carbon chain length with two to six methylene interrupted double bonds (Figure 3) (FAO, 1988). The PUFA

are classified into two groups. One is essential fatty acids and another is non-essential fatty acids. The two series of essential fatty acids, *n*-3 and *n*-6, are defined by the position of the first double bond closer to the methyl group of the fatty acids (Lauritzen *et al*., 2001). The *n*-3 PUFA have their first double bond at the third carbon atom, while n-6 fatty acids have their first double bond at the sixth carbon atom (Garcia *et al.,* 1992).

Eicosapentaenoic acid (EPA; C 20:5*n*-3)

Docosahexaenoic acid (DHA; C 22:6*n*-3)

Figure 3. Structure of EPA and DHA

Table 2. DHA and EPA contents of extracted marine fish oil

Type of fish	**DHA (MAG/100 g)**	**EPA (MAG/100 g)** Tuna
2,877	1,288	
Yellow Tail	1,785	898
Mackerel	1,781	1,214
Mackerel Pike	1,398	844

Eel	1,332	742
Sardine	1,136	1,381
Rainbow Trout	983	247
Salmon	820	492
Horse Mackerel	748	408
Conger Eel	661	472
Bonito	310	78
Sea Bream	297	157
Carp	288	159
Fleti Fish	202	210

Source: Jeyasahoke and Krisanungura (1999)

Table 3. Compositions of tuna oil

Composition	**wt%**
Triacylglycerols (TAG)	99.32
Diacylglycerols (DAG)	0.32
Monoacylglycerols (MAG)	0.00
Free fatty acids (FFA)	0.36

Source: Sarabok (2000); Wongsakul *et al*. (2004).

Table 4. Fatty acid compositions of tuna oil

Fatty acid	**%**
Myristic acid (C14:0)	4.02
Palmitic acid (C16:0)	20.78
Palmitoleic acid (C16:1)	5.76
Stearic acid (C18:0)	6.85
Oleic acid (C18:1)	10.73
Linoleic acid (C18:2)	1.68
Arachidonic acid (C20:4)	1.84
Eicosapentaenoic acid, EPA, (C20:5)	6.42
Docosahexaenoic acid, DHA, (C22:6)	27.18
Others	15.01

Source: Sarabok (2000); Wongsakul *et al*. (2004).

5.1 Sources of *n*-3 PUFA

Two of the important *n*-3 long-chain PUFA are eicosapentaenoic acid (EPA, C 20:5*n*-3) and docosahexaenoic acid (DHA, C 22:6*n*-3). Adult human could derive the long-chain PUFA, from alpha linolenic acid (ALA, C 18:3*n*-3) by chain elongation and desaturation. However, only a small amount of ALA is converted to DHA (less than 9%) (Burdage *et al*., 2002; Burdage and Wootton, 2002). These FA are synthesized mainly by both uni- and multi- cellular marine plants such as phytoplankton and algae (Shahidi and Wanasundara, 1998). They are eventually transferred through the food web and are incorporated into lipids of aquatic species such as fish and marine-animals thus fish oils are plentiful in *n*-3 PUFA (Harris, 2004). Generally speaking, the oilier the fish, the more EPA and DHA are present. Fish that tend to have high concentrations of oil include tuna, sardines, salmon, mackerel, and herring (Table 5).

Table 5. Amount of EPA and DHA in fish, crustacean and fish oils

	EPA+DHA content (g/g Oil)

Fish	
Tuna	
Light, canned in water, drained	0.26
White, canned in water, drained	0.73
Fresh	0.24-1.28
Sardines	0.98-1.70
Salmon	
Chum	0.68
Sockeye	0.68
Pink	1.09
Chinook	1.48
Atlantic, farmed	1.90-1.83
Atlantic, wild	0.90-1.56
Mackerel	0.34-1.54
Herring	
Pacific	1.81
Atlantic	1.71
Trout, rainbow	
Farmed	0.98
Wild	0.84
Halibut	0.40-1.00
Cod	

Table 5. (Continued)

Pacific	0.13
Atlantic	0.24
Haddock	0.20
Catfish	
Farmed	0.15

Wild		0.20
Flounder/Sole		0.42
Crustacean		
Oyster		
Pacific		1.17
Eastern		0.47
Farmed		0.37
Lobster		0.07-0.41
Crab, Alaskan King		0.35
Shrimp, mixed species	0.27	
Clam		0.24
Scallop		0.17
Fish Oil		
Cod liver oil*		0.19
Standard fish body oil		0.30
Omega-3 fatty acid concentrates	0.50	

Source: Adapted from Kris-Etherton *et al*. (2002)

*This intake of cod liver would provide approximately the Recommended Dietary Allowance of vitamins A and D.

5.1.1 Fish oils

Total world population of fish in 2005 was about 20 million metric tons (FAO, 2005). Most of this production has been used in various food and pharmaceutical formulations. Typical food uses hydrogenated or partially hydrogenated fish oils are production of salad oils, frying oils, table margarines, low caloric spreads and shortening in baker products (Bimbo, 1990). Most of the production from the US is exported overseas mainly to Europe, who is the major fish oil consumer. In 1998, U.S. Food and Drug Administration approved General Recognized as Self

(GRAS) status of partially hydrogenated menhaden oil (PHMO) and fully hydrogenated menhaden oil (FHMO). GRAS specifications of these products are given in Table 6.

Table 6. GRAS specification for hydrogenated menhaden oils

	Partially (PHMO)	**Fully (FHMO)**
Color	Opaque white	Opaque white
Odor	Odorless	Odorless
Saponification No.	180-200	180-200
Iodine value	85 max	10 max
Free fatty acids (%)	1.5	1.5
Unsaponificables (%)	0.10	0.10
Peroxide value (meq/kg)	5.0 max	5.0 max
Nickle (ppm)	0.5 max	0.5 max
Mercury (ppm)	0.5 max	0.5 max
Arsenic (ppm)	0.1 max	0.1 max
Lead (ppm)	0.1 max	0.1 max

Source: US Federal Register. (1989)

The lipid content of fish varies immensely with fish species, season and location of catch, food habitat and part of body. The fat content of low to high fat containing fish ranges from 0.5 to 24% (Kinsella, 1990). The fatty acids profile of selected fish oil is given in Table 7. Fish oil contains saturated, monounsaturated and polyunsaturated fatty acids. The predominant fatty acids are C 14:0, C 16:0, C 18:0, C 18:1, C 22:1, C 20:5 and C 22:6. The EPA and DHA content of fish oil usually varies from 5 to 24% and 4.1 to 37%, respectively (Kinsella, 1986). The fish oil used for concentration of *n*-3 PUFA were obtain from Pacific herring (Jangaard, 1996), cod liver (Haagsma *et al*., 1982; Rizvi *et al*., 1988), menhaden (Ratnayake *et al*., 1988; Rizvi *et al*., 1988) and red fish (Ackman, 1988). Ackmann *et al*. (1988) found that Atlantic red

fish, Atlantic herring and Atlantic dogfish could be used for enrichment of *n*-3 PUFA as the concentration of these fatty acids in all these fishes were of the same composition.

Table 7. Principal fatty acids of selected fish oils (wt%)

Fatty acids	**Menhaden**	**Rainbow trout liver**	**Atlantic herring**	**Cod liver oil**
C14:0	8.0	8.7	6.8	1.1
C16:0	24.2	18.2	14.8	18.5
C16:1	10.5	13.6	7.8	3.7
C18:0	3.0	5.5	1.1	5.3
C18:1	23.4	13.9	16.6	14.7
C18:2*n*-6	2.1	3.4	0.8	1.7
C20:1	1.8	1.8	16.0	9.8
C20:4*n*-6	2.1	1.2	0.2	0.4
C20:5*n*-3	14.0	15.5	2.8	6.4
C22:1	1.6	3.4	23.3	2.3
C22:6*n*-3	11.6	7.0	2.7	27.7
Total saturated	37.2	34.0	22.7	29.1
Total monosaturated	37.3	32.7	63.5	29.7
Total n6 (LA+AA)*	4.2	4.6	1.0	2.1
Total n3 (EPA+DHA)	25.6	22.5	5.5	33.8

Source: Kinsella (1990); Ackman (1980)

* AA: Arachidonic acid; LA: Linoleic acid

5.1.2 Seed oils

Flax, canola and soy bean constitute one of the largest sources of alpha-linolenic acid (ALA; C 18:3*n*-3), which can be converted to EPA and DHA in the human system. However, the conversion rate of ALA to physiologically more active EPA and DHA is extremely poor in the human (Dyerberg, 1986). Flax oil is currently being used as animal feed to increase the *n*-3 PUFA content of pond fish, pork and poultry eggs. By feeding 15% flax seed in poultry ration , Sim *et al*. (1992) report a six times increase in the total *n*-3 PUFA as compared to control (18% sunflower seeds) in the egg yolk. The ratio of n-6 to *n*-3 was reduced from 8.3 to 1.4% studied involving incorporation of flax seed in food formulations are ongoing (Anon, 1990).

5.1.3 Algal lipids

Marine fish feed and accumulate *n*-3 PUFA from marine phytoplanktons and sea weeds. Therefore, the latter are the ultimate source of these fatty acids. Advantages offered by these organisms as a source of lipids are their ability to grow on the artificial culture media and amenability for genetic manipulation for the production of desired end products. With the depleting fish stocks, it may be difficult to fulfill the demand of these FA by fish oil alone. Therefore, micro and macroalgae (seaweeds) are being considered as an alternative non-conventional source of *n*-3 PUFA (Ackman and McLachlan, 1979; Ackman, 1982; Choi *et al*., 1987; Polak *et al*., 1989; Radmer, 1990; Kyle, 1991). Although, the lipids content of algae generally ranges from 0.5 to 1.5% depending on the type, season and cultural condition (Ackman, 1980), the lipids in the marine algae are rich in *n*-3 PUFA. Table 8 shows the principle fatty acids of some selected algae. In some algae species, either DHA (e.g. *Gonyaulax cantanella*, about 34% DHA), or EPA (e.g. *Palmaria palmate* and *Schizymenia dubyi*, 71% and 50%, respectively) is preferentially accumulated. High *n*-3 fatty acids content of marine algae makes them a suitable alternative to fish oil as a source of these PUFA. However, some edible alga e.g. *Spirulina* sp. are more concentrated in medium chain n-6 fatty acids and palmitic acids (Ackman, 1980).

Table 8. *n*-3 fatty acid contents of some algae

Species	Fatty acid (wt%)			
	ALA (C 18:3)	EPA (C 20:5)	DPA (C 22:5)	DHA (C 22:6)
Amphidinium carterri	0.1	7.4	0.6	25.4
Dunaliella primoleeta	10.4	9.7	3.9	-
Cryptomonas sp.	7.0 16.0	10.0	-	
Schizymenia dubyi	2.6	49.8	-	-
Chrondrus crispus	5.8	26.4	-	-
Palmaria palmate	-	71.0	-	-
Skeletonema costatum	0.3	13.8	-	1.7
Gonyaulax cantanella	1.8	11.2	-	33.9

Source: Mishra *et al*. (1993)

6. Health Benefits of *n*-3 PUFA

Many researchers showed that fish oils (EPA and DHA) played a crucial role in the prevention of atherosclerosis, heart attack, depression, and cancer (Laugharne *et al.,* 1996). Moreover, the US National Institutes of Health recently published recommended data of total daily intakes of fatty acids as 650 mg/day of EPA and DHA, 2.22 g/day of alpha-linolenic acid and 4.44 g/day of gamma-linolenic acid. Saturated fat intake should not exceed 8% of total calories intake or about 18 g/day (Klinkesorn *et al*., 2004).

6.1 Brain development effects

The human brain is one of the largest consumers of DHA. A normal adult human brain contains more than 20 g of DHA. Low DHA levels have been linked to low brain serotonin levels which again are connected to an increased tendency to depression, suicide, and violence (Levine, 1997; Kalmin *et al*., 1999; Yehuda *et al*., 1996). Several studies have established a clear association between low levels of PUFA and depression (Edwards *et al*., 2005; Hibbeln, 1998; Stoll *et al*., 1999; Calabrese *et al*., 1999; Laugharne *et al*., 1996). Within mammalian brain, DHA containing phospholipids seem to correlate with development of synapses and may play an important role in the survival of mammalian neuronal cells. Some studies indicated that there might be possible effects on receptor properties. Whereas other studies indicated that *n*-3 PUFA content of membranes could exert an effect on receptor activity of signal transduction pathway (Lauritzen *et al*., 2001). In human, it may be possible for the nerve cells to transmit information to other nerve cells smoothly by glowing "off shoots" call dendrites. Growth of dendrite can be triggered by any stimulation. DHA is a membrane component required for the growth of dendrites (Suzuki *et al.,* 1988). A large number of animal studies have demonstrated that dietary *n*-3 PUFA increased learning acquisition and memory performance (Jansen *et al*., 1999; Greiner *et al*., 1999; Carrie *et al*., 2000; Morris *et al*., 2004).

6.2 Cardiovascular and heart disease effects

An enormous amount of medical literature testifies the fact that fish oils prevent and may help to ameliorate or reverse atherosclerosis, anginar, heart attack, congestive heart failure, arrhythmias, stroke, and peripheral vascular disease. Fish oil helps to maintain the elasticity of artery walls, prevents blood clotting, reduces blood pressure and stabilizes heart rhythm (Simopoulos, 1991; Pepping, 1999; Uauy and Valenzuela, 2000; Connor, 2000). Danish researchers have concluded that fish oil supplementation may help prevent arrhythmias and sudden cardiac death in healthy men. The Italian studies of 11,000 heart attack survivors found that patients supplementing with fish oils markedly reduce their risk of another heart attack, a stroke or death. A group of German researchers found that fish oil supplementation for 2 years cause regression of atherosclerotic deposits and American medical researchers report suddenly

cardiac event than do men who eat fish less than once a month (Christensen *et al*., 1999; Brow *et al*., 1999).

6.3 Tumor and cancer effects

Fish oils are particularly effective in reducing inflammation and can be of great benefit to people suffering from rheumatoid arthritis or ulcerative colitis. Daily supplementation with as little as 2.7 g of EPA and 1.8 g of DHA can markedly reduce the number of tender joints and increase the time before fatigue set in (Kremer *et al*., 1987; Kremer *et al.,* 2000; Fortin *et al*., 1995; Kremer *et al*., 1995). There is now also considerable evidence that fish oil consumption can delay or reduce tumor development in breast cancer. Studies have also shown that a high blood level of omega-3 fatty acids combined with a low level of omega-6 fatty acids reduces the risk of developing the breast cancer (Simonsen *et al*., 2000; Fernandez-Banares *et al*., 1996).

6.4 Visual function effects

DHA is efficiently retained in the retina as it is recycled via the pigment epithelium upon renewal of old rod outer segment (ROS) disk membrane. DHA is most highly concentrated in rods and molecular investigating has focused on the importance of DHA in ROS membrane for the function of rhodopsin, the visual pigment of rods, (Lauritzen *et al*., 2001). A number of studies have suggested that the content of DHA had a positive influence on the function of cell membranes. Preterm infants receiving human milk or infant formula with DHA have a better visual acuity than those receiving infant formula without DHA (Jonsbo *et al*., 1995).

7. Lipase-catalyzed synthesis of ST containing PUFA

ST are in the broadest sense defined as TAG that have been restructured to change the positions of FA and modified to change the FA compositions from the native state

(Akoh, 1995). Natural edible fats and oils are simply mixtures of the number of TAG that are different in terms of both FA species and their distribution along the glycerols backbone. In contrast to natural edible lipids ST are TAG modified either chemically or enzymatically in either type of FA or position of FA. In less broad sense, ST are SL containing either shot-chain fatty acids, or medium-chain fatty acids (MCFA), or both, and long-chain fatty acids, in the same glycerol molecule. A number of studies have been carried out for the synthesis of ST having (medium-chain)-(long-chain)-(medium-chain)-type FA (Christophe, 1998).

ST containing PUFA such as EPA, DHA or AA, have become of great interest because various pharmacological effect of these FA. These include several health benefits on cardiovascular diseases, immune disorders and inflammation, renal disorders, allergies, diabetes, cancer, etc (Akoh, 1995; Gill and Valivety, 1997). These FA may be also essential for brain and retina development in humans.

The absorption of PUFA into the body depends upon the position of PUFA along the glycerol backbone, i.e. at *sn*-1 (or 3) or *sn*-2 position (Christensen *et al*., 1999). ST containing PUFA at *sn*-2 position and MCFA at *sn*-1 and *sn*-3 positions can be hydrolyzed into 2-MAG containing PUFA and FA by pancreatic lipase and are efficiently absorbed into intestinal mucosa cells in normal adults. It is to be noted that mammalian pancreatic lipases hydrolyze the ester linkages at *sn*-1 and *sn*-3 positions with preference for MCFA over long-chain ones (Battino *et al*., 1967; Yang *et al*., 1990). ST can be synthesized either chemically or enzymatically. However, an enzymatic synthesis of ST is more advantageous over a chemical process with regard to several aspects. Enzymatic reactions have another advantage for the synthesis of ST containing PUFA because PUFA are very unstable (Battino *et al*., 1967).

Among a number of reactions to produce ST, only reactions involving PUFA biotransformation are discussed. These are classified in two types: (*i*) reaction involving edible oils (vegetable oil or fish oil); and (*ii*) reaction between chemically defined pure TAG and pure FA or FA esters. Reports are summarized in Table 9.

Table 9. Synthesis of ST containing PUFA from natural edible oils

Substrates	Lipases	Immobilization
Tuna oil+caprylic acid	*Rhizopus delemar*	Ceramic carrier
Borage oil+caprylic acid	*Rhizopus delemar*	Ceramic carrier
Single cell oil+caprylic acid	*Rhizomucor meihei*	Macroporous ionic resin
Fish oil+tricaprylin	*Candida antarctica*	-
Borage oil+caprylic acid	*Candida rugosa*	Ceramic carrier
Borage oil+EPA+caprylic acid	*Rhizomucor meihei*	-
Menhaden oil+C 10:0	*Rhizomucor meihei*	-
Single cell oil+caprylic acid	*Pseudomonas* sp.	Celite
Arachidonic acid-rich TAG+caprylic acid	*Rhizopus delemar*	Ceramic carrier
Fish oil+caprylic acid	*Rhizopus delemar*	Ceramic carrier

Source: Yamane and Iwasaki (2000); Wongsakul *et al*. (2004)

7.1 Production of ST enriched with PUFA by one-step process

Nutritional properties, application, benefits, and enzymatic approaches for the synthesis of ST have been recently reviewed, together with examples of already marketed, but chemically synthesized products (Soumanou *et al*., 1997). The first method for the production of medium-chain triglyceride is the chemical synthesis, which involved the fractionation of medium-chain fatty acid (C 8 and C 10), obtained from the hydrolysis of high-grade vegetable oil, and their subsequent esterification with glycerol by catalysis. But it would be difficult to control the fatty acid in the desired position (Soumanou *et al*., 1998). In addition, these conventional chemical products are very expensive due to the extreme conditions, thus enzymatic synthesis is focused on the esterification of this ST from fatty acids and glycerol using regiospecific lipases. Lipases-catalyzed interesterification reactions, including tranesterification and alcoholysis, offer the greater advantageous control over the position distribution of FA in the final products (Will and Marangoni, 1999).

There are three enzymatic routes to synthesis ST with PUFA; 1) direct esterification of glycerol with DHA and EPA; 2) ester interchange, or interesterification, between triacylglycerols and ethyl ester with contains *n*-3 PUFA; and 3) hydrolysis and acidolysis of TAG and monoesters (Haraldsson *et al.,* 2000).

There are many studies focused on ST synthesis with various processes and substrates. The study of Akoh (1995) indicated that lipids with specific FA can be enzymatically structured for varying special uses. They have used four-factor response surface optimization for enzymatic synthesis of structured triolein by incorporating capric acid (C 10) and were obtained excellent yields. In this study *Mucor meihei* (IM60) lipase in the presence of hexane was used to synthesize ST of defined structure containing 1 or 2 MCFA and a very little triolein. It has been shown that *Mucor meihei* (IM60) is capable of incorporating up to 10.5% EPA on to soybean oil with free EPA acid as acyl donor, and 29.2% with EPA ethyl ester as acyl donor.

7.2 Production of ST enriched with PUFA by two-step process

Will and Marangoni (1999) studied a two-step reaction to produces partial glycerides, which would subsequently be used as substrates in both lipase-catalyzed and chemical catalyzed esterification reactions with caprylic acid. The yields and kinetics of these two-step reactions were compared to lipase-catalyzed acidolysis and transesterification as well as to chemical transesterification reactions. Acyl migration did not occur during the hydrolysis or short-path distillation steps in the preparation of fatty acid free-partial glycerides for esterification reactions. No significant differences in final yields (59.9 to 82.8 wt% of total TAG) of new ST were detected among lipase-catalyzed (24 h) and chemical catalyzed (5 h) reactions. Thus, in contrast with other reports, they suggested that the chemical esterification using hydrolyzed oil could represent the best synthetic option.

Recently the two-step reactions for synthesis of the pure ST were suggested as effective method for MLM synthesis. In this study, ST with MCFA (caprylic acid) in *sn* 1- and *sn* 3-position and long-chain unsaturated fatty acid (oleic or linoleic acid) in the *sn*-2 position of glycerol were synthesized by lipase catalysis in two-step processes. First, pure 2-MAG were synthesized by alcoholysis of TAG (triolein, trilinolein, or peanut oil) in organic solvent with 1,3-regiospecific lipases (from *R. meihei*, *R. delemar*, and *R. javanicus*). The 2-MAG were then esterified in second reaction with caprylic acid in hexane to form almost MLM. For 2-MAG obtained from peanut oil, the final product contained more than 90% caprylic acid in the *sn* 1- and *sn* 3-positions, whereas, the *sn* 2-position was composed of 98.5% unsaturated LCFA. Highest initial rates and final concentration of MLM were found with Lypozyme (89.1%) and RDL immobilized on EP 100 (91.2%). In the esterification of 2-MAG with MCFA, the water content and/or activity must be low to avoid acyl migration and convert all 2-MAG to MLM. The effect of molar ratio between caprylic acid and 2-monoolein on the initial rate in the synthesis of MLM was studied with Lypozyme (with molar ratio between 2 and 6). The optimal ratio was 3:1. Comparison with the direct interesterification between peanut oil and caprylic acid, a two-step process allows the faster reaction and gives considerably higher final concentration of the desired MLM. The data obtained from the study show that the two-step reaction is superior to chemical methods and can also be applied to PUFA (or fats and oils) (Soumanou *et al*., 1998).

The study of Schmid *et al*. (1999) has shown that two-step process allows the synthesis of 1,3-dioleoyl-2-palmitoylglycerol in high yields and purity. In contrast to the direct

synthesis by a one-step interesterification, acyl migration can be suppressed efficiently in the alcoholysis reaction. In addition, alcoholysis enables simplified isolation of 2-monopalmitin by crystallization. To achieve the best results, it was very crucial to control the choice of solvent, carrier for lipase immobilization, and water activity which may need to be optimized of each new substrate.

To minimize acyl migration it is necessary to work in aprotic solvent, such as acetone, and to carefully control the water activity. Although acetone was not considered to be a suitable solvent for lipid modification, because the activity of lipase is very low in acetone, it was considered that high yield of 2-monopalmitin are possible in acetone. Thus the use of process preparation of food-related materials is very feasible. Another method to achieve constant water activity is a separate pre-equilibration of reaction component with saturated salt solution of known water activity (Schmid *et al*., 1999).

8. Enrichment of lipids with EPA and DHA by lipases

The long-chain *n*-3 type PUFA are characteristic of marine fat (Ackman, 1989) and commonly occur in triacylglycerols (TAG) (Holmer, 1989) and phospholipids (PL) (Vaskosky, 1989) of fish. They originate in the lipids of photosynthetic microalgae that constitute phytoplankton and accumulate through the food chain in fish, which are unable to biosynthesize them (Sargent *et al*., 1995). Microalgae of marine origin is another important source of *n*-3 PUFA which is being utilized to an increasing extent (Handerson, 1999). The most ubiquitous of *n*-3 PUFA are EPA and DHA (Ackman, 1982).

8.1 Recent developments and roles of lipase in *n*-3 PUFA field

Concentrates of EPA and DHA first appeared on the market in the early 1980s, max EPA was the first dietary *n*-3 PUFA supplement product on the market containing 18% of EPA and 12% of DHA in the natural TAG form (Sevan Seas, 1994). It has been widely used in various clinical studies for more than 15 years. TAG up to the level of 30% EPA+DHA can be

prepared directly from fish oils without splitting the fat by a careful selection of fish oils and various methods such as winterization, molecular distillation and solvent crystallization (Ackmann, 1988; Breivik and Dahl, 1992). Concentration beyond that level on the TAG form is difficult, as it requires a cleavage of fatty acids off the acylglycerols, either as free acids or monoesters. Various physical methods and combination of methods are available for concentrating EPA and DHA once released. Molecular distillation, supercritical fluid extraction and urea complexation can be used to concentrate them to 50-85% level. Concentration beyond the 90% level and separation and purification of EPA and DHA requires more specific methods based on HPLC (Breivik *et al*., 1997). A whole range of monoester concentrates of EPA and DHA are new commercially available, usually as ethyl ester, some of which have been registered as drugs in various countries (Haraldsson *et al*., 2000).

Resynthesis of TAG highly enriched with EPA and DHA from concentrates is by no means easy by traditional chemical esterification methods based on Lewis acid or alkoxide catalysis. The highly labile *n*-3 PUFA are very sensitive against the rather drastic conditions offered by these traditional methods. The all-cis *n*-3 PUFA framework makes them extremely prone to oxidation, *cis-trans* isomerization, double-bond migration, or polymerization. Despite that, some European companies have recently launched onto the market a whole variety of TAG concentrates comprising 50-70% EPA+DHA. These products usually constitute a mixture of roughly 55% TAG, 35-40% DAG and 5-10% MAG, which apparently reflects some compromise between efficiency and liability in their production (Haraldsson *et al.,* 2000).

Recently, lipases have been introduced to *n*-3 PUFA field to solve these problems (Haraldsson and Kristinsson*,* 1998). These enzymes offer a high efficiency and mildness, and their application in organic media is now firmly established (Bornscheuer and Kazlauskas, 1999). They offer TAG concentrates EPA and DHA of a whole range of composition including more than 80% of EPA or DHA, of high purity, highly efficiently, and in excellent yields. Neither chemicals nor organic solvents are required; thus these processes are highly feasible from industrialization as well as environmental hazard points of view. Nowadays, numerous industrial companies in the field are realizing the advantages and potential offered by the lipases, and some of them have marketed-or are about to market-TAG concentrates of EPA and DHA. It is interesting to know that the Japanese authorities have not allowed concentrated

ethyl esters or chemically modified *n*-3 PUFA concentrates to be sold as health food, only concentrated TAG produced by lipases having been allowed for general sale.

Lipases have also been employed to enrich various other lipid classes with *n*-3 PUFA, including phospholipids and ether lipids of 1-0-alkyl-2,3-diacyl-*sn*-glycerol type, which are characteristic of fat in shark liver oil (Haraldsson *et al.,* 1997). Lipases have also found applicability in concentrating EPA and DHA in fish oils. This application is based on their fatty acid selectivity, which can be utilized in concentrating EPA and DHA by kinetic resolution. Concentration levels of 50-70% EPA+DHA can easily be obtained in high recovery directly from fish oil by lipase-catalyzed hydrolysis or alcoholysis reactions. Higher levels above 90% can be obtained into two-step enzyme processes in high recoveries, and it is anticipated that lipases may be used purity EPA and DHA toward high purity levels. This is also highly important from an industrialization point of view, as some of these processes do not require solvents or chemicals (Haraldsson and Kristinsson, 1998).

8.2 Enrichment of TAG with EPA and DHA

This section is divided in to two parts. The first part deals with incorporation of EPA and DHA in to fish oil by lipase-catalyzed transesterification reactions. The second part involved the direct esterification of glycerol with PUFA as free acids to obtain TAG of identical composition to PUFA being used by a different lipase. The methodology also offers homogeneous TAG of 100% EPA and 100% DHA.

8.2.1 Enrichment of cod liver oil with EPA and DHA

It is relatively easy to concentrate EPA and DHA up to high levels as FFA or monoesters. However, the natural form of these fatty acids in fish oil is TAG, and the major challenge was to produce natural TAG highly enriched with EPA and DHA, far beyond the 30% level mentioned earlier. A highly successful solution to that problem was based on treating cod liver oil with free fatty acid or monoester concentrates of EPA and DHA in presence of lipase to

affect fatty acid exchange between the natural TAG and the concentrates (Haraldsson *et al*., 1997). For the marketing point of view, it was also important that cod liver oil already had a long history on the market as a vitamin A and D supplement (Hjaltason, 1989), and that the product could be claimed as being derived directly from cod liver oil.

Lipozyme, the immobilized 1,3-regiospecific lipase from the fungus *Rhizomucor meihei* (available commercially from Novo Nordisk in Denmark), was employed to transesterify cod liver oil with concentrates of EPA and DHA. The cod liver oil comprised approximately 9-10% each of EPA and DHA, and TAG highly enriched with *n*-3 PUFA was accomplished, of high purity and the virtually quantitative yields (Haraldsson *et al*., 1997). Interesterification and acidolysis with ethyl ester and free fatty acid concentrates, respectively, both comprising 85% EPA+DHA content, resulted in TAG containing 60-65% EPA+DHA and well over 70% total *n*-3 PUFA. At that time this presented by far the highest EPA-and DHA-enriched TAG product available. Both reactions were conducted in the absence of any solvent, using 10% dosage of lipase, as based on the weight of fat, 60-65°C, and the lipase preparation constituting 10% water. A 3-fold excess of FFA or ethyl ester was used, as based on number of molar equivalents of ester present in TAG (Arnar *et al*., 2003).

In both reactions the lipase displayed a significantly higher activity toward EPA than DHA, and the interesterification reaction took place considerably faster than the acidolysis reaction. Some hydrolysis side reaction was observed, especially for the interesterification reaction, but this could be reduced considerably by decreasing the water content of lipase (Harldsson *et al*., 1993).

Despite the 1,3-regiospecificity of lipase, the mid-position become enriched to an equal extent to the end-positions. Intramolecular nonenzyme-promoted acyl-migration processes (Kodali *et al*., 1990) were responsible for this, as was established by investigating the FA composition of individual positions of the acylglycerols when the reaction proceeded. This means that, at equilibrium, the FA composition of the TAG was reflected by a weighted average of the initial composition of cod liver oil TAG and the concentrates. This is exactly what was wanted, since the aim was to enrich the fish oil to the maximum levels. In order to allow that, prolonged reaction time was required because the acyl-migration processes was considerably

slower than the lipase-promoted processes and rate-limiting for the equilibrium. In order to obtain equilibrium for the interesterification, a 24-h reaction was needed, whereas the acidolysis reaction required 48 h.

8.2.2 Homogeneous TAG of EPA and DHA

Using the technology described previously, the EPA and DHA fatty acid composition of TAG product was determined by weighted average of initial fatty acid composition of the cod liver oil TAG and composition of the *n*-3 concentrates. Thus, in order to obtain elevated level of EPA and DHA into ordinary fish oil such as cod liver oil, excessive amounts of concentrates were needed. To avoid that limitation and obtain TAG of composition identical to the concentrates being used, a modified procedure based on a direct esterification of free fatty acid with glycerol was developed (Haraldsson *et al*., 1993; 1997). This also opened the possibility of synthesizing TAG homogenous with both EPA and DHA, i.e. 100% EPA or DHA.

A different lipase, this time an immobilized nonregiospecific yeast lipase from *Candida antarctica* (also available commercially from Novo Nordisk in Denmark (SP 382); has now been replaced by Novozym), was highly efficient in generating TAG of both 100% EPA and 100% DHA content. This was accomplished by a direct esterification of glycerol with stoichiometric amount of pure EPA and DHA, without any solvent, by stirring at 65° C under vacuum, with a 10% dosage of immobilized lipase, as based on the weight of substrates. The co-produced water was considered into liquid nitrogen-cooled trap during the progress of the reaction, thus driving the reaction to completing.

The resulting TAG, homogeneous with either EPA or DHA, was afforded in near quantitative yields of excellent purity. High-field ^{1}H- and ^{13}C-NMR spectroscopy was found extremely valuable as a probe to monitor the progress of the reaction. It also enabled experiment to followed the incorporation of EPA and DHA into glycerol to form the various intermediary acylglycerols participating in the direct esterification process, the 1- and 2-MAG, 1,2- and 1,3-DAG and TAG. The results implicated that the nonregioselective lipase acted considerably faster

at the end-position than the mid-position of the glycerol moiety. This accordance with general assumption that lipase normally display a strong preference for primary rather than secondary alcoholic substrates due to increased bulkiness of the secondary alcohols, rendering them less nucleophilic (Haraldsson *et al.,* 1993).

Interesterification involving tributyrin and pure EPA and DHA as ethyl ester under identical conditions offered similar product (Haraldsson *et al*., 1993; 1997). Similar treatment of glycerol with concentrated ethyl ester of EPA and DHA was observed to be inferior to the above mentioned processes in term of extent of conversion (Haraldsson *et al*., 1993). Finally, it is evident that immobilized *Candida antarctica* lipase is superior to other lipases in term of the direct esterification reaction between glycerol and free fatty acids, as can be witnessed by several literature report (Harldsson *et al*., 1993; Shahidi and Wanasundara, 1998).

8.3 EPA and DHA concentrate from fish oil by lipases

Based on their fatty acid selectivity and discrimination against *n*-3 PUFA, lipases can be used as an alternative means of concentrating EPA and DHA in fish oil by kinetic resolution. There are numerous reports in the literature describing the application of lipase to concentrate EPA and DHA in fish oil (Harldsson *et al*., 1997). There reactions involved hydrolysis and alcoholysis of TAG and monoesters, direct esterification of free acids with alcohols, and various transesterification reactions. In this respect lipases can be divided into three categories: (i) those which display no or low activity toward fish oils or fish oil FA as substrates; (ii) those discriminating against *n*-3 PUFA and can be use to concentrate EPA and DHA together; and (iii) those that offer a strong discrimination between EPA and DHA, usually in flavor of EPA. The lipase belong to third group can be used to concentrate EPA and/or DHA individually (Harldsson *et al*., 1999; Tanaka *et al*., 1992).

The lipase belonging to the first group clearly offers very little application in the fish oil field. The lipases in the second group act very well on the bulk of saturated and monosaturated fatty acids present in the fish oil, leaving EPA and DHA largely unaffected. This enables the preparation of concentrates of EPA plus DHA. *Pseudomonas* lipases belong to this category, as has been demonstrated by Haraldsson *et al*. (1997). Two commercially available *Pseudomonas* lipases were observed to afford a concentrate of approximately 50% EPA+DHA in very high recovery (80-90%) and highly efficiently without a solvent, since simply a 2-fold stoichiometric amount of ethanol was required. This demonstrates that lipases can be used as a valid alternative to conventional physical methods such as molecular distillation. One of the main advantages was the considerable reduction of bulkiness of the process, since no organic solvent was required and the ethyl esters produced were directly distilled off by short-path distillation from the residual acylglycerol mixture of EPA and DHA.

Geotrichum candidum lipase also belongs to this category of lipases not discriminating much between EPA and DHA. This lipase was used to concentrate EPA together with DHA in tuna oil by hydrolysis reaction up to 50% levels (Shimada *et al*., 1999), similarly with high recoveries of EPA and DHA (Shimada *et al*., 1994; 1995). It is interested that the residual acylglyceride mixture from the reaction comprised TAG to a large extent (85%) which was related to selectivity of that lipase favoring MAG and DAG rather than TAG. This lipase displays a somewhat lower activity toward DHA and EPA, which is usual behavior of lipase. An exception to that behavior was observed from *Pseudomonas* lipase mentioned above, which display higher activity toward DHA than EPA (Haraldsson *et al*., 1997).

Lipases belong to third class displaying moderate to strong discrimination between EPA and DHA, all in flavor of EPA. These lipases are produced by *Candida rugosa* (formerly named *Candida cylindracea*), *Rhizopus delemar*, and *Rhizomucor meihei*. Hydrolysis of tuna oil with the *Candida rugusa* lipase afforded an acylglycerol mixture highly enriched with DHA, up toward the 50% levels (Hoshino *et al*., 1990; Tanaka *et al*., 1992; McNeill *et al*., 1996; Moore and McNeill, 1996). With that lipase there are indications that TAG molecules in fish oil containing DHA may resistant to the lipase (Tanaka *et al*., 1992). Shimada *et al*. (1997a; b) have demonstrated that *Rhizopus delemar* lipase can be used to highly enrich free acid from tuna oil with DHA by direct esterification with long-chain alcohol. The residual FA contained 73% DHA

in very high recovery (83%), and no solvent was required. A second esterification afforded further purification of DHA up to almost 90% in 71% over all recovery.

Similar levels of DHA enrichment were obtained by Haraldsson and Kristinsson (1998) from tuna free acids in a direct esterification reaction with ethanol in the absence of solvent using Lipozyme. By the direct estericatión with long-chained alcohol they were also to purify EPA free DHA to above 90% purity level from a free fatty acid concentrate of EPA in high recoveries. These example demonstrate that enrichment level well beyond the urea crystallization method can be highly obtained efficiently by lipases. The fact that an immobilized lipase can be reused 20 to 40 times or more, with little or no deterioration of the lipase, suggests that the application of lipase in the field of fish oils is a highly feasible choice from the industrial point of view (Harldsson *et al*., 1997).

9. Use of bioreactors for production of fats and oils

Various possibilities exist for reactors containing immobilized lipase, but most situations fall within one of following cases: (*i*) a lipase contained in aqueous phase and confined by a surfactant liquid membrane within an organic liquid phase (*ii*) a lipase entrapped within a three dimensional polymeric matrix dispersed within an organic liquid phase, or dispersed within an aqueous phase, and (*iii*) a lipase contacted to a solid support and dispersed within an organic liquid phase, or dispersed within an aqueous phase (Balcao *et al*., 1996).

By the use of lipase in immobilized form for polyunsaturated fatty acids enriched *n*-3 PUFA production, the reactor can be used in one of several possibilities such as batch stirred tank reactor: BSTR (Mensah *et al*., 1998; Padmini *et al*., 1994; Carta *et al*., 1992),

packed bed reactor: PBR (Dossat *et al*., 1999; Arcos *et al*., 1999), continuous stirred tank reactor: CSTR (Carta *et al*., 1992), fluidized bed reactor: FBD (Kosuki *et al*., 1990; 1994), or membrane reactor: MR (van der Padt *et al*., 1990; Garcia *et al*., 1992; Bouwer *et al*., 1997).

9.1 Batch Stirred Tank Reactor (BSTR)

BSTR is the reactor most commonly used. This reactor operates batch wise and consists of a vessel in which the reactant fluid mixture is stirred by some mechanical means (e.g. magnetic bar, submerged impeller, reciprocal, or end-over-end rotator).

The immobilized lipase is separated from the reaction medium at the end of the reaction by filtration or centrifugation. This reactor is easy to operate (e.g. heat, cool, clean, and maintain) and normally requires a very limited set of auxiliary equipment. However, the overall volumetric throughput is relatively low because of dead time taken up by the operations of emptying, cleaning, and filling. So an economic constant must be considered for large scales use (Kaewthong and H-Kittikun, 2000).

Carta *et al*. (1992) studied the synthesis of esters by using a nylon-immobilized lipase from *Candida cylindracea* in batch and continuous reactors. They found that immobilized enzyme was effective in catalyzing the synthesis of ethylpropionate, isoamylpropionate, and isoamylbutylrate. With ethanol dissolved in hexane as a substrate, the maximum initial esterification rate was 0.02 mol/(h×g of immobilized protein), but enzyme was not stable when the substrate concentration was higher than 0.2 M. With isoamylalcohol in hexane as a substrate gave high rate of esterification. A continuous-flow, packed-bed reactor was operated successfully for the continuous synthesis of ester.

Mensah *et al*. (1998) focused on the removal of water formed during an enzymatic esterification in a batch reactor by a commercial immobilized lipase, Lipozyme, and propionic acid and isoamyl alcohol dissolved in hexane are the substrates. Reactions were carried out in screw-top glass vials with Teflon-lined caps slowly rotated end-to-end at 30 rpm. They found that the water formed would partition between the catalyst and the medium. The results

shown that, addition of the resin Dowex HCR-W2, a strong-acid cation exchange resin form, was effective to control the water accumulation without stripping the essential water.

9.2 Packed-Bed Reactor (PBR)

Because of their high efficiency, low cost and ease of construction, operation and maintenance, PBR, also known as fixed-bed reactor, has traditionally been used in most large-scale catalytic reactions. In this reactor, the granules of the immobilized enzyme are usually packed within a jacket thermostat column, thus providing a large surface area per unit volume of reactor. In the presence of a single phase, pumping may be made upward (to avoid compaction of the packed granules by passing) or downward (to take advantage of gravitation force), whereas in biphasic situation the two phase may be pumped in opposite directions (countercurrent flows) with the most dense flowing downward, or in the same direction (co-current flows). High pressure drops are normally associated with this kind of reactor, especially if diffusional limitations are alleviated by decreasing the mesh size of the bed granules (Kaewthong and H-Kittikun, 2000).

Arcos *et al*. (1999) studied enzymatic synthesis of mono-, di-, and triacylglycerols from polyunsaturated fatty acids (linoleic, oleic, and conjugated linoleic acids) as a solvent free reaction in a packed-bed reactor containing an immobilized lipase from *Mucor miehei*. The packed-bed reactor consisted of a length of tygon tubing sufficient to contain the weight (ca. 560 mg) of immobilized enzyme employed (20 cm for an internal diameter i.d. of 0.32 cm, 8.9 cm for an i.d. of 0.47 cm, 5 cm for an ID of 0.63 cm, and 3.2 cm for an ID of 0.79 cm). The tygon was packed manually with dry immobilized enzyme, which was then fixed in place using plugs of glass wool. The results, at the outlet of reactor, one observes excellent spontaneous separation of the glycerol and acylglycerol/fatty acids phase. At 50 $^{\circ}$C and a fluid residence time of 1 hour, as much as 90% of fatty acid can be esterified when the molar ratio of fatty acid to glycerol is 0.33 or less.

Dossat *et al*. (1999) studied the transesterification of high oleic sunflower oil with butanol by immobilized Lipozyme in hexane which was carried out in a continuous packed bed reactor. Oleic acid, butyl ester and glycerol were being formed as the main product. It was

found that glycerol, insoluble in n-hexane, remained in the reactor adsorbed onto the enzymatic support, leading to a drastic decrease in enzymatic activity. To recover enzymatic activity, several solutions were proposed. The addition of silica gel into the enzymatic bed to adsorb the produced glycerol did not enable this loss of activity to be avoided. In order to enhance the solubility of glycerol in the reaction medium as soon as it was produced, n-hexane amended acetone was used as solvent, but high conversion of sunflower oil was not restored. The glycerol was eliminated from the reactor, and conversion was maintained.

9.3 Membrane Reactor (MR)

Membrane or diaphragm reactor may be operated with one or two liquid phases. In this reactor, the enzyme is immobilized on to the membrane, which may take the form of either the flat sheet or hollow fiber. Because of the role of the membrane in the segregation of two immiscible fluids, MR is commonly employed for biphasic liquid systems. Prevention and elimination of the membrane pore plugging is considerably difficult than packed-bed plugging in a PBR, but the lower pressure drops for the given specific area of reaction usually compensate for such drawback (Kaewthong and H-Kittikun, 2000).

van der Padt *et al*. (1992) studied the enzymatic synthesis of MAG in MR with an inline adsorption column. The MR consisted of a cellulose hollow fiber membrane module, an internal oil circuit and an external glycerol-water circuit. Lipase from *Candida rugosa* was adsorbed at the inner fiber site. The oil circuit was operated batch wise. To recover the monoesters produced, a silica 60 column was placed in the oil phases of the MR. when no adsorption column is used; the MAG is about 18% w/w. The authors suggested that could be used in a continuous process using a sequence of columns. They estimated the production of 60 mol (15 kg) monoester/g enzyme. The half-life of the lipase from *Candida rugosa* was given as 50 days.

Bouwer *et al*. (1997) studied the immobilized enzymes in a bioreactor, which often lead to decrease in the enzyme activity. Two-phase membrane reactors were constructed with lipase immobilized on membrane of various thicknesses. The reactions studied were

hydrolysis of triglycerides and peroxidation of FA. It appeared that in the case of hydrolysis, the membrane activities are all of the same order of magnitude about 20 μmole/m^2S). Independent of membrane type or thickness; however, with hydrophilic membrane, the enzyme activities are much higher. The thickness of the reaction layer and the degree of enzyme utilization could be estimated. The thickness of the reaction layer was 0.5-7 μm and the enzyme utilization was 0.2-14% depending on condition.

Objective of study

1. To purify tuna oil and determine compositions of glycerides and fatty acids of refined tuna oil.
2. To optimize synthesis of monoacylglycerols by immobilized lipases.
3. To optimize synthesis of polyunsaturated fatty acids rich free fatty acids by two steps enzymatic process.
4. To synthesize of PUFA enriched triacylglycerols by immobilized lipases.