Chapter 1

INTRODUCTION

Monoacylglylcerols (MAG) are the most widely used as emulsifiers in food, pharmaceutical and cosmetic industries (Thude *et al*.,1997). They have excellent emulsifying properties, are low odor and taste. MAG are biodegradable and generally recognized as safe (GRAS). At present, they are manufactured by continuous chemical glycerolysis of fats and oils at high temperatures (220-250 °C) employing inorganic alkaline catalysts under nitrogen gas atmosphere. The result is a crude mixture of mono-and diglycerides (roughly equal amounts) and some unreacted triglycerides (overall conversion 90 %). The product has several drawbacks, e.g., low yield, dark color and burnt test etc. (Thude *et al*., 1997). Further separation is generally carried out by molecular distillation yielding high-purity MAG (>90 %) (Diks and Bosley, 2000).

Due to very high temperature applied during glycerolysis, decomposition and oxidation reactions take place which often result in a dark-colored and burnt-flavor product. Obviously, this requires extensive purification during further downstream processing. Because of the ambient reaction temperature applied, biocatalysis can potentially yield a higher quality product with lower energy consumption in a more 'natural' type process.

Lipases or glycerol ester hydrolases (EC 3.1.1.3) were originally employed for the hydrolysis of ester bonds of triacylglycerols (TAG) to produce free fatty acids (FFA), glycerol and partial acylglycerols (e.g., MAG and diacylglycerols: DAG). In addition to plants and animals, these enzymes are widespread in many microorganisms which are also capable of actively producing these enzymes both in endogenous and exogenous forms (Balcao *et al*., 1996).

By immobilizing the enzyme, it is possible to operate enzymatic processes continuously with all the attendant advantages. Enzyme immobilization has been accomplished by chemical and physical attachment to solid surfaces (Prazeres and Cabral, 1994). Several approaches for the synthesis of MAG by immobilized lipase have been reports. They include selective hydrolysis using 1,3-specific lipases (Holmberg and Osterberg, 1988), esterification of FFA with glycerol (Kwon *et al*., 1995) and especially glycerolysis of fats and oils (McNeill *et al*., 1991), which high yield of MAG was obtained. Reactions were performed in reverse micelles, water-organic biphasic systems, organic solvents, solvent free systems and in solid-state with several reactors namely, batch stirred-tank reactors (BSTR), continuous stirred-tank reactors (CSTR), packed-bed reactors (PBR), fluidized-bed reactors (FBR) or membrane reactors (MR)(Thude *et al*., 1997).

The oil palm (*Elaeis guineensis*) is widely cultivated in Southern of Thailand. The yield of palm olein is more than domestic food oil consumption so the price is low. Production of MAG from palm olein is an alternative that increases advantages and value added of palm olein. Consequently, the aim of this research is to investigate the continuous glycerolysis of palm olein for MAG production by immobilized lipase in suitable bioreactors and scale up the system.

Literature Review

1. Palm Oil

The oil palm produces two distinct oils: palm oil from the fleshy mesocarp and palm kernel oil from the fruit kernel. The usage of both oils is extended by fractionation. Palm oil gives a more valuable olein (an excellent frying oil) and a less valuable stearin (used in part as a replacement for tallow in the oleochemical industry). The stearin is the major fraction of palm kernel oil. The oils and their fractions can be further modified by blending with other oils, by partial hydrogenation, or by interesterification. Further fractionation of the palm oil fractions yields an intermediate fraction (PMF, palm midfraction) that can be used as a cocoa butter extender (Gunstone, 1997). Palm oil is characterized by high levels of carotene and of tocopherols and tocotrienols (vitamin E), which can also be isolated or concentrated as products of considerable value. Palm oil fatty acid distillate (PFAD), a by-product of the principal refining procedure, is an important component of animal feed (Gunstone, 1997).

Palm oil is widely used as a food oil with limited non-food uses also. It differs from other commercially available oils in its fatty acid and TAG composition. It contains almost equal amounts of saturated acids (mainly palmitic with some stearic) and unsaturated acids (mainly oleic with some linoleic acid). The proportion of palmitic and oleic acids in the major TAG leads to stability of the crystals. These are very desirable in the production of margarines and shortenings, especially those with high levels of unsaturated acids. Some information on TAG composition is given in Table 1 (Gunstone, 1997). The major fatty acids in palm oil are palmitic acid and stearic acid. While the major triacylglycerols are POP and POO or PLSt.

Table 1 Triacylglycerol composition of palm oil

(i)	Major triacylglycerols (%)							
	PPP	POP	PLP	POSt	POO, PLSt	PLO	OOO	Other
	7	33	7	6	23	7	3	14
	5	43	8	4	29	8	2	1
	7	31	9	6	23	9	4	11

(ii)	Distribution of fatty acids between *sn*-1, -2, and –3 positions (%)			
	16:0	18:0	18:1	18:2
TAG	48.4	3.7	36.3	10.0
Sn-1	60.1	3.4	26.8	9.3
Sn-2	13.3	0.2	67.9	17.5
Sn-3	71.9	7.6	14.4	3.2

Source : Gunstone (1997)

2. Lipases

Lipases or glycerol ester hydrolases (EC 3.1.1.3) were originally employed for the hydrolysis of ester bonds of TAG to produce FFA, glycerol and partial acylglycerols (MAG and DAG)

2.1 Sources of lipases

Lipases occur widely in animals, plants and microorganisms. Numerous mammalian tissues, organs and fluids, such as pancreas, kidney, adipose tissue, heart, brain, muscle and serum, have been known to contain lipases. Among animal lipases pancreatic lipase has been studied most extensively. The hog pancreatic lipase has been studied most extensively, presumably because of its high concentration (2.5% of the total protein in the pig pancreatic juice) and high turnover number. Moreover, the presence of

esterolytic enzymes in the milk of cow, sow, goat, sheep, and human has been known for a long time (Shahani, 1975).

Numerous fruits, vegetables, plant tissues, and particularly plant seeds, such as wheat, oat, rye, cotton, soybean, castor bean, and *Vernonia anthelmintica*, have been known to exhibit lipase activity. Among the lipases of plant origin, the acid lipase of castor bean (*Ricinus communis*) is the most extensively investigated lipase that exists in dormant seeds (Shahani, 1975). In general, the germ portions of wheat, rye, and barley contain a much higher level of lipolytic enzyme activity than the endosperm, and the scutellum section and aleurone layer are also reportedly rich in lipase. (Shahani, 1975)

During recent years considerable attention has been devoted to lipases produced by microorganisms, presumably because of their stability and their practical medical and industrial applications. Several microorganisms produce intracellular or extracellular lipases. Especially, extracellular microbial lipases have high potential for application and are appropriated for mass production. A variety of microorganisims produce lipases. These include the genera of *Candida* yeast; *Rhizopus, Penicillium*, *Aspergillus*, *Geotrichium* and *Mucor* molds; and *Pseudomonas*, *Achromobacter*, and *Staphylococcus* bacteria (Godtfredsen, 1993).

2.2 Function of lipases

Lipases catalyze three types of reaction. The catalytic action of lipases is reversible. It catalyzes ester synthesis in a microaqeous system. However, in view of biotransformation of oleochemical industry yielding value-added products, the transesterification action seems more worthwhile than hydrolysis and ester synthesis. The difference in free energy involved in TAG hydrolysis is quite small and the net free energy of transesterification is zero. Consequently, transesterification reaction takes place easily. Transesterification is categorized into four subdivisions according to the chemical species with which the ester reacts. Some researchers designate these four types of reaction by

"interesterification" but Yamane (1987) prefers transesterification to interesterification as the technical term covering all four types of reaction because in biochemistry transfer of a group from one chemical species to another is called "trans", such as transglycosylation, transpeptidylation, transphophatidylation. Therefore, the author confines the term interesterification only to the type 3 reaction (ester exchange) (Yamane, 1987).

However, Gandhi (1997) suggested that lipase catalyzed reaction has classically been divided into two main categories: (i) hydrolysis, and (ii) synthesis. Reactions under synthesis category can be further separated: (a) esterification, (b) interesterification, (c) alcoholysis and (d) acidolysis (Figure 1). The last three reactions are often grouped together into a single term, viz, transesterification.

(i) Hydrolysis

$$RCOOR' + H_2O \longleftrightarrow RCOOH + R'OH$$

(ii) Synthesis

(a) Esterification

$$RCOOH + R'OH \longleftrightarrow RCOOR' + H_2O$$

(b) Interesterification

$$RCOOR' + R''COOR^* \longleftrightarrow RCOOR^* + R''COOR'$$

(c) Alcoholysis

$$RCOOR' + R''OH \longleftrightarrow RCOOR'' + R'OH$$

(d) Acidolysis

$$RCOOR' + R''COOH \longleftrightarrow R''COOR' + RCOOH$$

Figure 1 Types of reaction catalyzed by lipases

Source : Gandhi (1997)

2.3 Specificity of lipases

Macrae (1983) divided lipases into three groups according to their specificity. The first group shows no marked specificity both as regards the position on the glycerol molecule which is attacked and the nature of the fatty acid released. The second group of lipases catalyzes the release of fatty acids specifically from the outer 1- and 3-positions of glycerides. The third group of lipases catalyses the specific release of a particular type of fatty acid from glyceride molecules.

However, Malcata *et al*. (1992) divided lipases into five major types : (i) lipid class, (ii) positional, (iii) fatty acid, (iv) stereochemical and (v) combinations thereof.

Lipid class selectivity has been observed in animal plasma which apparently contains separate lipoprotein lipases for the hydrolysis of TAG, DAG and MAG. A lipase produced by a strain of *Penicillium cyclopium* has been shown to display its highest activity on MAG and much lower activity toward DAG and TAG. This type of selectivity is dependent on temperature for a lipase from *Pseudomonas fluorescens* (Malcata *et al*.,1992).

Lipases obtained from natural sources can be positionally nonspecific or display one of two kinds of positional specificity: *sn*-1,3 specific or *sn*-2 specific. Non-specific lipases hydrolyse all three ester bonds of triglycerides equally well. Nonspecificity has been observed for lipases from *Chromobacterium viscosum*, *Pseudomonas fluorescens*, *Candida cylindracea*, *Geotrichum candidum*, and *Penicillium cyclopium*, and also for hepatic lipase. Specificity of the *sn*-1,3 type is associated with the preferential release of fatty acid residues from the terminal positions of the glycerol backbone rather than from the central carbon atom, whereas *sn*-2 specificity refers to preferential release from the central carbon atom. The *sn*-1,3 type of specificity has been observed for pancreatic and adipose tissue lipases and lipase from microorganism such as *Rhizopus arrhizus*, *Aspergillus niger*, *Rhizopus delemar*

and *Mucor miehei*. The *sn*-2 specificity is extremely rare, and it has been ascribed to a lipase from *Geotrichum candidum* which has a particular ability to hydrolyse oleic and linoleic acids from the *sn*-2 location. A more general classification states that the positional specificity of lipases is not divided clearly into the above categories; instead it changes continuously from highly specific an-1,3 activity to a very weakly specific or completely nonspecific activity (Malcata *et al*.,1992).

Lipase often exhibit a particular ability to release fatty acids whose chain lengths or degrees of unsaturation fall within well-defined ranges. This situation has been explored in the lipase-catalysed production of cheese-type flavors. The lipase from *Mucor miehei* exhibits similar activities for release of C_4 and C_6 acids at either pH 5.3 or 8.0; however, longer chain fatty acids are released more slowly at the acidic pH. Experimental data for the fatty acid specificities of lipases from *Chromobacterium viscosum* and *Geotrichum candidum* indicate that the rate of attack by these lipases follows a bell-shaped distribution in the number of carbon atoms in the hydrocarbon backbones of unsaturated fatty acid residues. The distribution is centered at C_8-C_{10} (Malcata *et al*.,1992).

Although early studies did not report evidence of any kinds of stereospecificty for the catalytic action of lipases on fats and oils, a rather large body of literature dealing with the preparation of chiral esters and alcohols via lipase-mediated kinetic resolution of racemic (nontriglyceride) substrates is currently available. A number of researchers have observed stereoselectivity for the catalytic action of lipases on such substrates as straight-chain secondary alcohols, acetonide and butyric acid optically active esters, oxazolidones, glycidyl ethers, cyclohexanols, 2-benzylglycerol ether, sugar alcohols, and several esters of ibuprofen. Stereoselectivity has also been observed with serum lipoprotein lipase for hydrolysis of the enantiomeric esters at the *sn*-1 position, and with human and rat lingual lipases for the hydrolysis of enantiomeric esters

at the *sn*-3 position. Finally, combinations of fatty acids and stereoselectivities have been found with rat lingual and a human lipoprotein lipase preparation. (Malcata *et al*.,1992)

Moreover, Diks and Bosley (2000) divided lipases into three classes based on their specificity and selectivity (Table 2): 1) Regio- or positional specific; 2) Fatty acid type specific; 3) Specific for a certain class of acylglycerols, i.e., MAG, DAG or TAG.

Table 2 Major specificities of lipases and their applications

Specificity	Lipases	Production of
Regio specificity		
1,3-Regio specific	*Rhizomucor miehei* *Rhizopus oryzae*	Triglyceride synthesis
	Rhizopus arrhizus	1,2(2,3)-diglycerides by triglyceride hydrolysis
	Rhizopus delemar *Rhizopus niveus*	1,3-diglyceride by fatty acid (directed) esterification
	Porcine pancreatic lipase	2-monoglycerides by triglyceride hydrolysis 1(3)-monoglycerides by fatty acid esterification
Non-specific	*Candida rugosa* *Chromobacterium viscosum*	Fatty acid production by hydrolysis
	Pseudomonas fluorescens *Pseudomonas cepacia*	mono- and diglycerides by directed glycerolysis
Fatty acid specific		
Long chain poly-unsaturated acids	*Geotrichum candidum* *Candida rugosa*	Selective hydrolysis
Saturated acids	*Fusarium oxysporum*	Selective hydrolysis
cis-Δ9 unsaturated acids	*Geotrichum candidum* B	Selective hydrolysis
Short acids	*Cuphea* sp.	Selective hydrolysis
Acylglycerol specific		
Monoacylglycerols	Potato acylhydrolase (patatin)	Monoglycerides by fatty acid esterification
Mono-and diacylglycerols	*Penicillium camembertii* *Penicillium cyclopium* M1 *Fusarium* sp.	Monoglycerides and diglycerides by fatty acid esterification
Triacylglycerols	*Penicillium roquefortii* *Penicillium cyclopium* M1 *Penicillium expansum*	1,2-Diglycerides by triglyceride hydrolysis or alcoholysis

Source : Diks and Bosley (2000)

2.4 Application of lipase

Lipase can be employed in the production of pharmaceuticals, cosmetics, leather, detergents, foods, perfumery, medical diagnostics, and other organic synthetic materials (Gandhi, 1997). Table 3 lists major application areas for lipases as well as the most important uses of the enzymes in the food sector (Godtfredsen, 1993). Moreover, lipases have found applications in various fields of biotransformations. These can be classified according to the nature of the substrates into three main categories: (i) modification of fats and oils, (ii) acylation/deacylation of carbohydrates and protecting/deprotecting of peptides and (iii) synthesis of chiral compounds. The major focus will be given to the first field. These can be classified depending on the targeted product into the synthesis of MAG, an important class of emulsifiers and the synthesis of structured triglycerides, which are used as, e.g., cocoa-butter equivalents or in nutrition. Furthermore, lipases have found some special applications such as in the selective enrichment of specific fatty acids.

3. Immobilized lipases

Lipases are spontaneously soluble in aqueous solutions (as a result of their globular protein nature), but their natural substrates (i.e., lipids) are not. Although 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 pseudohomogeneous reaction systems poses technological difficulties (viz. contamination of the products with residual enzymatic activity) and economic difficulties (viz. use of the enzyme for a single reactor pass). The former leads to constraint on the product level because the final characteristics of the product depend on such postprocessing conditions as storage time and temperature. The latter leads to constraints on the process level because the useful life of the enzyme is restricted to the space time of the reactor (on the assumption that the space time is small compared with the time scale associated

with deactivation of the enzyme). 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).

Table 3 Industrial application areas for microbial lipases

Industry	Effect	Product
Dairy	Hydrolysis of milk fat	Flavor agents
	Cheese ripening	Cheese
	Modification of butter fat	Butter
Bakery	Flavor improvement and shelf life prolongation	Bakery products
Beverage	Improved aroma	Beverages
Food dressing	Quality improvement	Mayonnaise, dressings, and whipped toppings
Health food	Transesterification	Health foods
Meat and fish	Flavor development and fat removal	Meat and fish products
Fat and oil	Transesterification	Cocoa butter, margarine
	Hydrolysis	Fatty acids, glycerol, mono- and diglycerides
Chemical	Enantioselectivity	Chiral building blocks and chemicals
	Synthesis	Chemicals
Pharmaceutical	Transesterification	Specialty lipids
	Hydrolysis	Digestive aids
Cosmetic	Synthesis	Emulsifiers, moisturing agents
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper products
Cleaning	Hydrolysis	Removal of cleaning agents, e.g., surfactants

Source: Godtfredsen (1993)

Lipases are normally used in an immobilized form in industry because reuse or continuous use of the immobilized lipase is made possible and the separation of the product from the enzyme is easy. The stability of lipase is often increased by immobilization. The advantages of the various types of available enzyme reactors can also be more readily exploited by using immobilized lipases, especially the use of packed-bed reactors. Brady *et al*. (1988) searched for adsorbents suitable as supports for lipases. Adsorbents, such as celite, cellulose, ethyl cellulose, silica gel, kieselguhr, clay, alumina, CPG-100, carbon, Accurel, Celgard 2500, Profax PP, Microthene HDPE, etc., were screened as possible immobilization supports. Most of hydrophilic materials were found to decrease tremendously the lipase activity upon immobilization. On the other hand, hydrophobic microporous materials such as Accurel and Celgard 2500 were found to provide better performances (Brady *et al*., 1988). In the Table 4, several immobilized lipases for MAG production are listed.

4. Kinetics of reaction catalysed by immobilized lipases

The simplest kinetic model of lipase-catalysed reactions of ester bonds can easily be derived from the classic Michaelis-Menten mechanism (Malcata *et al*., 1992).

$$E + S \underset{k_{-1}}{\overset{k_1}{\longleftrightarrow}} ES \xrightarrow{k_{cat}} E + P + Q \qquad (1)$$

where E denotes the immobilized enzyme, S the substrate (glyceride), ES the enzyme-substrate complex, P product species containing one more alcohol moiety than reactant S, and Q a free fatty acid. The rate of disappearance of glyceride per unit volume of reacting fluid ($-dC_S/dt$) can be represented in term of this mechanism as,

$$\frac{-dC_S}{dt} = \frac{V_{max}\, C_S}{K_m + C_S} \qquad (2)$$

The kinetic parameters appearing in this expression are defined as $V_{max} = k_{cat}C_{E(tot)}$ and $K_m = (k_{cat} + k_{-1})/ k_1$. The parameter V_{max} is the rate observed when the lipase is saturated with substrate, K_m is the Michaelis-Menten constant, and the subscript *tot* denotes the total amount of enzyme present in either the E or ES form (Malcata *et al*., 1992).

For a simple enzymatic reaction in soluble, the maximum intrinsic rate of reaction is limited by the rate at which lipase and substrate come together in the proper orientation. For the case of hydrolysis reaction, the substrate is often part of the disperse phase of an emulsion, a micelle, or a monolayer which contacts water. These structures may be orders of magnitude larger in size than the supported enzyme for the case where the carrier exists in powdered form. Thus, the maximum attainable rate is limited by the amount of lipase which can interact with the substrate continuum. This phenomenon, which is similar in nature to an adsorption process, can be schematically represented by equation (1) provided that k_1 and k_{-1} are interpreted as adsorption (k_{ads}) and desorption (k_{des}) constants, respectively (Malcata *et al*., 1992).

In the case of lipases immobilized on continuous supports, or on discrete supports larger in size than the individual droplets of substrate, the above reasoning remains valid provided that the spacing between neighboring molecules of immobilized lipase is still larger than the area of contact between the droplet and the lipase carrier. For this situation, in contrast to the classic Michaelis-Menten rate expression where the reaction rate increases linearly with the total enzyme concentration, a limiting rate is approached as the lipase concentration is increased. In the present case a balance on the total number of adsorption sites is more relevant than a balance on the total number of active sites. This approach leads one to the following rate expression :

$$\frac{-dC_S}{dt} = \frac{V_{max}\ C_E}{K_m + C_E} \qquad (3)$$

where the constants are defined as $V_{max} = k_{cat}C_{S(tot)}$ and $K_m = k_{des} / k_{ads}$, and where the physical interpretations of the constants are as follows: V_{max} is the rate when the adsorption sites on the surface of the fat globule are saturated with lipase, and K_m is a pseudo-Michaelis-Menten constant for the above rate expression. When a lipase from *Candida rugosa* was immobilized by adsorption on cellulose, values for V_{max} and K_m changed from ca. 6.48 to 2.92 mol/min, and from ca. 3.88 to 0.54 mg/mL, respectively. The primary mechanistic distinction between this mechanism and the simple Michaelis-Menten mechanism is that in the present case adsorption of lipase at the fluid solid interface (i.e., contact with the substrate molecules) is independent of catalysis in the interfacial plane. Observed K_m values for lipases may thus reflect the extent of adsorption of the lipase at the lipid/water interface rather than the affinity between enzyme and substrate at the active site (Malcata *et al*., 1992).

5. MAG production and application

MAG are the most widely used as emulsifiers in the food, pharmaceutical, and cosmetic industries (Thude *et al*.,1997). In phamaceuticals, MAG are used as binders in tablets and as emollients for transdermal, slow-release drugs (Jackson and King, 1997). Li and Ward (1993) reported that MAG containing n-3-polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may have positive preventive effects on people with different cardiovascular disorders. In the food industry, MAG and DAG are the most common food emulsifiers (McEvily and Zaks, 1991). They serve to stabilize emulsions in sauces and baked goods (Jackson and King, 1997). In cosmetic industries, they used as texturising agents in cosmetics (Stevenson *et al*., 1993). Monopentadecanoylglycerol is used as a hair care additive (Bornscheuer, 1995). MAG of octanoic and decanoic acid can act as dyes and perfume bases in cosmetics, toiletries, and phamaceuticals. They are also known to dissolve gallstones in humans (Gandhi, 1997). Furthermore, oleyl monooleate is used in bath oils, cosmetic creams and lotions, hair preparations, makeup, skin preparations and pharmaceuticals, etc (Gandhi, 1997).

In the current method, MAG are manufactured on an industrial scale by continuous chemical glycerolysis of fats and oils at high temperature (220-250°C) employing inorganic alkaline catalysts under a nitrogen gas atmosphere (Sonntag, 1982). The product produced by this strategy has several drawbacks (Bornscheuer, 1995). A molar excess of glycerol is used and because the reaction temperature is greater than 220 °C, dark-colored by-products with an undesirable flavor are formed. Moreover, the yield of MAG is rather low (30-40%) (McNeill and Yamane, 1991). Molecular distillation is necessary because MAG need to be highly pure in the food industry, since they have better emulsifying properties than a mixture of different acylglycerols (Bornscheuer, 1995).

Unlike ordinary chemical catalysts, enzymes have ability to catalyze reactions under very mild conditions in aqueous solution at normal temperature and pressure reducing the possibility of damage to heat-sensitive substrates and also reducing the energy requirements and corrosion effects of the process. Enzymes have high substrate specificity leading to a low content of undesirable by-products in the reaction solution, thus decreasing not only material costs but also downstream environmental burdens (Kennedy and Cabral, 1987).

Three lipase-catalyzed routes to MAG have been described: (a) hydrolysis or alcoholysis of triglycerides, (b) glycerolysis of triglycerides and (c) esterification or transesterification of glycerol with fatty acids or esters. Method (a) yields an 2-MAG, while methods (b) and (c) usually yield an equilibrium mixture of MAG, from which the predominant 1(3)-MAG can be isolated in good yield (Bornscheuer, 1995). There are several MAG production by lipases listed in Table 5.

5.1 Hydrolysis or alcoholysis of triglycerides to produce 2-monoglycerides

Hydrolysis or alcoholysis of triglycerides catalyzed by a 1,3-selective lipase yield 2-MAG (Figure 2). Hydrolysis gave moderate yield of 2-MAG (78%, corresponding to 26% per mol TAG) (Holmberg and Osterberg, 1988). Acyl migration probably limited the yield by forming 1(3)-MAG which underwent further hydrolysis. On the other hand, alcoholysis can be carried out in non-polar solvents where acyl migration is slower. For this reason, alcoholysis of triglycerides gave higher yield (75-97%, corresponding to 25-32 % per mol TAG) than hydrolysis. In addition, alcoholysis reaction is often faster because there is no change in pH during the reaction and less inhibition of lipase by free fatty acids.

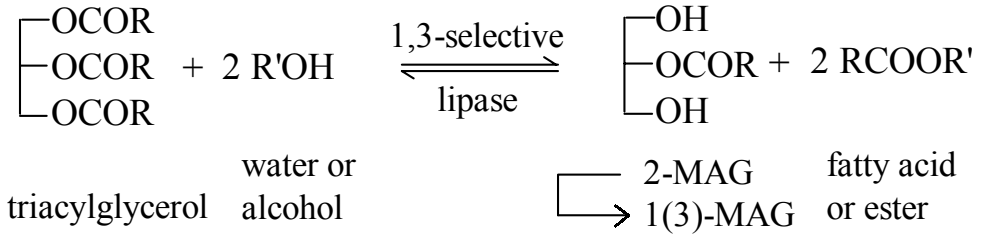

Figure 2 Hydrolysis or alcoholysis of a triacylglycerol to produce 2-MAG (acyl migration as indicated by the arrow might lead to 1(3)-MAG)

Source: Bornscheuer (1995)

5.2 Glycerolysis of triglycerides with glycerol

Compared to hydrolysis or alcoholysis, a glycerolysis of TAG is the most economic method, because all three fatty acids bond to the TAG are converted into product (Figure 3) (Yamane *et al*., 1986). One can use non-selective lipases for this reaction because even a 1,3-selective lipase yield one 2-MAG and two 1(3)-MAG. In practice, reaction conditions usually promote acyl migration so that an equilibrium mixture of 9:1, 1(3)-MAG : 2-MAG is formed.

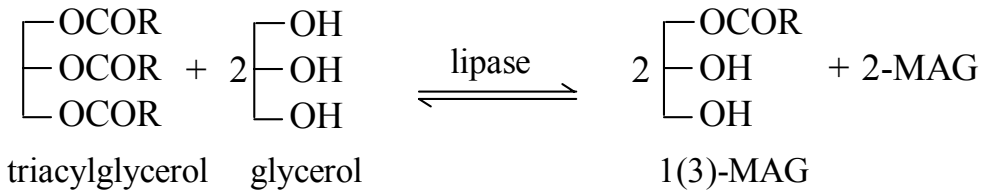

Figure 3 Glycerolysis of a triacylglycerol produces monoacylglycerol

Source: Bornscheuer (1995)

Glycerolysis of triglycerides in the liquid phase typically yield only 30-50% MAG, due to an unfavored equilibrium. A substantial shift of the equilibrium in the glycerolysis can be achieved by performing the reaction in a solid-phase system (McNeill *et al*., 1990). Initially, the reaction is carried out in a liquid-liquid-emulsion of glycerol and triglyceride, then cooled to

crystallize 1(3)-MAG. Yield with this method increased to 70-99% depending on the triglyceride serving as substrate (McNeill *et al*., 1990; McNeill and Yamane, 1991; Bornscheuer and Yamane, 1994). This crystallization also increases the relative amount of 1(3)-MAG over 2-MAG. Reaction temperature is critical and must be kept just below the melting temperature of the monoglycerides, e.g., for beef tallow, optimum temperature was 42 °C yielding 72% 1(3)-MAG using PFL or CVL (McNeill *et al*., 1990).

The disadvantage of glycerolysis in the solid state is the difficulty to perform the reaction in a continuous manner, which requires a liquid phase. Under these conditions, considerably lower yields of MAG are achieved. To circumvent this problem, off-line extraction of MAG into hexane, filtration of the enzyme and recycling of the non-monoglyceride portion with fresh tallow for the next reaction cycle gave 69% MAG yield (Stevenson *et al*., 1993). Performing the reaction in a continuous stirred vessel bioreactor with polysulfone membrane for the glycerolysis of olive oil in AOT/isooctane reverse micelles using a lipase immobilized on liposomes resulted in a high operational stability of 45 days, however, six days were necessary to reach the steady state (Chang *et al*., 1991).

5.3 Esterification of glycerols with fatty acid or fatty acid esters

Esterification of glycerol with a fatty acid or a fatty acid ester also yields MAG without wasting fatty acids (Figure 4). Reaction mixtures contain polar protic reactants that promote acyl migration, so esterification yields an equilibrium mixture of MAG. To shift the reaction toward MAG formation, water or alcohol was removed using vacuum or molecular sieves (Miller *et al*., 1988).

The low solubility of the glycerol in non-polar organic solvents slows the reaction and promotes a side reaction –further acylation of the more soluble MAG to DAG. To minimize this problem different solvents (Akoh *et al*., 1992; Kwon *et al*., 1995), reverse micelles (Hayes and

Gulari, 1991; Bornscheuer *et al.,* 1994), or hollow-fiber membrane reactors (Padt *et al*., 1992) have been used.

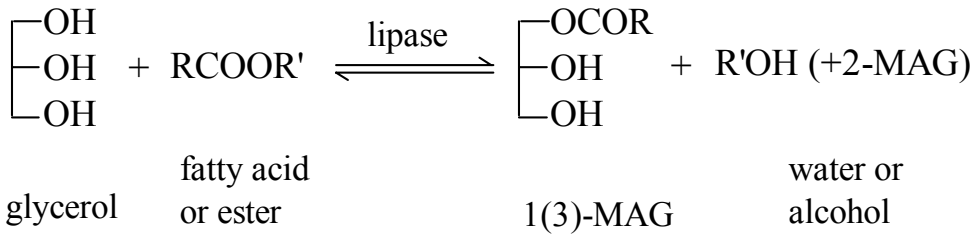

Figure 4 Esterification of glycerol yield a mixture of 1(3)- and 2-MAG

Source: Bornscheuer, 1995

6. The influence of reaction condition on glycerolysis

6.1 Sources of lipases

Various lipases of commercial origin were tested with respect to their glycerolysis activities by Yamane *et al*. (1986). The amounts of enzyme giving the same hydrolytic activity were used in all of the experiments. They found that lipases produced by *Alcaligenes* sp., *Arthrobacter ureafaciens*, *Phycomyces nitens*, *Pseudomonas fluorescens* and *Rhizopus delemar* gave relatively high conversion, while lipases produced by *Aspergillus niger*, *Candida cylindracea*, *Mucor javanicus* and *Penicillium cyclopium* gave vary low conversions. Especially, *Chromobacterium viscosum* lipase gave the highest conversion.

Chang and Rhee (1991) also found that although in all experiments the amounts of hydrolytic activity were equivalent their glycerolysis activity were quite inconsistent. Lipases from *C.viscosum*, *P. fluorescens*, *Pseudomonas* sp. gave high conversion, while lipases from *R. delemar* and *R. javanicus* gave relatively low conversion. Among them, lipase from *C. viscosum* showed the highest glycerolysis activity. Bornscheuer and Yamane (1991) concluded that high concentration of MAG was obtained with bacterial lipases. Yeast and mold lipases were unsuitable for the reaction because of the rapid inactivation in the reaction mixture.

6.2 Initial water content

To activate the lipase catalyst, it is essential to dissolve a trace amount of water in the glycerol phase (Li and Ward, 1993). A similar requirement has been previously reported for other lipase catalyzed reaction where a low water concentration was desired (Macrae, 1983). However, the moisture content of the glycerol phase must be maintained at low levels to avoid excessive production of FFA. As much as 12 % FFA is produced when greater than 8 % water is dissolved in the glycerol phase. Moreover, the yield of MAG is considerably reduced when 12 % water was used (McNeill *et al*., 1990). For glycerolysis of hydrogenated beef tallow, FFA content at equilibrium depended on the water concentration in the glycerol phase. The initial rate of FFA formation was low and hardly affected by the moisture content between 0.5 and 4 %, but at higher water content (4-6.7 %), there was a small increase in the rate (Yamane *et al*., 1994). Yang and Parkin (1994) found that yield of MAG formation by glycerolysis of butter oil using a gel-entrapped lipase increased with increasing water content up to 10 % in glycerol.

6.3 Glycerol/triacylglycerols molar ratio

Theoretically, 3 mol MAG were obtained when 1 mol TAG and 2 mol glycerol were used as substrates for glycerolysis. Bornscheuer and Yamane (1994) found that 96 % MAG was achieved when glycerol/triolein molar ratio of 2.7 was used for glycerolysis of triolein in solid-phase system.

A yield of 70 % MAG was obtained when glycerol/tallow mole ratio ranging from 1.5 to 2.5 was used in glycerolysis of tallow. The yield of MAG was independent of the glycerol/fat mole ratio at 5:1 or greater (McNeill *et al*, 1990). Furthermore, Yamane *et al*. (1994) reported that at low glycerol to tallow molar ratio (1:2), the main DAG product was obtained, Brady *et al.* (1988) and Yang and Rhee (1991) also reported that glycerol stabilized the lipase dramatically as the glycerol concentration was increased.

6.4 Temperature

McNeill *et al*. (1990) reported that the yield of MAG was greatly influenced by the reaction temperature. At higher temperature (48-50 °C), a yield of approximately 30 % MAG was obtained, while at lower temperature (38-46 °C), a yield of approximately 70 % MAG was obtained. A sharp transition was observed between the high and low yield equilibrium states. The critical temperature (Tc) was found to be 46 °C in the case of tallow. Moreover, McNeill and Yamane (1991) tried to improve the yield of MAG during enzymatic glycerolysis. They found that temperature programming was the most effective. With an initial temperature of 42 °C for 8-16 h followed by incubation at 5 °C for up to 4 days, a yield of approximatelly 90 wt% MAG was obtained from beef tallow, palm oil and palm stearin. For high melting point fats, Tc is 35-45 °C, but for liquid oils Tc is 5-10 °C (McNeill and Yamane, 1991).

6.5 Organic solvents

Fukui *et al*. (1990) suggested that for bioconversion of various lipophilic or water-insoluble compounds, it is essential to introduce organic solvents into reaction systems to improve the solubility of these reactants. Furthermore, organic solvents can shift the reaction equilibrium towards synthesis in the case of hydrolytic enzyme by reducing the water content in the systems. However, organic solvents produce various physicochemical effects on enzyme molecules and the effects differ depending upon the kinds of organic solvents and enzyme used.

Nonpolar solvents such as isooctane were used for hydrolysis (Holmberg and Osterberg, 1988; Kosugi *et al*., 1990; Yang and Rhee, 1992), esterification (Li and Ward, 1993) and glycerolysis (Chang *et al*., 1991). Furthermore, Li and Ward (1993) found that a higher extent of esterification was observed in isooctane and hexane as compared to other solvents but more

polar solvents, such as benzene and acetone were unsuitable for the synthetic reaction. However, Yang and Rhee (1992) found that immobilized lipase was less stable in hexane than in isooctane.

7. Bioreactors for MAG production

Various possibilities exist here for reactors containing immobilized lipase, but most situations fall within one of the following cases: a) a lipase contained in an aqueous phase and confined by a surfactant liquid membrane within an organic liquid phase (Chane *et al*., 1991; Hayes and Gulari, 1991; Singh *et al.*, 1994), b) a lipase entrapped within a three dimensional polymeric matrix dispersed within an organic liquid phase or an aqueous phase (Yang and Parkin, 1994), and c) a lipase attached to a solid support and dispersed within an organic liquid phase or an aqueous phase (Stevenson *et al*., 1993; Bonscheuer and Yamane, 1994; Padt *et al*., 1992)

By the use of lipases in immobilized form for MAG production, the biorectors (Figure 5) can be used in one of serveral possibilities, namely, in batch stirred-tank reactors : BSTR (Figure 5a) (Stevenson *et al*., 1993; Bornscheuer and Yamane, 1994; Yang and Parkin, 1994; Myrnes *et al*., 1995; Kamlangdee and Yamane, 1996; Thude *et al.*, 1997), packed-bed reactors : PBR (Figure 5b) (Gancet, 1990; Stevenson *et al*., 1993; Jackson and King, 1997), continuous stirred-tank reactors : CSTR (Figure 5c) (Chane *et al*., 1991), fluidized-bed reactors : FBR (Figure 5d) (Kosugi *et al*., 1990; Kosugi *et al*., 1995) or membrane reactors : MR (Figure 5e) (Padt *et al*., 1992; Ohta *et al*., 1989). There are several reactors for MAG production by lipases listed in Table 6.

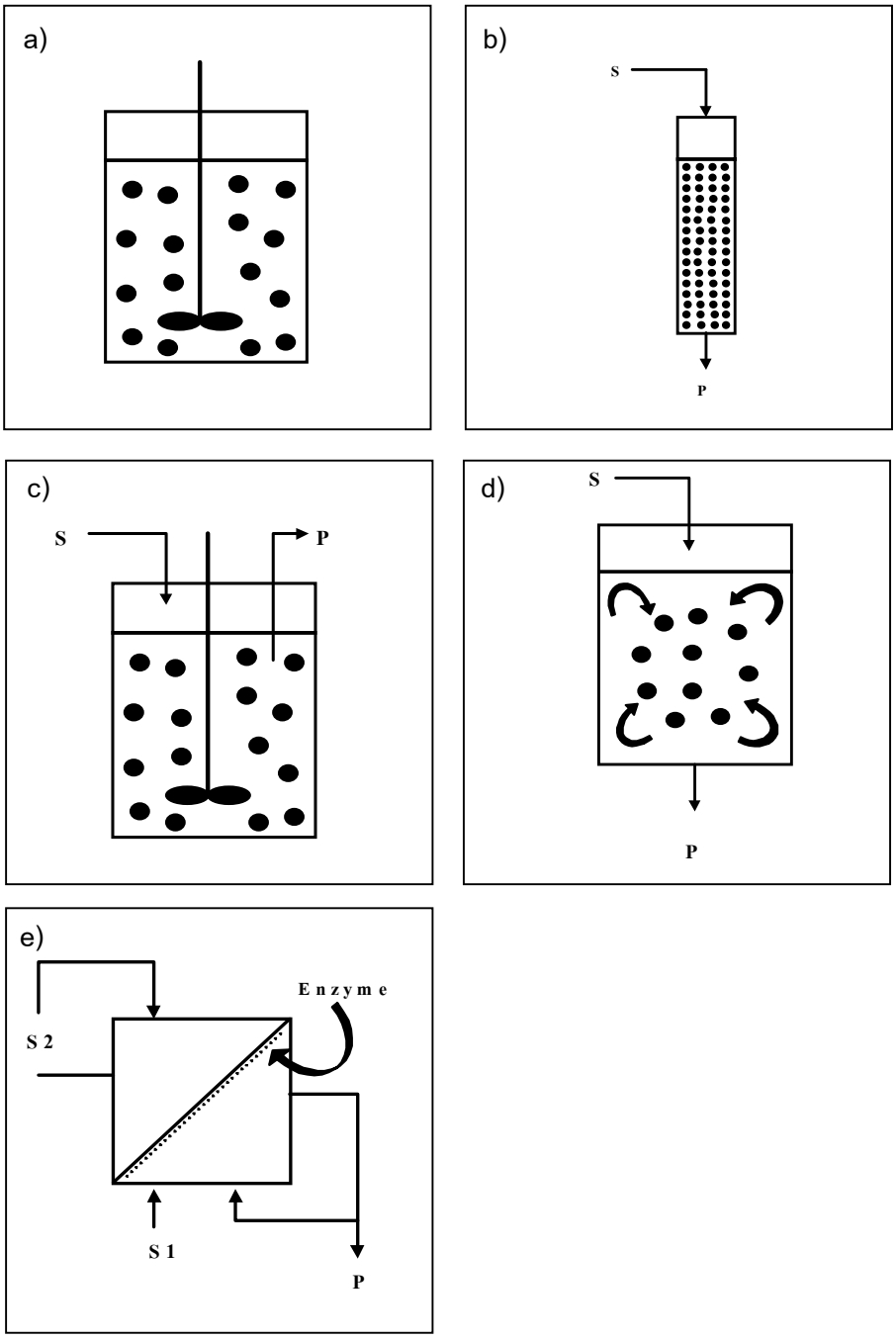

Figure 5 Enzyme reactor designs

a) BSTR b) PBR c) CSTR

d) FBR e) MR

•, immobilized enzyme; S, substrate; P, product

7.1 Batch stirred-tank reactors (BSTR)

BSTR is the reactor most commonly used. This reactor operates batchwise and consists of a vessel in which the reactant fluid mixture is stirred by some mechanical means (e.g., magnetic bars, submerged impellers, reciprocal oscillators, or end-over-end rotators). The immobilized enzyme is separated from the reaction medium at the end of the reaction by filtration or centrifugation. These reactors are easy to operate (e.g., heat, cool, clean, and maintainance) and normally require a very limited set of auxiliary equipment. However, overall volumetric through put is relatively low because of dead times taken up by the operations of emptying, cleaning, and filling. So an economic constrain must be considered for large scale use (Balcao *et al*., 1996).

Stevenson *et al*. (1993) studied the glycerolysis of tallow by lipozyme (immobilized *Mucor meihei* lipase) in BSTR at the reaction temperature of 50 °C for 24 h with a maximum yield of 35 % MAG. Cooling the reaction below 42 °C after 48 h resulted in MAG crystallisation which improved the yield to up to 50 %. An increase in the overall MAG yield up to 69 % was achieved by recycling of the non-MAG portion with fresh tallow in other reaction. Bornscheuer and Yamane (1994) studied the glycerolysis of triolein by immobilized lipase in BSTR. It was found that lipase immobilized on celite enhanced the long-term stability of the pure lipase from *Pseudomonas cepacia*. The reaction temperature was 25 °C for the first 8 h, followed by cooling to 8 °C and the yield of 90 % MAG was obtained after 48 h. Yang and Parkin (1994) studied MAG production from butteroil by glycerolysis with a gel-entraped *Pseudomonas* sp. lipase in microaqueous media. They found that optimum conditions for gel-entraped lipase-mediated glycerolysis of butteroil were 35-40 °C, acyl group:glycerol molar ratio 0.30-0.70, and 4-6% water in the glycerol phase, resulting in the formation of 60 % MAG after 72 h. Myrnes *et al*. (1995) employed glycerolysis of marine oils by lipase AK from *Pseudomonas* sp. in solvent-free BSTR with magnetic stirring. The yield of

MAG 42-53 % was obtained. Thude *et al.* (1997) studied lipase-catalyzed glycerolysis of Campher tree seed oil and Cocoa-butter in BSTR. From nine microbial lipases, lipases from *Pseudomonas cepacia* (PCL) and *Chromobacterium viscosum* (CVL) were suitable for the synthesis of MAG. The glycerolysis of Campher tree seed oil was performed initially at 25 °C followed by cooling to 7 °C resulted in 86% and 90 % MAG by PCL and CVL, respectively. Maximum concentration of MAG from cocoa-butter was 89 % with both lipases, when the reaction was performed at 25 °C.

7.2 Continuous stirred-tank reactors (CSTR)

In CSTR, no gradients of temperature or concentration exist, because of an efficient stirring which promotes intimate contact of the enzyme with the reaction mixture. The immobilized enzyme is retained within the reactor by means of a filter at the outlet. Low costs of construction are normally associated with these reactors, but to achieve similar degrees of conversion, a CSTR must be larger than a PBR, or a cascade should be used instead of a single unit (with concomitant problems of layout space requirements) (Balcao *et al*., 1996).

Chane *et al*. (1991) employed CSTR with polysulfone membrane for the glycerolysis of olive oil in bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT)/iso-octane reverse micelles using immobilized lipase from *C. viscosum*. The lipase was immobilized on liposome (small unilamellar vesicle) as a matrix. After a 1-day delay, a steady state was achieved, resulting in 80 % conversion of olive oil during 6 days of operation. The highest productivity of 1-monoolein (90 μmol mL^{-1} h^{-1} outlet) was obtained at optimum condition and the operational stability of the liposome-lipase was given as 45 days.

7.3 Packed-bed reactors (PBR)

Because of its high efficiency, low cost and ease of construction, operation and maintenance, PBR, also known as fixed-bed reactor, has traditionally been used for most large-scale catalytic reactions. In this reactor,

the granules of immobilized enzyme are usually packed within a jacketed 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 extensive bypassing) or downward (to take advantage of gravitational forces), whereas in a biphasic situation the two phases may be pumped in opposite directions (countercurrent flows) with the most dense flowing downward, or in the same direction (cocurrent flows). Great 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 (Balcao *et al*., 1996).

Gancet (1990) developed a two-fixed bed segment for the continuous glycerolysis of beef tallow. At a residence time of 75 min, a yield of 38.8 g MAG/100 g beef tallow was obtained. The reactor system was run for several months with a rather good operational stability of the mycelial lipase from *Rhizopus arrhizus*. In continuous system, Stevenson *et al*. (1993) reported the glycerolysis of tallow by lipozyme (immobilized *Mucor meihei* lipase) in a column reactor without the addition of surfactants or emulsifiers at 50 °C. The reaction was completed within 8 h and the final yield of MAG was 17- 19 %. Jackson and King (1997) described the transesterification of soybean oil with glycerol, 1,2-propanodiol, and methanol by an immobilized lipase from *Candida antarctica* in flowing supercritical carbon dioxide for the synthesis of MAG. MAG was obtained at up to 87 wt%.

7.4 Fluidized-bed reactors (FBR)

A FBR is, in a sense, a hybrid of a CSTR and a PBR, for which the upward linear velocity of reacting fluid is above the terminal velocity of the bed of enzyme. The usually large pump size and power requirement, nevertheless prevent extensive use of this type of reactor.

The process that used immobilized lipase in FBR for MAG production has not been reported. However, FBR with immobilized lipase has been used

for fatty acid and glycerol production. For example, Kosugi *et al*. (1990) described the FBR with immobilized lipase for fatty acid and glycerol production by continuous hydrolysis of oil. The FBR system consists of settling compartment and stirring compartment. Immobilized lipases are placed in stirring compartment. The agitation is provided by a paddle-type stirring blade. Kosugi *et al*. (1995) used counter-current FBR with immobilized lipases for EPA and DHA production by hydrolysis of sardine oil. Pulse agitation was provided from the bottom of the reactor by the pump.

7.5 Membrane reactors (MR)

Membrane or diaphragm reactor may be operated with one or two liquid phases. In this reactor, the enzyme is immobilized onto the membrane, which may take either a flat sheet (Garcia *et al*., 1992) or hollow fiber (Pronk *et al*., 1992; Padt *et al*., 1992). 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 membrane pore plugging are considerably more difficult than packed-bed plugging in a PBR, but the lower pressure drops for a given specific area of reaction usually compensate for such drawback (Balcao *et al*., 1996).

Padt *et al*. (1992) studied the enzymatic synthesis of MAG in MR with an in-line adsorption column. The MR consists of a cellulose hollow-fiber membrane module, an internal oil circuit and an external glycerol-water circuit. Lipase from *Candida rugosa* is adsorbed at the inner fiber site. The oil circuit is operated batch wise. To recover the monoesters produced, a silica 60 column is placed in the oil phase of MR. When no adsorption column is used, the MAG production is about 18 %w/w. The authors implied that MR can be used in a continuous process using a sequence of column. They estimated a production of 60 mol (15 kg) monoester g^{-1} enzyme. The half-life time of the lipase from *Candida rugosa* was given as 50 days. Ohta *et al*. (1989) used the microporous hydrophobic MR to study inhibition and inactivation of lipase by fat peroxide in

continuous glycerolysis of fat by lipase. However, MR with immobilized lipases was used for another application, for example, fatty acid production by hydrolysis of tallow (Taylor *et al*., 1992) and lipolysed butteroil production by hydrolysis of milkfat (Garcia *et al*., 1992)

8. Scale up of MAG production

Transferring to an industrial scale processes successfully developed at the laboratory scale is not a simple procedure. Scale up is defined as the successful startup and operation of a commercial size unit whose design and operating procedures are in part based upon experimentation and demonstration at a smaller scale of operation (Sola and Godia, 1995).

The purpose of scale-up is the selection of design conditions and operational procedures to insure that the effect of the different variables on the process is the same in units of different size. The objective is then to obtain similar yields with the same product distribution (quality). This process starts at the laboratory scale and frequently must continue at the smallest possible scale, for practical and economic reasons. When knowledge of the process is incomplete or when relatively important amounts of product are needed (for market evaluation, regulatory issues, etc.) it is necessary to employ pilot plant of bigger sizes, currently referred to as demonstration units (Sola and Godia, 1995).

Sweere *et al*. (1987) classified scale-up methods as

1. Fundamental method
2. Semi-fundamental method
3. Dimensional analysis/ regime analysis
4. Rules of thumb
5. Scale-down approach/ regime analysis
6. Trial and error
7. Multiplication of elements

Scale-up often results in both technical and economic compromises. When developing a process, it is never possible to achieve a perfect anticipation of its industrial performance, and time and budget limitations make it necessary to take calculated risk in building the first industrial unit (Sola and Godia, 1995).

9. Recovery of MAG

In the microemulsion system to produce MAG will end up with a mixture of nonionic emulsifier, enzyme, solvents, fatty acids and esters. Downstream processing involves several extraction steps and, unfortunately, denaturation of the enzyme takes place. Therefore, the enzyme can be used for one batch only (Padt *et al*., 1992).

Kwon *et al*. (1995) tried to use the difference in solubility of reaction products in hexane. The solubility of MAG and DAG in hexane is very low in comparison with that of fatty acid and TAG, as MAG and DAG have the hydrophilic hydroxyl groups. Hence, hydrophilic products formed from esterification reaction were allowed to precipitate in the reactor.

Stevenson *et al*. (1993) used hexane to recover MAG. The reaction mixture was dissolved in hexane. Unreacted glycerol was separated out and could be removed. After cooling to 4 °C, most of the MAG crystallized out and could be filtered off and dried.

In the solid-phase system to produce MAG, downstream precessing as presented by McNeill and Yamane (1991) involves a heating step to melt the MAG products, which also results in activating the enzyme. In the same system, Thude *et al*. (1997) used chloroform as solvent to suppress a lipase-catalyzed back reaction. Isolation was performed at 4 °C. The organic layer was dried with anhydrous sodium sulfate, filtered and excess solvent was evaporated. Almost pure MAG (purity >95%) was isolated by silica gel chromatography using chloroform for elution.

Padt *et al*. (1992) found that a silica 60 column has shown preferential adsorption of monocaprinate. The adsorption of a mixture of decanoic acid, MAG and DAG is based on two different mechanisms. The decanoic acid will interact with hydroxyl groups at the silica gel surface, which results in a noncompetitive decanoic acid adsorption onto 25 % of the silica gel surface. On the remaining part of the silica gel surface, MAG and DAG adsorb competitively. When a mild eluant is used, such as 5 % ethanol in hexane, only the competitively adsorbed molecules are desorbed. This results in a purification factor of approximately 90 % after desorption.

Objective

To produce MAG using continuous glycerolysis of palm olein with glycerol by immobilized lipase in reactor

Scope of research works :

1. Selection of suitable commercail lipase for MAG production
2. Selection of suitable support to immobilize selected lipase
3. Selection of suitable reactor type for continuous MAG production
4. Optimization of continuous MAG production in selected reactor
4. Scale-up selected reactor for continuous MAG production
5. Selection of suitable method for MAG recovery

Table 6 Reactor for enzymatic MAG production

Reactor	Reaction	Acyl donor	Acyl aceptor	Source of lipase	Reference
BSTR					
	Glycerolysis	Olive oil	Glycerol	*Pseudomonas* sp.	Rosu *et al*., 1997
	Hydrolysis	Palm oil		*Rhizopus delemar*	Holmberg and Osterberg, 1988
	Esterification	Oleic acid	Glycerol	*Penicillium* sp.	Akoh *et al*., 1992
	Glycerolysis	Triolein	Glycerol	*Pseudomonas cepacia*	Bornscheuer and Yamane, 1994
	Glycerolysis	Butter oil	Glycerol	*Pseudomonas* sp.	Yang and Parkin, 1994
	Glycerolysis	Campher tree seed oil	Glycerol	*Pseudomonas cepacia*	Thude *et al*., 1997
	Glycerolysis	Cocco butter Trilaurin	Glycerol	*Pseudomonas cepacia*	Bornscheuer *et al*., 1994
	Glycerolysis	Tallow	Glycerol	*Pseudomonas fluorescens*	McNeill *et al*., 1990
	Glycerolysis	Palm oil	Glycerol	*Pseudomonas* sp.	McNeill and Yamane, 1991
	Glycerolysis	Palm stearin Palm oil	Glycerol	*Pseudomonas fluorescens*	McNeill *et al*., 1991
CSTR					
	Glycerolysis	Olive oil	Glycerol	*Chromobacterium viscosum*	Chang *et al*., 1991
MR					
	Esterification	Decanoic acid	Glycerol	*Candida rugosa*	Padt *et al*., 1992
	Glycerolysis	Butter oil	Glycerol	*Pseudomonas cepacia*	Garcia *et al*., 1996
PBR					
	Glycerolysis	Tallow	Glycerol	*Mucor meihei*	Stevenson *et al*., 1993
	Esterification	Linoleic acid	Glycerol	*Mucor miehei*	Arcos *et al*., 2000

BSTR = batch stirred-tank reactor , CSTR = continuous stirred-tank reactor, PBR = pack-bed reactor, MR = membrane reactor

Table 5 Enzymatic MAG production

Reaction	Acyl donor	Acyl aceptor	MAG Content (%)	Source of lipase	Reference
Hydroysis	Palm oil		80	*Rhizopus delemar*	Holmberg and Osterberg, 1988
Esterification	Palmitic acid	Glycerol	60	*Rhizopus delemar*	Kwon *et al*., 1995
	Decanoic acid	Glycerol	18	*Candida rugosa*	Padt *et al*., 1992
	Oleic acid	Glycerol	98.5	*Penicillium* sp.	Akoh *et al*., 1992
	Linoleic acid	Glycerol	30	*Mucor miehei*	Arcos *et al*., 2000
	Oleic acid	Glycerol	70	*Chromobacterium viscosum*	Berger and Schneider, 1992
	Lauric acid	Glycerol	60	*Rhizopus delemar*	Hays and Gulari, 1991
	Oleic acid	Glycerol	42	*Mucor miehei*	Singh *et al*., 1994
	Oleic acid	Glycerol	44	*Chromobacterium viscosum*	Hoq *et al*., 1984
Glycerolysis	Tallow	Glycerol	35	*Mucor miehei*	Stevenson *et al*., 1993
	Olive oil	Glycerol	90	*Pseudomonas* sp.	Rosu *et al*., 1997
	Trilaurin	Glycerol	99	*Pseudomonas cepacia*	Bornscheuer *et al*., 1994
	Butter oil	Glycerol	61	*Pseudomonas* sp.	Yang and Parkin, 1994
	Triolein	Glycerol	96	*Chromobacterium viscosum*	Bornscheuer and Yamane, 1994
	Olive oil	Glycerol	83	*Chromobacterium viscosum*	Chang *et al*., 1991
	Palm oil	Glycerol	68	*Pseudomonas fluorescens*	McNeill *et al*., 1991
	Tallow	Glycerol	72	*Chromobacterium viscosum*	McNeill *et al*., 1990
	Rapseed oil	Glycerol	28	*Mucor miehei*	Elfman-Borjesson and Harrod, 1999
	Plam oil	Glycerol	79	*Pseudomonas* sp.	Hongpattarakere, 2001
	Butter oil	Glycerol	22	*Pseudomonas* sp.	Garcia *et al*., 1996

Table 4 Lipases immobilization for MAG production

Method of immobilization	Source of lipase	Support or Barrier	Reaction	Reference
Adsorption	*Mucor miehei*	Synthetic resin	Glycerolysis	Stevenson *et al*., 1993
Adsorption	*Chromobacterium viscosum* *Pseudomonas cepacia* *Pseudomonas* sp.	$CaCO_3$ Celite $Ca_2P_2O_7$ $CaSO_4.2H_2O$	Glycerolysis	Rosu *et al*., 1997
Adsorption	*Candida rugosa*	Cellulose	Esterification	Padt *et al*., 1992
Entrapment	*Pseudomonas* sp.	Polyethaleneglycol Polypropyleneglycol	Glycerolysis	Yang and Parkin, 1994
Adsorption	*Pseudomonas cepacia*	Celite Sepharose	Glycerolysis	Bornscheuer and Yamane, 1994
Adsorption	*Chromobacterium viscosum*	Liposome	Glycerolysis	Chang and Rhee, 1991
Adsorption	*Chromobacterium viscosum* *Pseudomonas pseudoalkali*	$CaCO_3$	Glycerolysis	Kamlangdee and Yamane, 1996
Adsorption	*Mucor miehei*	Synthetic resin	Glycerolysis	Elfman-Borjesson and Harrod, 1999
Adsorption	*Rhizopus arrhizus*	Celite	Alcoholysis	Millqvist *et al*., 1994
Adsorption	*Mucor miehei*	Synthetic resin	Esterification	Singh *et al*., 1994
Adsorption	*Pseudomonas* sp.	$CaCO_3$ Celite	Glycerolysis	Hongpattarakeree, 2001
Adsorption	*Pseudomonas* sp.	Polypropylene	Glycerolysis	Garcia *et al*., 1996