**UNIVERSITY OF GAZİANTEP**
**GRADUATE SCHOOL OF**
**NATURAL & APPLIED SCIENCES**

# ENZYME CATALYZED SYNTHESIS OF LOW CALORIE TRIGLYCERIDES

**M. Sc. THESIS**
**IN**
**FOOD ENGINEERING**

BY

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# Enzyme Catalyzed Synthesis of Low Calorie Triglycerides

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**University of Gaziantep**

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Hasene KESKİN

**ABSTRACT**

**ENZYME CATALYZED SYNTHESIS of LOW CALORIE TRIGLYCERIDES**

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**M.Sc. in Food Engineering**
**Supervisor: Prof. Dr. Sibel FADILOĞLU**
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Acidolysis reaction of triolein with caprylic and palmitic acids was performed using immobilized *sn*-1,3 specific lipase from *Mucor miehei* to produce a reduced calorie spreadable structured lipid (SL).

Response surface methodology was applied to model and optimize the reaction conditions using a four factor five level central composite rotatable design. The selected factors were time (10-24 h), enzyme load (10-25 wt%), substrate mole ratio (Triolein:Caprylic acid:Palmitic acid), (1:1:1-1:2.5:2.5) and temperature (45-60°C). Best-fitting models were successfully established for the produced triacylglycerols ($R^2$=0.92-0.96) and residual triolein ($R^2$=0.95) established by multiple regressions with backward elimination.

The produced SLs were compared to fat extracts of commercial soft and hard margarines in terms of melting profile and solid fat content (SFC). The caloric values of produced structured lipids were determined using mathematical equations. SL with a melting peak of 42°C and SFC of 40.69% at 0°C was found very similar to soft margarines. The caloric value of this SL was determined as 37.74 kJ/g, theoretically. The optimum reaction conditions were found as reaction time 14 h; substrate mole ratio 1:2.1:2.1; temperature 58°C; and enzyme load 15 wt%. Under these optimum conditions, the product contained 29.68% COC, 25.47% POC, and 3.80% POP.

The results of this system revealed that it is possible to produce a reduced calorie spreadable fat from vegetable oils rich in triolein.

**Key words:** Enzymatic acidolysis, triolein, caprylic acid, palmitic acid, *Mucor miehei*, response surface methodology

## ÖZ

### ENZİM KATALİZÖRLÜĞÜNDE DÜŞÜK KALORİLİ TRİGİLİSERİT SENTEZİ

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**Tez Yöneticisi: Prof. Dr. Sibel FADILOĞLU**
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Kalorisi düşürülmüş sürülebilen bir yapılandırlmış yağ üretmek amacıyla triolein ile kaprilik ve palmitik asitlerinin asidoliz reaksiyonları immobilize *sn*-1,3 spesifik *Mucor miehei* lipaz enzimi kullanılarak gerçekleştirildi.

Reaksiyon koşullarının modellemesinde ve optimizasyonunda, dört faktörlü beş seviyeli merkezi bileşik dairesel tasarım kullanılarak yüzey tepki metodu uygulandı. Seçilen faktörler; zaman (10-24 h), enzim miktarı (10-25 w%), substrat mol oranı (Triolein:Kaprilik asit:Palmitik asit), (1:1:1-1:2.5:2.5) ve sıcaklıktı (45-60°C). En iyi uyan modeller üretilen triasilgliseroller için ($R^2$=0.92-0.96) ve kalan triolein ($R^2$=0.95) için geri elimineli çoklu regrasyon ile başarıyla uygulandı.

Üretilen yeniden yapılandırılmış yağlar ticari olarak mevcut yumuşak ve sert margarinlerin yağ ekstreleri ile erime profili ve katı yağ miktarı açısından karşılaştırıldı. Reaksiyon sonucu oluşan ürünlerin kalori değerleri matematiksel olarak hesaplandı. Erime piki 42°C olan ve 0°C'de %40.69 katı yağ içeren yapılandırılmış yağ yumuşak margarinlere benzer özellik göstermiştir. Bu yağın kalori değeri 37.74 kJ/g olarak hesaplanmıştır. Kalorisi düşürülmüş sürülebilen bir yapılandırılmış yağ üretmek için optimum reaksiyon koşulları, reaksiyon zamanı 14 h; substrat mol oranı 1:2.1:2.1; sıcaklık 58°C; ve enzim miktarı 15% olarak bulundu. Optimum reaksiyon koşullarında, ürün 29.68% COC, 25.47% POC, ve 3.80% POP içermektedir.

Çalışmanın sonucu, trioleince zengin bitkisel yağlardan kalorisi düşürülmüş sürülebilir bir yağ elde edilmesinin mümkün olabileceğini göstermiştir.



**Anahtar Kelimeler:** Enzimatik asidoliz, triolein, kaprilik asit, palmitik asit; *Mucor miehei,* yüzey tepki metodu

To my family

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## LIST of SYMBOLS/ABBREVATIONS

| | |
|---|---|
| TAG | Triacylglycerols |
| CA | Caprylic acid |
| CCRD | Central composite rotatable design |
| COC | 1,3-capryloyl-2-oleoyl-glycerol |
| COO | 1-capryloyl-2,3-dioleoyl-glycerol |
| DAG | Diacylglycerols |
| DSC | Differential scanning calorimeter |
| FA | Fatty acid |
| HPLC | High performance liquid chromatography |
| LCFA | Long chain fatty acid |
| LCT | Long chain triacylglycerols |
| MAG | Monoacylglycerols |
| MCFA | Medium chain fatty acid |
| MCT | Medim chain triacylglycerols |
| OOO | Triolein |
| PA | Palmitic acid |
| POC | 1-palmitoyl-2-oleoyl-3-capryloyl |
| POO | 1-palmitoyl-2,3-dioleoyl-glycerol |
| POP | 1,3-dipalmitoyl-2-oleoyl-glycerol |
| RI | Refractive index |
| RSM | Response surface methodology |
| SCFA | Short chain fatty acid |
| SFC | Solid fat content |
| SL | Structured lipid |

# CHAPTER 1

# INTRODUCTION

Fats and oils are the major food components and the main energy source of the human body (Adamczek, 2004). Natural oils are mainly triacylglycerols (TAGs) which differ in their physical and nutritional properties and do not always meet all nutritional recommendations or possess desirable physicochemical properties. Fats and oils are used in human nutrition directly as natural products or, more often, after appropriate modifications (Gunstone, 2004; Adamczek, 2004). SLs are defined as triacylglycerols which are modified to change fatty acid composition and positional distribution in the glycerol backbone (Iwasaki and Yamane, 2000). SLs combine the unique characteristics of component fatty acids such as melting behaviour, digestion, absorption, and metabolism in one triacylglycerol molecule to enhance the role of fats and oils play in food, nutrition, therapeutics and health applications (Akoh and Min, 2002; Osborn and Akoh, 2002; Ciftci et al., 2009).

The fatty acid (FA) composition and their position in the TAG determine the functional and physical properties, metabolic rate, and health benefits of the structured lipid (Koh et al., 2010). The principal variation in fatty acid composition of oils and fats is the chain length and degree of unsaturation of the component FA. This variation in FA composition can dramatically affect the bioavailability and digestibility of oils and fats in infants and adults (Tan and Che Man, 2000). The position of FA in the TAG molecules (*sn*-1, *sn*-2, and *sn*-3) has a significant impact on their metabolism in the body. In general, FAs at the terminal positions of TAG (*sn*-1 and *sn*-3) are hydrolyzed by pancreatic lipase and absorbed, while those at the *sn*-2 position of TAG remain unchanged and are used in the synthesis of a new TAG (Zarevucka and Wimmer, 2009).

The modification of fatty acid composition as well as the regio- and stereo-chemical structure of triacylglycerols changes their physicochemical properties (Adamczek, 2004). The desired melting behavior can be achieved through interesterification of suitable triacylglycerol mixtures with the use of *sn*-1,3 specific lipases (Schmid and Verger, 1998). When short or medium chain fatty acids and long chain fatty acids are incorporated, they can produce TAGs with good spreadability and temperature stability (Osborn and Akoh, 2002).

These modifications can be done chemically or enzymatically. However, enzymatic transesterification is more preferable than chemical synthesis due to its milder reaction condition requirements. Moreover enzymatic modification is providing incorporation of specific fatty acid at specific positions of triacylglycerols in order to target specific diseases,

metabolic conditions and for optimal nutrition for particular population groups (Fomuso and Akoh, 1997; Xu et al., 1998). There are several studies reporting synthesis of structured lipids (Ferreira and Foresti, 2010; Koh et al., 2010; Zhou et al., 2001).

Response surface methodology (RSM) has been an effective and a powerful statistical method for optimizing experimental conditions and investigation of critical processes by reducing the number of experimental trials (Myers and Montgomery, 2002). The optimization of a process by RSM is a faster and more economical method for gathering research results than classical one-variable-at-a-time or full-factorial experimentation. RSM has been successfully applied to optimize enzymatic transesterification reactions in organic solvents (Alim et al., 2008; Wanasundara and Shahidi, 1999). In this study, response surface methodology was used to evaluate the effect of several variables (enzyme load, temperature, substrate mole ratio, time) on the acidolysis reaction and to optimize reaction conditions.

The objective of this study is to synthesize a reduced calorie spreadable SL using triolein. In this respect, *sn*-1,3 regiospecific lipase catalyzed acidolysis reaction of triolein with caprylic and palmitic acids were carried out. Caprylic acid (CA) and palmitic acid (PA) were targeted to *sn*-1 and *sn*-3 positions of structured lipids while oleic acid was maintained at *sn*-2 position. Caprylic acid was added to reduce the caloric value and palmitic acid was used to improve melting characteristic of the lipid in the acidolysis reaction. As a prior study, different kinds of enzymes were screened in terms of their ability to incorporate fatty acids. Subsequently, the most effective lipase, *Mucor miehei*, was used in the synthesis of structured lipid. The products were investigated in terms of their TAG content, melting profiles and solid fat content. The melting profile and solid fat content of SLs were compared with those of fats extracted from commercially available soft and hard margarine products. The caloric values of the produced SLs were determined theoretically.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Structured Lipids

Fats and oils are essential dieatery requirements, providing energy, essential nutrients (essential fatty acids and fat soluble vitamins), adding palatability and flavor to cooked food. Fat (~9 kcal/g) is more energy rich than carbohydrate or protein (~4 kcal/g) (Gunstone, 2001).

The major sources of fats and oils include animal fats (butter, lard, tallow and fish) and vegetable oils (soy, cotton, corn, palm, palm kernel, coconut, olive, rape, sunflower, groundnut, and linseed) (Gunstone, 2001). Natural oils are mainly triacylglycerols (TAGs) which differ in their physical and nutritional properties and do not always meet all nutritional recommendations or possess desirable physicochemical properties. Fats and oils are used in human nutrition directly as natural products or, more often, after appropriate modification (Gunstone, 2004; Adamczek, 2004).

Structured lipids (SLs) can be defined as triacylglycerols restructured or modified to change the fatty acid composition and/or their positional distribution in glycerol molecules by chemical or enzymatic processes (Lee and Akoh, 1998). Generally, SLs are triacylglycerols containing short-chain (SCFAs) or medium-chain (MCFAs), or both, and long chain fatty acids (LCFAs), preferably on the same glycerol molecule to exhibit maximum efficiency (Lai et al., 2005). SLs combine the unique characteristics of component fatty acids such as melting behaviour, digestion, absorption, and metabolism in one triacylglycerol molecule to enhance the role of fats and oils play in food, nutrition, therapeutics and health applications (Akoh and Min, 2002; Osborn and Akoh, 2002).

Structured lipids are tailor-made fats and oils with improved nutritional or physical properties because of modifications to incorporate new fatty acids or to change the position of existing fatty acids on the glycerol backbone (Osborn and Akoh, 2002).

SLs can be designed for use as medical or functional lipids and as nutraceuticals. They can be considered as ‘nutraceuticals’: food or part of food that provide medical or health benefits, including the potential for the prevention and/or treatment of disease. Sometimes, they are referred to as functional foods or functional lipids that describe lipids that provide specific health benefits (Akoh and Min, 2002). Moreover, industrial processing technologies use fat

modification to control spreadability and improve oxidative/flavor stability and palatability in margarines, shortenings, and cooking fats (Yankah and Akoh, 2000).

Some commercially available modified fats include SALATRIM/Benefat (Nabisco Foods Group, Parsippany, NJ/Cultor Food Science, Inc., New York, NY), and caprenin (Procter & Gamble Co., Cincinnati, OH) (Yankah and Akoh, 2000).

**2.2 Why are Structured Lipids Required?**

An oil or fat should have the optimum physical, chemical, nutritional properties dictated by its end use (Gunstone, 2006). Nutritional properties are important in structured lipids, functional foods, infant formula, dietary supplements, and in formulations for the maintenance of good health as well as for the treatment of disease. Physical properties are important in spreads, cooking and baking fats, frying oils, creams (Gunstone, 2001).

Fats can be modified to meet a nutraceutical need including medical/therapeutic, nutritional, low caloric value, or fortification with a desired fatty acid (Yankah and Akoh, 2000)**.** Molecular structure of triacylglycerols influences their metabolic fate in organisms i.e. digestion and absorption as well as their physical characteristics e.g. melting points. Consequently, when designing SLs with particular chemical structure, it is possible to control the behavior of triacylglycerols, thereby improving the nutritional and pharmaceutical properties of TAGs (Iwasaki and Yamane, 2000).

Fats and oils can be modified to attain a certain functionality such as improved spreadability, a specific melting point, or a particular solid fat content and temperature profile (Osborn and Akoh, 2002). Industrial processing technologies use fat modification to control spreadability and improve oxidative/flavor stability and palatability in margarines, shortenings, and cooking fats (Yankah and Akoh, 2000). For example, salad oils do not contain lipids that will crystallize during storage in a refrigerator. Most frying oils and oils used as food coatings (and lubricants) should also be free of solid components. Few natural oils meet this requirement and appropriate modification has to be carried out (Gunstone, 2006).

Fats can be also modified to obtain certain desired performance characteristics or to provide to the food processor a fat similar to a more expensive alternative (Yankah and Akoh, 2000).

Consequently the modification of the composition, structure and properties of triacylglycerols aims at:

- Reducing the consumption of saturated fatty acids and their trans isomers

- Increasing the content of polyenoic fatty acids

- Limiting the caloric value of fats and oils

- Improving the physicochemical properties of fats and oil

- Synthesis of fats and oils useful in different industry branches

- Production of fats and oils characterized by health promoting properties

- Production of fat and oil substitutes characterized by desirable physicochemical and nutritional benefits (Adamczek, 2004).

### 2.3 Synthesis of Structured Lipids

Structured lipids can be produced chemically or enzymatically. Through enzymatic synthesis, it is possible to incorporate a desired acyl group onto a specific position of the triacylglycerol, whereas chemical catalysis does not possess this regiospecificity due to the random nature of the reaction (Kawashima et al., 2001). Enzymatic interesterification is more desirable due to the advantages of requiring mild reaction conditions, producing well defined products and less waste. It is ideally suited for the production of nutritionally superior fats (Yankah and Akoh, 2000).

#### 2.3.1 Chemical Synthesis

Chemical synthesis of SLs usually involves hydrolysis of a mixture of medium chain triacylglycerols (MCTs) and long chain triacylglycerols (LCTs) and then reesterification after random mixing of the medium chain fatty acids and long chain fatty acids has occurred approximately equally on each position (*sn*-1, *sn*-2, *sn*-3), such that one-third of each fatty acid is on the *sn*-1 position, one-third on the *sn*-2 position, and one-third on the *sn*-3 position of the triacylgcerol, by a process called transesterification (Akoh and Min, 2002; Berry, 2009).

In the process of random chemical synthesis, the fat is heated (at about 100–140°C) for a long time period. Oil blends need to be dried under vacuum at a temperature higher than 90°C for 0.5–1 hour. However, the reaction is completed in a short time (e.g., 30 min) at temperatures as low as 50°C in the presence of a catalyst (usually sodium methoxide; about 0.05–0.15%). The reaction can be initiated by the addition of catalyst after the blend is cooled to ~50°C. At the beginning of the reaction, the reaction mixture becomes brown, which indicates the start of the reaction. Once such colour change occurs, reaction can be completed within 30 minutes. The reaction is stopped by the introduction of water, and subsequent processing usually includes washing, bleaching and deodorization to obtain the final randomized product. Alkali metals and alkali metal alkylates are effective low-temperature catalysts, with sodium methoxide being the most popular (Belitz et al., 2009; Gunstone, 2006; Berry, 2009). In the chemical synthesis, mineral acids are most commonly used to catalyze the esterification. Other agents such as tin salts, organotitanates, silica gel, and cation-exchange resins are also employed. The classical acid catalysis may lead to unwanted side reactions (Rajendran et al., 2009). Higher concentrations may cause excessive losses of oil resulting from the formation of soap and methyl esters (Belitz et al.,

2009; Berry, 2009).

Chemical interesterification is inexpensive and easy to scale up; however, the reaction lacks specificity and offers little or no control over the positional distribution of fatty acids in the final product. In addition to the desired randomized TAGs, a number of unwanted products are also obtained from this reaction and may be difficult to remove (Osborn and Akoh, 2002). Positional specificity of fatty acids on the glycerol molecule is not achieved by chemical transesterification, and this is a key factor in the metabolism of SLs. A possible alternative is the use of enzymes (specifically lipases) (Akoh and Min, 2002).

### 2.3.2 Enzymatic Synthesis

Enzymatic modification of fats and oils for the production of specific structured triacylglycerols has been of great interest for a long time (Xu, 2000). Chemical interesterification is not capable of modifying specific positions due to the random nature of the reactions. In contrast, the reactions catalyzed by *sn*-1,3 specific lipases are more promising for positionally specific modification of lipids (Iwasaki and Yamane, 2000). The application of selective lipases can produce pure structured triacylglycerols (Figure 2.1a) and it makes them advantageous to chemical catalysts and chemical synthesis, whose products are a mixture of TAGs (Figure 2.1b) (Adamczek, 2004).

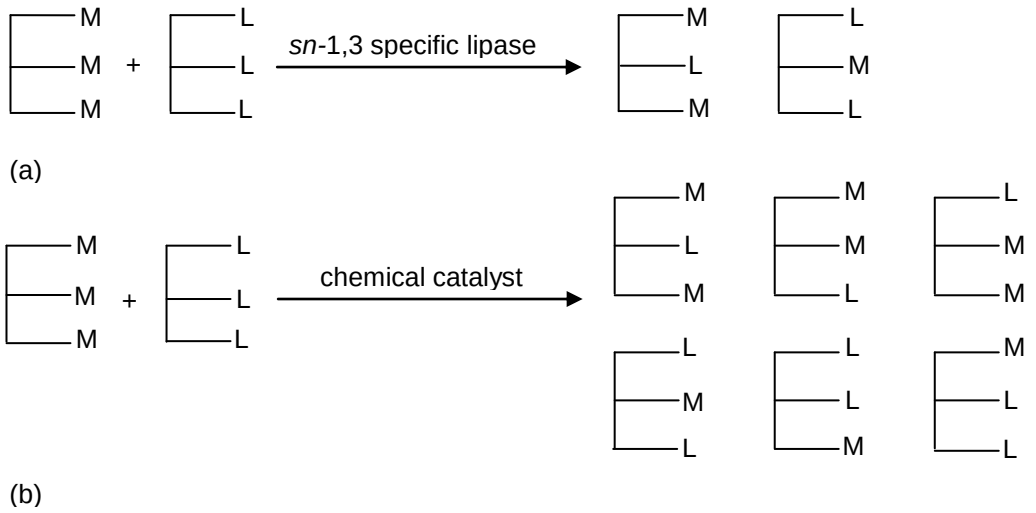



Figure 2.1. (a) Synthesis of SL catalyzed by *sn*-1,3 specific lipase and (b) a chemical catalyst M: medium chain fatty acid; L: long chain fatty acid. Figure presents only the main reaction products (Adamczek, 2004)

The use of lipases as biocatalysts for the production of structured lipids has many more potential benefits for future developments besides the specificity of lipases. The most important and also obvious merits are

- Efficiency of lipases under mild reaction conditions

- Utility in 'natural' reaction systems and products

- Reduced environmetal pollution

- Availability of lipases from a wide range of sources

- Ability to improve lipases by genetic engineering, and in special situations

For these reasons, many nutritional and functional SLs have been produced enzymatically (Xu, 2000).

Different methods can be used for lipase catalyzed production of SLs depending on the types of substrates available:

**2.3.2.1 Direct Esterification**

The reaction consists in the direct condensation of a fatty acid and an alcohol, that may be glyceride, a mono- or diglyceride, or other alcohol (Figure 2.2). (Rodriguesa and Lafuenteb, 2010) The major problem is that the water molecules formed as a result of the esterification reaction must be removed as they are formed to prevent them from hydrolyzing back to product, leading to low product yield.

*Glycerol + MCFA + LCFA* $\xrightarrow{lipase}$ *SL + Water*

Where MCFA: Medium chain fatty acid LCFA: Long chain fatty acid SL: Structured lipid moieties (Akoh and Min, 2002).

It was found that the water activity could be lowered to extreme values to favor the synthesis, without any sacrifice on the productivity of the process. Esterification may be used to obtain desired glycerides or to reduce the acidity of an acid oil (Rodriguesa and Lafuenteb, 2010; Ciftci, 2003)

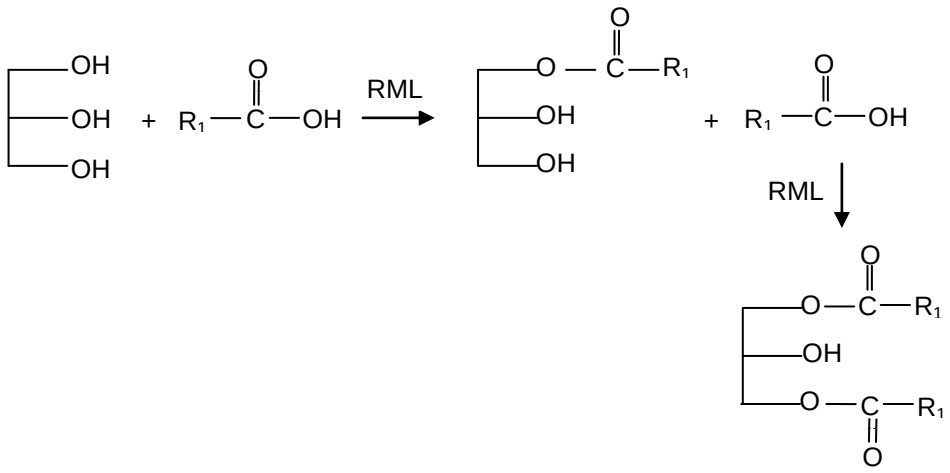



Figure 2.2. General scheme of direct esterification of fatty acids and alcohols catalyzed by *Rhizomucor miehei* lipase

### 2.3.2.2 Interesterification

Interesterification is one of the major reactions used by industry for the modification of natural fats and oils. Reactions under this category can be classified as further and the last three reactions are often grouped together into the single term interesterification.

#### 2.3.2.2.1 Acidolysis

Acidolysis is a type of interesterification reaction involving the exchange of acyl groups or radicals between ester and a free acid:

$$MCT + LCFA \xrightarrow{lipase} SL + MCFA \quad \text{or} \quad LCT + MCFA \xrightarrow{lipase} SL + LCFA$$

where MCT: Medium chain triacylglycerol and LCT: Long chain triacylglycerol.

Figure 2.3 shows an example of acidolysis reaction between long chain triacylglycerol and medium chain fatty acid catalyzed by *sn*-1,3 specific lipase (Akoh and Min, 2002).

Acidolysis is based on incorporation of the fatty acids specifically at *sn*-1 and *sn*-3 positions of desired TAGs by an *sn*-1,3 specific lipase, remaining the fatty acid at *sn*-2 position unchanged. The acidolysis reaction proceeds in two steps. The TAGs are hydrolyzed into diacylglycerols (DAG) or even to monoacylglycerols (MAG), followed by the esterification of new fatty acids into the TAGs. Acyl migration may occur between these two steps and results in the formation of by-products that may affect product quality (Koh et al., 2010).

Theoretically, these reactions give mixtures of TAGs and fatty acids (or their esters) (Figure 2.3). These fatty acids or esters can be removed by molecular distillation or alkali extracions. Eventually the triacylglycerol species with specific structure can be obtained with high purity (Iwasaki and Yamane, 2000).

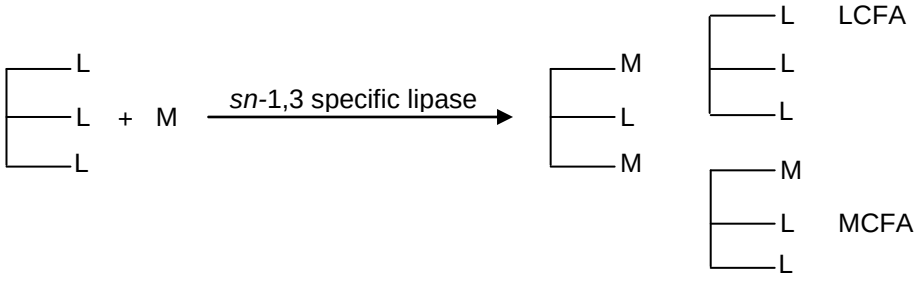



Figure 2.3. Acidolysis of long chain triacylglycerol with medium chain fatty acid (Akoh and Min, 2002)

#### 2.3.2.2.2 Transesterification

In its simplest form, transesterification corresponds to an exchange of acyl residues between two triacylglycerols, resulting in the formation of new TAGs that have chemical and physical properties distinct from the starting triacylglycerols (Mangos et al., 1999).

The first stage of transesterification involves hydrolysis of triacylglycerols with consumption of water to produce diacylglycerols, monoacylglycerols, and free fatty acids. Accumulation of hydrolysis products will continue during transesterification until equilibrium is established (Akoh and Min, 2002). If we want to introduce a new fatty acid into the glycerol moiety, the ester bond between the native fatty acid residue (the original substituent group) and the glycerol moiety must first be hydrolyzed. This reaction liberates the native fatty acid and produces a lower (less substituted) glyceride containing at least one hydroxyl group. The hydrolysis step is followed by the formation of a new ester bond by reaction of the newly created hydroxyl group with the incoming replacing fatty acid (that needs to be also released from the ester) (Rodriguesa and Lafuenteb, 2010). Figure 2.4 shows an example of transesterification reaction between long chain triacylglycerol and medium chain triacylglycerol catalyzed by *sn*-1,3 specific lipase.

In this reaction, all the substances in the reaction mixture are TAGs theoretically, if the hydrolysis of the substrates suppressed. However, the reaction always gives a nonhomegenous mixture of many TAG species, each of which is very difficult to isolate by practical means (Iwasaki and Yamane, 2000).

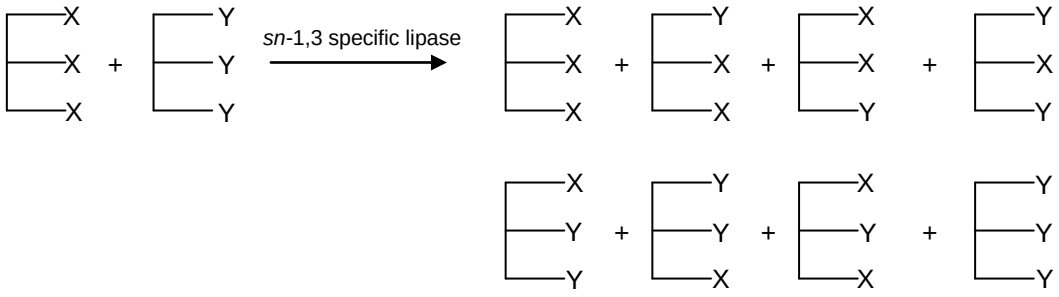



Figure 2.4. Generale scheme of enzyme catalyzed transestrification reaction X: medium chain fatty acid; Y: long chain fatty acid (Xu, 2000)

**2.3.2.2.3 Alcoholysis**

When the original ester is reacted with an alcohol, the interesterification process is called alcoholysis (Figure 2.5) (Schuchardt et al., 1998).

Alcoholysis involves the reaction of a triacylglycerol and an alcohol and has several commercial applications, primarily the production of monoacylglycerols and diacylglycerols. Alcoholysis must be avoided in the interesterification of food lipids; however, since monoacylglycerols and diacylglycerols are undesirable by-products (Akoh and Min, 2002).

Two examples of alcoholysis widely used with lipids have their own specific names (methanolysis and glycerolysis). Reaction with methanol (methanolysis) to convert glycerol esters to methyl esters is carried out on a mg scale as a preliminary to gas chromatographic analysis and on a tone scale for the production of methyl esters used as solvents, as biofuels

or as intermediates in the production of alcohols (Gunstone, 2006). Glycerolysis is an alcoholysis reaction in which glycerol acts as the alcohol. Glycerolysis is the exchange of acyl groups between glycerol and a triacylglycerol to produce monoacylglycerols, diacylglycerols, and triacylglycerols (Akoh and Min, 2002).

Mono- and diglycerides were the first fatty emulsifiers to be added to foods. Monoglycerides with only one fatty acid attached to a glycerol molecule and two free hydroxyl groups on the glycerol take on the properties of both fats and water. Monoglycerides not only are used as surfactants as produced, but also can be further modified to produce other surface-active products suitable for use in prepared foods (O'Brien, 2009). The intermediate product of 1,3-specific lipase-catalyzed alcoholysis, 2-monoglycerides, can also be used as starting materials for the synthesis of other structured lipids (Gunstone, 2006).

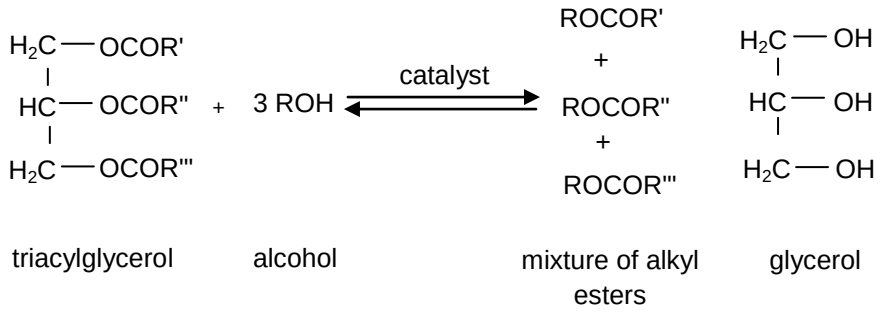



Figure 2.5. Alcoholysis of triacylglycerols (Schuchardt et al., 1998)

**2.4 Component Fatty Acids**

A fatty acid is an organic acid- a chain of carbon atoms with hydrogen attached- that has an acid group (COOH) at one end and a methyl group ($CH_3$) at the other end. The types of fatty acids in a triacylglycerol molecule play an important role in the nutritional and functional properties of fats and oils (Yankah and Akoh, 2000).

Three types of acyldonors can be used for the production of structured triacylglycerols: triacylglycerols, ethyl fatty acid esters, and free fatty acids (short chain fatty acids, medium chain fatty acids and long chain fatty acids, polyunsaturated fatty acids, saturated long chain fatty acids, and monounsaturated fatty acids) (Akoh and Min, 2002).

The choice of acyl donors depend on the product purity, reactivity, purification techniques and price. Generally triacylglycerols as acyl donors give lower reactivity and free fatty acids give higher reactivity. The choice of ethyl esters as acyl donors are mainly due to their easy separation by distillation because they are more volatile than free fatty acids, but more diacylglycerol formation may occur. No clear difference of reactivity between free fatty acids and their ethyl esters have been obtained. Free fatty acids have been most often used acyl donors so far due to their easy availability, low price, and high reactivity (Xu, 2000).

### 2.4.1 Short Chain Fatty Acids

Short chain fatty acids (SCFAs) range from C2:0 to C6:0 and are also known as volatile fatty acids. Due to their water soluble nature, molecular size and short chain length, they are more rapidly absorbed in the stomach than other fatty acids and provide low energy (4-8 kcal/g) (Osborn and Akoh, 2002). On the basis of heats of combustion, SCFA provide fewer calories per unit weight than MCFAs or LCFAs. Butyrate provides 6 kcal/g and caproate about 7 kcal/g, compared to 9 kcal/g for LCFA (Fomuso and Akoh, 1997). SCFAs are useful ingredients in the synthesis of low-calorie SLs.

They diffuse freely across the mucosalcytosol and enter venous blood remaining as free fatty acid, whereas long-chain fatty acids are absorbed through the lymphatic system and transported in the form of chylomicron and lipoprotein triacylglycerols (Zhou et al., 2006). In the human diet, SCFAs are usually taken in during consumption of bovine milk, which has a triacylglycerol mixture containing approximately 5-10% butyric acid and 3-5% caproic acid (Breckenridge and Kuksis, 1967; Garton, 1968). Butyric acid is found in butterfat, where it is present at about 30% of the TAGs (Hawke and Taylor, 1983).

### 2.4.2 Medium Chain Fatty Acids

Medium chain fatty acids (MCFAs) are saturated fatty acids with a carbon chain length ranging from 6 to 12, and are prepared mostly from tropical plant oils such as coconut and palm kernel oils and have unique nutritional characteristics different from those of long-chain fatty acids (Hamam and Shahid, 2004; Takeuchi et al., 2008).

MCFAs are preferentially transported via the portal vein to the liver, because of their smaller size and greater solubility compared to longchain fatty acids (LCFAs) (Hamam and Shahid, 2004). The following characteristics were already reported in the 1950–60s: 1. medium-chain fatty acids are readily digested and absorbed, 2. transported to the liver and easily utilized as energy, and 3. less likely to accumulate as body fat (Takeuchi et al., 2008).

For entry into mitochondria of all tissues, MCFAs are not carnitine dependent. Additionally, MCFAs are metabolized as rapidly as glucose in the body with little tendency to deposit as stored fat, because they are not readily reesterified into triacylglycerols (Osborn and Akoh, 2002). Although MCFAs may be useful in the control of obesity, a study by Cater et al. (1997) showed that medium chain triacylglycerols indeed raised plasma total cholesterol and triacylglycerol levels in mildly hypercholesterolemic men fed with MCT, palm oil, or high oleic acid sunflower oil diets (Akoh and Min, 2002). Pure MCT have a caloric value of 8.3 kcal/g. Therefore, it appears that MCFAs are most useful in structured lipid that combines their inherent mobility, solubility, and ease of metabolism with more healthful polyunsaturated fatty acids (Osborn and Akoh, 2002).

Beside, for its use as cooking oil, it has disadvantages: it has a low smoking point, and easily

foams during deep frying. Since the properties of accumulating less body fat and safety were confirmed, medium long chain triacylglycerols (MLCT) oil was approved as a food for specified health use (trade name: Healthy Resetta) in 2002 as a cooking oil, less likely to lead to body fat accumulation, and sold in most supermarkets in Japan (Takeuchi et al., 2008). MCFAs are used as a source of quick energy and rapid absorption, especially for immature neonates, hospitalized patients, and individuals with lipid malabsorption disorders. (Yankah and Akoh, 2000)

### 2.4.3 Long Chain Fatty Acids

LCFAs, ranging from C14 to C24, are absorbed and metabolized more slowly than either medium or short chain acids; much of LCFAs may be lost as calcium-fatty acid soap in the feces (Osborn and Akoh, 2002).

Structured lipids, which contain long chain fatty acids and medium-chain fatty acids, respectively, where each group is located specifically at the *sn*-2 position or *sn*-1,3 positions, have also attracted great attention for their special nutrition (Xu et al., 2000)

Long chain saturated fatty acids are generally believed to increase cholesterol levels (Osborn and Akoh, 2002). However, studies on oleic acid-rich diets have shown that, although a LCFA, oleic acid can be credited with reducing total cholesterol and low density lipoprotein (LDL) cholesterol levels. It also reduces the ratio of LDL to high-density lipoprotein (HDL) cholesterol. Stearic acid (18:0) has also been reported not to raise plasma cholesterol levels (Bonanome, 1988). TAGs containing high amounts of LCFAs, particularly stearic acid, are poorly absorbed in man, partly because stearic acid has a melting point higher than body temperature; they exhibit poor emulsion formation and poor micellar solubilization (Hashim and Babayan, 1978). Indeed, Nabisco Foods Group used this property of stearic acid to make the group of low-calorie SLs called Salatrim (now Benefat), which consist of short-chain aliphatic fatty acids and LCFAs, predominantly C18:0 (Finley et al., 1994).

## 2.5 Fatty Acids and Melting Points

Long chain fatty acids have a higher melting point than short chain fatty acids, because there is more potential for attraction between long chains than there is between short chains. A second factor that determines melting point is the number of double bonds. As the number of double bonds increases, the melting point decreases when the carbon numbers are same. Double bonds introduce kinks into the chain and it is harder for molecules to fit together to form crystals; thus, the attractive forces between the molecules are weaker (Vaclavik and Christian, 2007). Some selected properties of some alkanoic acids are given in Table 2.1.

T**able 2.1 Names and selected physical properties of some alkanoic acids**

| Chain Length | Systematic Name | Trivial Name | Fatty Acid | | mwt (g/mol) |
|---|---|---|---|---|---|
| | | | mp (°C) | bp (°C) | |
| **4** | butanoic | Butyric | -5.3 | 164 | 88.1 |
| **6** | hexanoic | Caproic | -3.2 | 206 | 116.2 |
| **8** | octanoic | Caprylic | 16.5 | 240 | 144.2 |
| **10** | decanoic | Capric | 31.6 | 271 | 172.3 |
| **12** | dodecanoic | Lauric | 44.8 | 130 | 200.3 |
| **14** | tetradecanoic | Myristic | 54.4 | 149 | 228.4 |
| **16** | hexadecanoic | Palmitic | 62.9 | 167 | 256.4 |
| **18** | octadecanoic | Stearic | 70.1 | 184 | 284.5 |
| **20** | eicosanoic | Arachidic | 76.1 | 204 | 312.5 |
| **22** | docosanoic | Behenic | 80.0 | - | 340.6 |
| **24** | tetracosanoic | Lignoceric | 84.2 | - | 368.6 |

### 2.6 Lipases

Lipases are ubiquitous enzymes and can be found in various sources, such as plants, animals and microorganisms (Reis et al., 2009). Lipases of microbial origin, mainly bacterial and fungal, represent the most widely used class of enzymes in biotechnological applications and organic chemistry.

Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of microorganisms on inexpensive media. Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Hasan et al., 2006). Microbial lipases have been the most attractive ones as they are thermostable, without co-factor requirements, and of different specifications that have been extensively described (Xu, 2000).

#### 2.6.1 Lipases as Biocatalysts in the Fats Modification Industry

The particular benefits offered by enzymes are specificity and selectivity, mild conditions and reduced waste producing oils or fats with desired composition. It may be possible, by choosing the right enzyme, to control which products are produced, and unwanted side reactions are minimized due to specificity of enzymes that appear in the waste stream (Hasan et al., 2006; Rodriguesa and Lafuenteb, 2010).

Lipases do not require cofactors. They are often used in both, free or immobilized forms, and are commercially available, relatively inexpensive, and display relatively high stability (Yahya et al., 1998). They act at the lipid-water interface and, therefore, they do not require water-soluble substrates. This function distinguishes lipases from other hydrolytic enzymes, and their efficiency in conducting transformations in organic solvents under mild conditions

increases their importance as useful tools in organic synthesis (Zarevúcka and Wimmer, 2008).

In addition to lipases, carboxylic esters bonds can be hydrolyzed by esterases. The distinction between lipases and esterases has been based for a long time on the interfacial activation and presence of a lid for the former enzyme (Houde, et al., 2004). Lipases act at the interface between hydrophobic and hydrophilic regions, a characteristic which distinguishes lipases from esterases (Yahya et al., 1998).

The cost of the enzyme is generally so high that their use is only economic for high value products but these difficulties are being overcome as enzyme producers develop immobilized enzymes of greater stability with a longer useful life. Beyond this there is growing willingness to pay more for fats for which approved health claims can be made (Gunstone, 2004).

### 2.6.2 Reaction of Lipases

Lipases have been found to be able to catalyze various hydrolytic and synthetic reactions concerning ester bonds, using natural and synthetic substrates in aqueous and non-aqueous phases (Song et al., 2008) (Figure 2.6). The two main categories into which lipase catalyzed reactions may be classified are as follows;

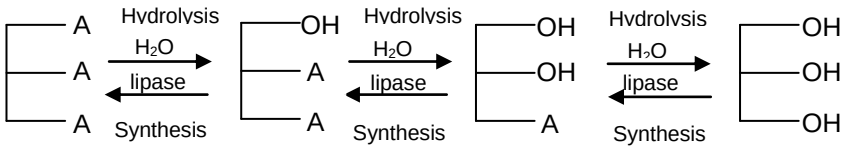



Figure 2.6. Enzymatic reaction of nonspecific lipase catalyzing hydrolysis or synthesis of a TAG substrate. A: fatty acid (Panesar, et al., 2010).

#### 2.6.2.1 Hydrolysis

Hydrolysis reactions (Figure 2.7) involve an attack on ester bond of fats in the presence of water to produce glycerols, fatty acids, and partial acylglycerols, depending on the positional specificity of enzymes. This refers to the splitting of a fat/ester into its constituent acid and glycerol/alcohol in the presence of water. The product of interest may be either a specific acid or alcohol that is formed. There are three major routes currently used for the hydrolysis of fats and oils in the production of fatty acids; high pressure steam splitting, alkaline hydrolysis and enzymatic hydrolysis (Gandhi et al., 1997).

The usual method of hydrolysis of oils to fatty acids and glycerol uses high temperature and pressure (Rodriguesa and Lafuenteb, 2010). Fat splitting is normally effected by a continuous, high-pressure, uncatalysed, counter-current process at 250°C and 20±60 bar. Under these high-temperature conditions the products become discoloured and both the fatty acids and the glycerol may have to be distilled (Gunstone, 1999). There are also

difficulties associated with alkaline hydrolysis, namely high energy costs and the need to acidify the soaps formed, to produce the fatty acid products (Gandhi et al., 1997).

Enzymatic hydrolysis is a good alternative to overcome these disadvantages as the use of enzymes for the hydrolysis not only gives colorless pure products but also reduces the by-product formation, due to enzyme specificity. In other cases, the target of the reaction is the production of di- or monoglycerides with a special composition (Rodriguesa and Lafuenteb, 2010). When the concentration of water in the reaction medium is reduced, e.g, by working in an organic solvent containing a limited amount of water, a drastic shift of the chemical equilibrium can be observed (Ciftci and Fadıloğlu, 2010).

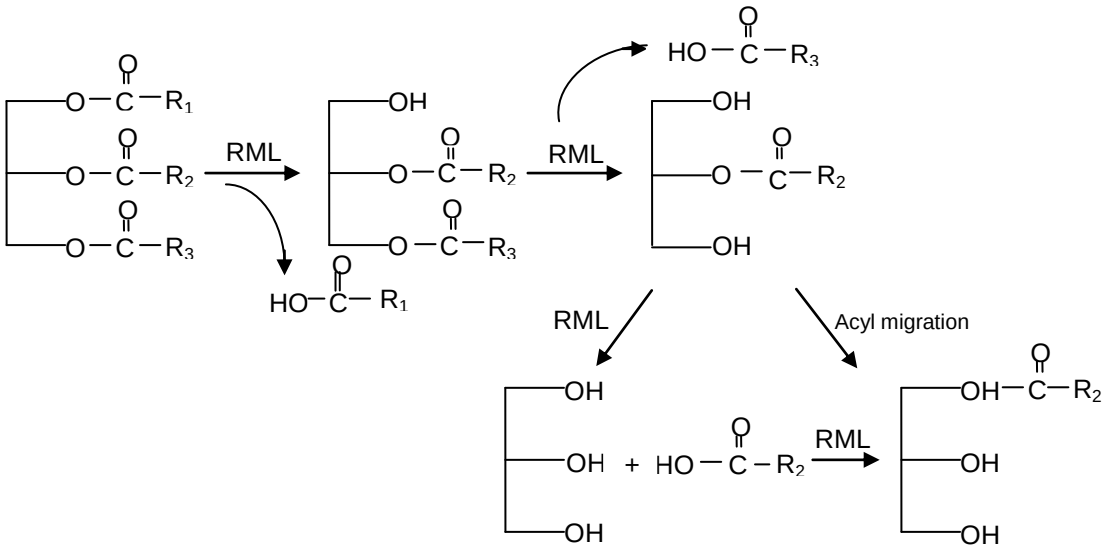



Figure 2.7. General scheme of the full hydrolysis of triacylglycerols catalyzed by *Rhizomucor miehei* lipase to produce free fatty acids

### 2.6.2.2 Synthesis

#### 2.6.2.2.1 Esterification

Esterification mixtures generally contain only the substrates and enzyme and water is the only by-product of the reaction. Because the latter is generally easy to remove, this process is quite superior to other synthetic processes, which entail the use of hazardous solvents, corrosive acid catalysts, etc., especially because esterification can proceed efficiently without the use of solvent and because almost complete conversion is possible by adopting means such as continuous water removal. This enables an equilibrium shift in favor of the synthetic reaction (Gandhi et al., 1997).

#### 2.6.2.2.2 Interesterification

While esterification produces water and ester (usually the desired product), interesterification processes, such as alcoholysis, acidolysis and transesterification give rise to alcohol, acid, or ester instead of water. Hence, interesterification becomes more lucrative when any of these are the desired products (Gandhi et al., 1997) Transesterification, acidolysis, alcoholysis are often grouped together into the single term interesterification. The detailed explanations are given in Section 2.3.2.

### 2.6.3 Specificity of Lipases

The main advantage of lipases that differentiates enzymatic interesterification from chemical interesterification is their specificity (Akoh and Min, 2002) with respect to the glyceride position and fatty acid type, which could seldom be constructed by chemical catalysis (Quinlan and Moore, 1993).

There are three main types of lipase specificity: positional, substrate, and stereo.

#### 2.6.3.1 Nonspecific Lipases

Some lipases show no positional or fatty acid specificity during interesterification with respect to the position of the acyl group on the glycerol backbone. Nonspecific lipases break down acylglycerol molecules at random positions, producing free fatty acids and glycerol with monoacylglycerols and diacylglycerols as intermediates. The products would be similar to those by chemical catalysts with the exception of lower product thermodegradation due to the much lower reaction temperatures in biocatalysis (Yahya et al., 1998). Some examples for nonspecific lipase sources are: *Candida cylindracea, Corynebacterium acnes*, and *Stapyhlococus aureus* (Ciftci and Fadıloğlu, 2010).

#### 2.6.3.2 Positional Specificity

Lipases can be divided according to their regioselectivity: *sn*-1,3 regiospecific or non-regiospecific. Regioselectivity is the ability of lipases to distinguish between the two outer positions (*sn*-1 or *sn*-3 position) and the inner position (*sn*-2 position) of glycerides results from an inability of lipases to act on position *sn*-2 on the triacylglycerol, due to steric hindrance (Pfeffer, 2008; Akoh and Min, 2002). Figure 2.8 represents reactions catalyzed by positional specific and fatty acid specific lipases.

Lipases that are 1,3 specific include those from *Aspergillus niger*, *Mucor miehei*, *Rhizopus arrhizus,* and *Rhizopus delemar* (Macrae, 1983). The specificity of individual lipases can change due to microenviromental effects on the reactivity of functional groups or substrate molecules (Pabai et al., 1995). Beside *sn*-1,3 specific lipases, there are a few lipases available possessing *sn*-2 specificity or *sn*-1 and *sn*-3 specificity (Xu, 2000). (e.g. lipase A from *Candida antarctica*, the lipase from *Candida parapsilosis* or a lipase from the bovine

rumen metagenome) (Pfeffer, 2008). In Table 2.2, selectivity of some lipases are given.

### 2.6.3.3 Stereospecificity

In triacylglycerols, these *sn*-1 and *sn*-3 positions are sterically distinct. Very few lipases can distinguish between the *sn*-1 and *sn*-3 position in triacylglycerols, e.g. the human tongue lipase and some other lipases from *Pseudomonas fluorescens* and *Humicola languinosa*. (Pfeffer, 2008). Porcine pancreatic lipase have shown stereoselectivity when certain acyl groups are hydrolyzed (Ciftci and Fadıloğlu, 2010).

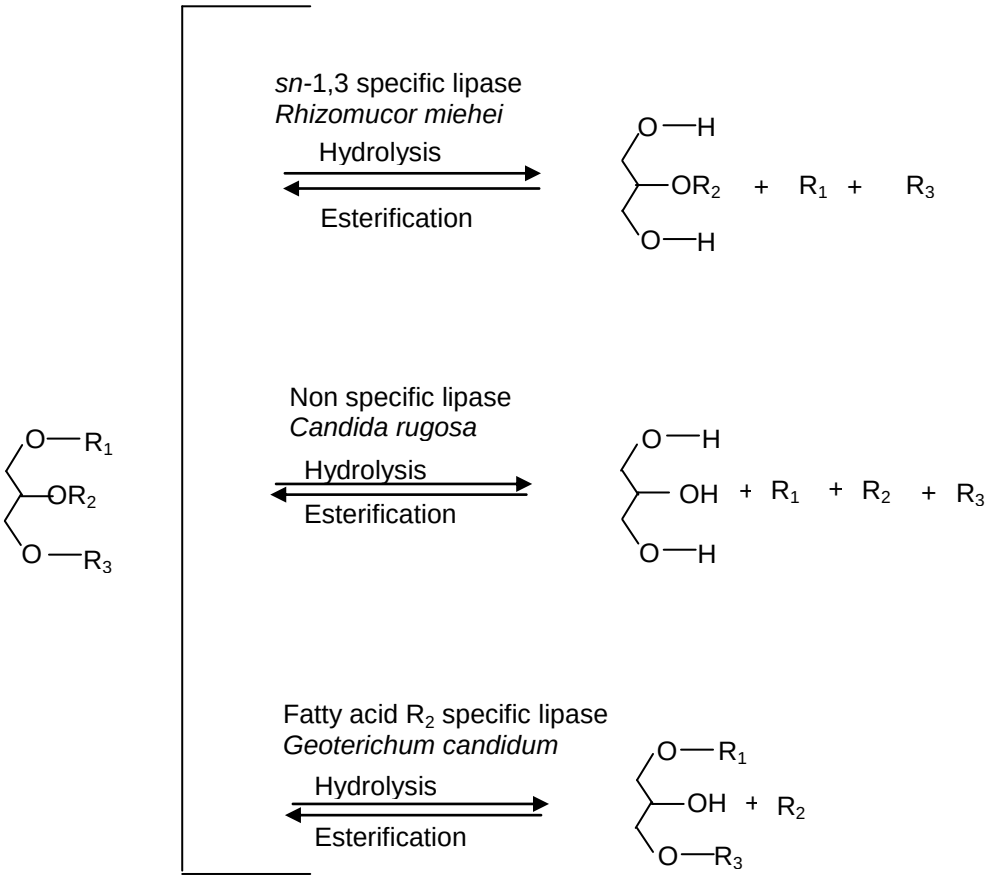



Figure 2.8. Specificity of triacylglycerol lipases in hydrolysis and esterification: $R_1$, $R_2$, $R_3$, fatty acids/ acyl moieties (Akoh and Min, 2002)

### 2.6.3.4 Fatty Acid Specificity

Many lipases show preference for a specific fatty acid or chain length range. The most extreme example is a lipase from *Geotrichum candidum* which mainly reacts with fatty acids with cis-9 configuration (Reis et al., 2009).

### 2.6.4 Factors Affecting Lipase Activity in Reaction Systems

Enzyme activity is a mesaure of the rate at which an enzyme converts substrate to products in a biochemical reaction. pH, temperaure, water content and water activity, enzyme purity, substrate concentration, enzyme concentration are the main factors that affect enzyme

activity

#### 2.6.4.1 pH

Enzymes are extremely sensitive to the pH of the reaction medium. All enzymes have a characteristic pH at which their activity is maximum. At pH lower or higher than the optimal pH, the activity of enzyme decreases (Ciftci and Fadıloğlu, 2010). The pH optima for most lipases lies between 7 and 9, although lipases can be active over a wide range of acid and alkaline pHs, from about pH 4 to 10 (Kuo and Gardner, 2005).

Table 2.2 The selectivity of lipases used for synthesis of TAG (Xu,2000; Akon and Min, 2002)

| **Lipases Source** | **Fatty acid specificity** | **Regiospecificity** |
|---|---|---|
| *Rhizomucor miehei* | S>M, L | 1>>3, 2 |
| *Candida antarctica* | M, L>S | 2 |
| *Rhizopus oryzae* | M, L>S | 1, 3>>>2 |
| *Psuedomonas fluorescens* | M, L>S | 1>>3, 2 |
| *Rhizopus arrhizus* | S, M>L | 1,3 |
| *Porcine pancreatic* | S>M, L | 1,3 |
| *Aspergillus niger* | S, M, L | 1,3>>2 |
| *Rhizopus delemar* | S, M, L | 1, 2, 3 |
| *Candida rugosa* | S, L> M | 1, 2, 3 |

S: short chain fatty acid, M: medium chain fatty acid, L: long chain fatty acid

Running an interesterification reaction with lipases at a pH well removed from the optimum can lead to rapid inactivation of the enzyme (Akoh and Min, 2002). pH may affect binding of substrate to enzyme and the ionization of catalytic groups such as carboxyl or amino groups that are part of the enzyme's active site. The stability of the tertiary or quaternary structure of enzymes is also pH dependent and affects the velocity of the enzyme reaction, especially at extreme acidic or alkaline pHs (Nielsen, 2010).

#### 2.6.4.2 Temperature

Temperature is a measure of the kinetic energy of molecules. Higher temperatures mean molecules are moving faster and colliding more frequently. This concept applies to collisions between substrate molecules and enzymes. As the temperature of an enzymatically catalyzed reaction increases, so does the rate (velocity) of the reaction (Stoker, 2010).

Temperature affects not only the velocity of catalysis of acidolysis, but also stability of the enzyme; the equilibria of all association/dissociation reactions (ionization of buffer, substrate, product and cofactors (if any); association/disassociation of enzyme- substrate complex; reversible enzyme reactions; solubility of substrates, especially gases; and ionization of prototropic groups in the active site of the enzyme and enzyme-substrate complex (Belitz et al., 2009).

The optimal temperature for most immobilized lipases falls within the range of 30-62 °C,

whereas it tends to be slightly lower for free lipases (Kuo and Gardner, 2005). Immobilized lipases are more stable to thermal deactivation because immobilization restricts movement and can reduce the degree of unfolding and denaturation.

#### 2.6.4.3 Water Content and Water Activity

The water content in a reaction system is the determining factor as to whether the reaction equilibrium will be toward hydrolysis or ester synthesis. Ester synthesis depends on low water activity. Too low a water activity prevents all reactions from occurring because lipases need a certain amount of water to remain hydrated, which is essential for enzymatic activity (Briand et al., 1994; Svensson et al., 1994). The optimal water content for interesterification by different lipases ranges from 0.04% to 11% (w=v), although most reactions require water contents of <1% for effective interesterification (Bornaz et al., 1998; Malcata et al., 1992; Li et al., 1995). A water content higher than 1% can produce high degrees of hydrolysis, whereas water levels lower than 0.01% can prevent full hydration of the lipase and reduce the initial rate of hydrolysis (Akoh and Min, 2002).

#### 2.6.4.4 Substrate Concentration and Composition

Enzyme activity increases up to a certain point with increasing substrate concentration and there after remains constant. As substrate concentration increases, the point is eventually reached where enzyme capabilities are used to their maximum extent. The rate remains constant from this point.

The reactions take place at the active sites of the enzymes, once they are all accupied, the reaction is proceeding at its maximum rate. Increasing the substrate concentration can no longer increase the rate because the excess subtrate cannot find any active sites to which to bind (Stoker, 2010).

#### 2.6.4.5 Product Accumulation

Lipase loss its activity at high concentration of free fatty acids, this can be attributed to several factors. High levels of free fatty acids would produce high levels of free or ionized carboxylic acid groups, which would acidify the microaqueous phase surrounding the lipase or cause desorption of water from the interface. In addition, with short- and medium-chain fatty acids, there could be partitioning of fatty acids away from the interface into the surrounding water shell due to their increased solubility in water. This would limit access by the substrate to the interface (Kuo and Parkin, 1993).

#### 2.6.4.6 Solvent type

The main reasons to apply enzymes in organic media are; (1) to reverse enzymatic hydrolytic reactions (2) to suppress side- reactions that require water (3) to increase the substrate solubility (4) to simplify product recovery.

Partition coefficient, P, between octanol and water can be used as parameter for selecting the organic solvents based on their hydrophobicity and correlated with enzyme activity in organic solvent. Solvents with high log-P values (3.5-4.5) are more hydrophobic and exhibit high enzyme activity while solvents with log-P values less than 2 exhibit little activity. Polar solvents may remove the essential water from the enzyme and distrupt the active conformation. Log-P values of selected organic solvents are: iso-octane (4.5), n-hexane (3.5), benzene (2.0), and acetonitrile (-0.03) (Lee and Akoh, 1998; Polaina and MacCabe, 2007). Lipases are more active in n-hexane and isooctane than other solvents, such as toluene, ethyl acetate, and acetylnitrile (Akoh et al., 1995; Miller et al., 1988).

### 2.7 Application of Structured Lipids

The constituent FAs and their locations in the glycerol backbone determine the functional and physical features, the metabolic fate, and the health benefits of SLs. Therefore, designing SLs with selected FAs at specific locations in the TAGs for medicinal applications has attracted much attention (Zarevúcka and Wimmer, 2008). This section outlines some current commercial applications and potentially interesting uses of lipase-catalyzed reactions for the production of specialty products from oils and fats (Akoh and Min, 2002).

#### 2.7.1 Medical and Nutraceutical Application of Structured Lipids

##### 2.7.1.1 Reduced Calorie Lipids

Although fats possess a high-calorie intake, their good taste and smoothness make them difficult to be circumvented (Houde et al., 2004). However, a high intake of fats has been blamed for the high incidence of cardiovascular disease, hypertension, and obesity, especially in industrialized countries (Akoh and Min, 2002). Obesity is the result of an imbalance between energy intake and energy expenditure, by which surplus energy intake is stored as triacylglycerols in adipose tissues (Yang et al., 2004). So that, with increasing consumer awareness of the risks associated with high fat intake, a market for reduced calorie fats or fat replacers has opened up (Koh et al., 2010).

SLs have many industrial applications and have recently attracted the attention of food manufacturers for production of low-caloric lipids that are characterized by a mixture of short-chain fatty acids (SCFAs) and/or MCFAs and LCFAs in the same glycerol moiety (Zarevúcka and Wimmer, 2008). Increasing interest in such products stems from the fact that they contain 5-7 kcal/g energy compared to 9 kcal/g for usual fats and oils; this is because of the lower caloric content of SCFA or MCFA compared to LCFA.

Restructuring fat to inhibit absorption or to achieve a lower caloric density is the principle underlying production of low calorie fats because the metabolism and absorption of the fatty acid depend on their position in the TAGs (Yankah and Akoh, 2000). Structured lipids, in which long chain (mainly essential) fatty acids locate at the *sn*-2 position of glycerol

backbone and short- or medium- chain fatty locate at the *sn*-1,3 positions, have attracted increasing attention for nutritional development (Xu, 2000). These triacylglycerols provide rapid delivery of energy via oxidation of the more hydrophilic medium-chain fatty acids, while at the same time providing an adequate supply of essential fatty acids from the remaining 2-monoglyceride (Soumanou et al., 1998).

A considerable number of papers on MLCT structured lipids (Swift et al., 1992; Matsuo et al., 2001; Bendixen et al., 2002; Kasai et al., 2003; Matsuo and Takeuchi, 2004) had been published and the results obtained showed that long-term substitution of MLCT for LCT will result in reduction in body weight, body fat, and total serum cholesterol. The data obtained from a few Japanese studies on MLCT suggested that enhancement of energy expenditure and medium-chain fatty acids oxidation without activating de novo lipogenesis are responsible for the lower fat accumulation (Shinohara et al., 2002, 2005).

Kanjilal et al. performed an interesterification of sunflower oil with a lipase and incorporated behenic acid at the *sn*-1 and *sn*-3 positions. The resulting oil, Bohenin, produced by Fuji Oil, is a commercial triacylglycerol containing behenic acid at *sn*-1 and *sn*-3 positions with oleic acid at the *sn*-2 position and has 5.36 kcal/g. Bohenin has a taste very close to sunflower oil (Osborn and Akoh, 2002).

#### 2.7.1.2 Infant Formulas

Infant formula offers a good alternative to breast milk and ideally tends to mimic human milk as much as possible. The major triacylglycerol present in human milk is unsaturated at the *sn*-1,3 positions and saturated at the *sn*-2 position. Palmitic acid (C16:0) represents 20–33% of the total fatty acids with one-third located at the *sn*-2 position (Houde et al., 2004). SLs with high proportions of palmitic acid at the *sn*-2 position would provide a fat with improved absorption capability in infants and decrease the loss of calcium in the feces (Gunstone, 2001). Care must be taken with regard to the concentrations of the saturates at the *sn*-2 position, because palmitic acid is the only saturate that has been studied extensively, and other long chain saturates may have hypercholesterolemic effects (Pai and Yeh, 1997). Betapol™ (Loders Croklaan) was the first commercial product made by the 1,3-specific lipase treatment of tripalmitin with unsaturated fatty acids that resulted in 1,3-diunsaturated-2-saturated triacylglycerols intended for infant formula (Houde et al.,2004).

#### 2.7.1.3 Enteral and Porentral Nutrition

Physical mixtures of MCTs and LCTs have proven useful in the past for enteral and parenteral nutrition. More recently, structured TAGs comprised of LCFAs and MCFAs have emerged as the preferred alternative to physical mixtures for treatment of patients, although both products provide identical fat contents (Osborn and Akoh, 2002).

It may be desirable to develop a SL containing PUFAs at the *sn*-2 position with medium-

chain fatty acids (MCFAs) at the *sn*-1,3 positions for patients with maldigestion as well as cystic fibrosis (Zarevúcka and Wimmer, 2008). For instance, incorporation of linoleic, arachidonic, or eicosapentaenoic acid at the *sn*-2 position is being evaluated for the specific objective of modulating membrane fatty acid composition and essential fatty acid absorption in models of cancer, burns, and immune dysfunction (Stein, 1999). Another commercial product of this type is Unichemas Betapol (OPO), a diet additive for premature infants. It is prepared by interesterification of tripalmitin with oleic acid with use of immobilized Rhizomucor miehei lipase (Fomuso and Akoh, 1997).

### 2.7.2 Functional Structured Lipids

#### 2.7.2.1 Plastic Fats

Margarine, modified butters, and shortenings are “plastic”, that is, they have the appearance of solids in that they resist small stresses, but yield to a deforming stress above a certain minimal value (the yield stress) to flow like liquid (Osborn and Akoh, 2002). The desired plastic fat should be solid in refrigerator, but should be spread easily and melt quickly in the mouth. The proportion of solid to liquid crystals is the key factor that determines the hardness of the mixture (Houde et al., 2004). The spreadability of margarine at refrigerator temperatures is related to its content of solid fats at 2 and 10°C. The solid content at 25°C influences plasticity at room temperature (Brekke 1980). A solid fat content between 15 and 35% characterizes plastic fats (desired spreadability) that can be produced enzymatically or chemically (Houde et al., 2004).

The techniques of modification in regular use are well defined in terms of how they alter triacylglycerols structure by recolating the fatty acids available (interesterification), by concentrating the higher and lower melting triacylglycerols (fractionation), or by modifying the fatty acids present by way of reduction of unsaturation and generation of trans isomers (hydrogenation) (Gunstone, 2001).

Hydrogenation reduces double bonds to single bonds, thereby increasing the melting point of the fat (Seriburi and Akoh, 1998a). This is the consequences of reaction between a liquid (fatty oil) and a gas (hydrogen) occurring at a solid surface (catalyst) (Gunstone, 2004). However, this method results in the formation of geometric isomers, i.e., trans rather than cis configuration (Seriburi and Akoh, 1998a). Trans fatty acids have been suggested to raise blood low-density lipoprotein cholesterol and lower high-density lipoprotein cholesterol, thereby leading to coronary heart disease (Houde et al., 2004).

Concern about the health effects of trans unsaturated acids has raised interest in an alternative way of producing fats with the required melting behaviour. This can be achieved by interesterification of blend of natural or fractioned fats (Gunstone, 2004). Fractionation is limited by the sources of oils and the varieties of products (Piska et al., 2006).

In recent years, chemical interesterification is increasing in the industrial margarine production for the benefit of avoiding trans fatty acid formation and providing zero trans margarine in which fully hydrogenated fats are blended with liquid oils as the feedstocks of interesterification (Piska et al., 2006).

Alternatively, the desired melting behavior can be achieved through interesterification of suitable triacylglycerol mixtures with the use of *sn*-1,3-specific lipases or a combination of both procedures (Schmid and Verger, 1998). When short or medium chain fatty acids and LCFAs are incorporated, they can produce TAGs with good spreadability and temperature stability (Osborn and Akoh, 2002).

Chemical interesterification is a method with complete positional randomization of acyl groups in triacylglycerols (Piska et al., 2006). However, enzymatic approaches present several advantages: (1) no modification of the chemical properties of the original fat by interesterification, (2) constant fatty acids unsaturation levels and, (3) no cis-trans isomerization (Houde et al., 2004). The development of lipase catalyzed transesterification has given spread manufacturers yet another method for fat restructuring. This will expand further their ability to restructure fats and may even lead to 'designer' fats (Gunstone, 2001).

#### 2.7.2.2 Cocoa Butter

Cocoa butter is a mixture of oil and fat composed of triacylglycerols possessing palmitic acid, stearic acid, and oleic acid as the major components. Cocoa butter is a fat with a high commercial value for the confectionery industry, in particular chocolate (Houde et al., 2004). The low availability of cocoa butter, which causes high cost, has prompted much research on alternatives that can be used as cocoa butter replacements or extenders in chocolate and confectionery coatings. There are no naturally occurring fats with similar physical properties to cocoa butter; all alternatives are made by blending and/or modifying fats (Osborn and Akoh, 2002). Some typical applications of lipase-catalyzed interesterification reactions include the preparation, from in expensive starting materials, of properties (Akoh and Min, 2002). Consequently, interesterification of abundant and less expensive fats, including illipe fat, shea butter, sal fat, and kokum butter, offers a good alternative for the production of cheaper cocoa butter substitutes (Houde et al., 2004).

Production of cocoa butter equivalents by enzymatic acidolysis can be done by using *sn*-1,3 specific lipases that catalyze incorporation of palmitic acid and stearic acid to the *sn*-1,3 positions of a source oil containing oleic acid at *sn*-2 position until a similar composition of cocoa butter is obtained (Ciftci et al., 2009).

### 2.8 Experimental Design and Response Surface Methodology

Response surface methodology (RSM) has been an effective and a powerful statistical method for optimizing experimental conditions and investigation of critical processes by

reducing the number of experimental trials (Myers and Montgomery, 2002). The optimization of a process by RSM is a faster and more economical method for gathering research results than classical one-variable-at-a-time or full-factorial experimentation. RSM has been successfully applied to optimize enzymatic transesterification reactions in organic solvents in several studies (Haas et al., 1993; Huang and Akoh, 1996; Uosukainen et al., 1999).

The objective of studying RSM can be accomplish by

(1) understanding the topography of the response surface (local maximum, local minimum, ridge lines), and

(2) finding the region where the optimal response occurs. The goal is to move rapidly and efficiently along a path to get to a maximum or a minimum response so that the response is optimized (Bradley, 2007).

In RSM, both mathematical and statistical techniques were used together in the modeling and analysis of situations in which a response is affected by several variables, alone or in combination. The relationship between a set of independent variables and the response is determined by a mathematical model called *regression model*. When there are more than two independent variables the regression model is called *multiple-regression model*. There are many designs available for fitting a second-order model. The most popular one is the *central composite design* (CCD). This design was introduced by Box and Wilson. It consists of factorial points (from a 2*q* design and 2*q-k fractional factorial design*), central points, and axial points (Can and Ozcelik, 2005).

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

Triolein (OOO, purity ≥99%) and caprylic acid (CA) (purity ≥99%) were obtained from Sigma Chemical Co. (St. Louis, MO). Palmitic acid (PA) (purity ≥98%) was obtained from Merck (Darmstadt, Germany). Immobilizied *sn*-1,3 specific lipases (*Rhizopus oryzae*, 367 U/g; *Candida antarctica*, 2.1 U/mg; *Mucor miehei*, 140 U/mg; *Pseudomonas fluorescens*, 40 U/g) were purchased from Fluka Chemie Gmbh. Acetone, acetonitrile and *n*-hexane were purchased from Sigma Aldrich. All solvents used were HPLC grade. All other reagents and solvents were of analytical or chromatographic grade.

## 3.2 Lipase Screening

Acidolysis of triolein with caprylic and palmitic acids were carried out using four kinds of lipases from different sources and analyzed for their ability to incorporate CA and PA into triolein to produce desired TAGs. Immobilized *sn*-1,3 specific lipases (*Rhizopus oryzae*, *Candida antarctica*, *Mucor miehei*, *Pseudomonas fluorescens*) were used as biocatalysts. The reaction conditions of the screening study were chosen as enzyme load, 10 wt%; time, 24 h; reaction temperature, 55°C and substrate mole ratios (Triolein:CA:PA), 1:1:1. *sn*- 1,3 specific lipase from *Mucor miehei* producing desired TAGs at higher amounts was selected for further studies.

## 3.3 Effect of Caprylic Acid: Palmitic Acid Mole Ratio on the Acidolysis Reaction

A preliminary study was implemented prior to the RSM work to understand the effect of different CA:PA mole ratios on the lipase-catalyzed acidolysis reaction. The acidolysis reactions were performed as enzyme load, 10 wt%; time, 24 h and temperature, 55°C with variable substrate mole ratios (Triolein:CA:PA). Amount of triolein was fixed at 0.1 mmol in each reaction. The molar ratio of CA:PA was changed from 1:1 to 1:4 and from 1:1 to 4:1. The samples were analyzed using reversed-phase high performance liquid chromatography (RP-HPLC). The percentage of TAG obtained was calculated and optimum CA:PA ratio was chosen on the basis of higher incorporation of fatty acids to produce desired TAG content. The molar ratio of CA:PA was chosen as 1:1 to study in reaction systems. The selected CA:PA ratio was fixed and their ratio to triolein (Triolein:CA:PA) was ranged between 1:1:1 and 1:3:3 at RSM design.

### 3.4 Synthesis of Structured Lipids

Acidolysis of triolein was performed in 100- ml tightly closed-screw capped flasks. Triolein (0.1 mmol) and the correspondent ratio of caprylic and palmitic acids were mixed in 2 ml of *n*-hexane. The specified amount of enzyme (wt%) was added as given in (Table 1). The values of time and temperature were set according to an experimental central composite design Table 1. The reactions were carried out in a rotary incubator (New Brunswick Scientific, Nova 40, USA) at 200 rpm. At the end of the reaction, the product was separated from the enzyme by decanting. The *n*-hexane was evaporated in water bath at 60°C and the mixtures were stored at -20°C for subsequent analysis. All reactions were performed duplicate and average values were reported.

### 3.5 Removal of Free Fatty Acids

The reaction products were neutralized to remove free fatty acids in a similar manner as in de Araújo (2011). Reaction mixture (0.5 mg) was mixed with 20 ml *n*-hexane and 0.5 ml phenolphthalein. Then 0.5 N KOH in 20% (v) ethanol was added until the pink colour was observed. The upper layer was decanted. The lower layer was washed with *n*-hexane and the upper layer was again decanted. Remained *n*-hexane was evaporated and fatty acid free reaction product was obtained.

### 3.6 Separation of Fat Phase of Margarine

10-15 g margarine was incubated at 55°C for 40 min in a separatory funnel. The upper phase was separated and used in DSC analysis.

### 3.7 Methods of Analysis

#### 3.7.1 Analysis of Triacylglycerol Content

The TAG composition of reaction product was determined by reversed phase HPLC using the method proposed by AOCS Official Method Ce 5b-89 (AOCS, 1993). The analyses were carried out isocratically with a mobile phase consisting of 64:36 (v/v) acetone/acetonitrile. Oil was diluted in acetone, filtered and injected into the column (Sphereclone 5 µ ODS (2), 250 x 4.6 mm; Phenomenex, USA) with an accompanying guard column (40 x 3-mm id) of the same phase and eluted at a flow rate of 1.0 ml/min. The column temperature was set at 50°C and elution was monitored with a Schimadzu LC RID-10A refractive index detector. The total analysis time was 30 min. All triacylglycerol contents were expressed as weight percent of the total weight of the sample. All analyses were performed in duplicates, and average values were reported.

### 3.7.2 Analysis of Thermal Characteristics

#### 3.7.2.1 Melting Profile

Melting profiles of the samples were performed with a Perkin Elmer 6 DSC (Norwalk, CN, USA) instrument. The DSC instrument was calibrated with indium (m.p. 156.6°C, ΔHf = 28.45 J/g). Nitrogen was used as purge gas and flowed at 40 mL/min. A sample was completely melted at 80°C before being weighed (5-10 mg) into an aluminium pan which was then sealed. An empty, hermetically sealed aluminum pan was used as reference The previous thermal history of the sample was erased by heating the sample to 80°C in the DSC instrument and holding it for 10 min. The sample was then cooled to -60°C at a rate of 5°C/ min and waited at -60 °C for 10 min. At the end of the cooling, the sample was heated at 5°C/ min to 80°C. The onset temperature ($T_o$), and peak temperatures were determined. The melting and cooling temperature ranges were obtained by determining the difference between $T_o$ and $T_f$ . All DSC values reported are the average of two scans.

#### 3.7.2.2 Solid Fat Content

Percentage of SFC of the samples were analysed by DSC using the procedure explained in section 3.7.2.1. The SFC was calculated at various temperatures from the DSC heating thermogram data by partial integration according to Nassu and Goncalves (1995). The partial areas were obtained directly from calculations made using the DSC software (Pyris version 7.0). The percentages corresponding each temperature represent the liquid fraction of the sample. Solid fat fraction for each temperature was obtained by subtracting value of liquid fraction from 100.

### 3.8 Experimental Design

A five-level, four factorial central composite rotatable design (CCRD) was employed to study the responses, weight percent of TAG yields (COC, 1,3-capryloyl- 2- oleoyl-glycerol; COO, 1-capryloyl-2,3-dioleoyl-glycerol; POC, 1-palmitoyl-2-oleoyl-3-capryloyl; OOO, triolein; POO, 1-palmitoyl-2,3-dioleoyl-glycerol and POP, 1,3-dipalmitoyl-2-oleoyl-glycerol), effect of reaction factors on the production of TAGs and to optimize reaction conditions to obtain desired TAGs mixture. The independent variables were selected as reaction time (Ti), enzyme load (En), substrate molar ratio (Sr) and reaction temperature (Te). Levels of the independent variables were defined as Ti, 10-24 h; En, 10-25 wt%; Te, 45-60°C and Sr, (Triolein:CA:PA), 1:1:1-1:2.5:2.5. Although the substrate mole ratio (Triolein:CA:PA) in the reaction mixtures varied from 1:1:1 to 1:2.5:2.5, the molar ratio between fatty acids (CA:PA) was fixed at 1:1 among themselves. The employed CCRD was composed of 30 experiments consisting of 16 axial points, 8 star points, 6 center points (Table 3.1). The star points provide estimation of curvature of the models. Six replicate runs at the center point of the design were performed to allow the estimation of pure error.

CCRD consists of three parts: factorial points (−1, 1), center points (0, 0), and axial points (−1.68, 1.68). Each variable to be optimized was coded at these levels, and the ranges are shown in Table 3.2.

Table 3.1 A five-level, four factorial central composite rotatable design (CCRD) generated for acidolysis of triolein with caprylic and palmitic acids

| Run | Enzyme load (wt%) | Reaction temperature (°C) | Time (h) | Substrate mole ratio[1] |
|---|---|---|---|---|
| **1** | 10.0 | 45.0 | 10 | 1.00 |
| **2** | 25.0 | 45.0 | 10 | 1.00 |
| **3** | 10.0 | 60.0 | 10 | 1.00 |
| **4** | 25.0 | 60.0 | 10 | 1.00 |
| **5** | 10.0 | 45.0 | 24 | 1.00 |
| **6** | 25.0 | 45.0 | 24 | 1.00 |
| **7** | 10.0 | 60.0 | 24 | 1.00 |
| **8** | 25.0 | 60.0 | 24 | 1.00 |
| **9** | 10.0 | 45.0 | 10 | 2.50 |
| **10** | 25.0 | 45.0 | 10 | 2.50 |
| **11** | 10.0 | 60.0 | 10 | 2.50 |
| **12** | 25.0 | 60.0 | 10 | 2.50 |
| **13** | 10.0 | 45.0 | 24 | 2.50 |
| **14** | 25.0 | 45.0 | 24 | 2.50 |
| **15** | 10.0 | 60.0 | 24 | 2.50 |
| **16** | 25.0 | 60.0 | 24 | 2.50 |
| **17** | 2.5 | 52.5 | 17 | 1.75 |
| **18** | 32.5 | 52.5 | 17 | 1.75 |
| **19** | 17.5 | 37.5 | 17 | 1.75 |
| **20** | 17.5 | 67.5 | 17 | 1.75 |
| **21** | 17.5 | 52.5 | 3 | 1.75 |
| **22** | 17.5 | 52.5 | 31 | 1.75 |
| **23** | 17.5 | 52.5 | 17 | 0.25 |
| **24** | 17.5 | 52.5 | 17 | 3.25 |
| **25** | 17.5 | 52.5 | 17 | 1.75 |
| **26** | 17.5 | 52.5 | 17 | 1.75 |
| **27** | 17.5 | 52.5 | 17 | 1.75 |
| **28** | 17.5 | 52.5 | 17 | 1.75 |
| **29** | 17.5 | 52.5 | 17 | 1.75 |
| **30** | 17.5 | 52.5 | 17 | 1.75 |

[1] Substrate mole ratio refers to Triolein:Caprylic acid:Palmitic acid, ranged between 1:1:1 and 1:2.5:2.5.

Table 3.2 Independent variables and their levels for central composite design in optimization of lipase-catalyzed esterification process

| Independent Variables | Symbol | Coded variable levels | | | | |
|---|---|---|---|---|---|---|
| | | -1.68 | -1 | 0 | +1 | +1.68 |
| Reaction time | Ti | 3 | 10 | 17 | 24 | 31 |
| Enzyme load | En | 2.5 | 10 | 17.5 | 25 | 32.5 |
| Substrate molar ratio | Sr | 0.25 | 1 | 1.75 | 2.5 | 3.25 |
| Reaction temperature | Te | 37.5 | 45 | 52.5 | 60 | 67.5 |

### 3.9 Data Analysis and Optimization by RSM

The data obtained were analyzed using RSM (Stat-Ease, Design-Expert software, version 7). Analysis of variance (ANOVA), regression analysis and model generation were used to evaluate the effects of factors and to optimize reaction conditions. The level of significance for all tests was set at 95% confidence level. The goodness of the models established were determined using coefficient of determination, $R^2$, together with the absolute average deviation (AAD) values and ANOVA (Arifin et al., 2010).

The first- or second-order coefficients were generated by regression analysis with backward elimination. The quadratic response surface model was fitted to the equation (3.1)

$$Y_i = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + \varepsilon \quad (3.1)$$

where $Y_i$ (i = 1–6) are the responses for weight percent of produced TAGs, namely, COC ($Y_1$), COO ($Y_2$), POC ($Y_3$), OOO ($Y_4$), POO ($Y_5$), POP ($Y_6$). $\beta_0$, intercept, $\beta_i$ first-order model coefficients, $\beta_{ii}$ quadratic coefficients for the ith variable, $\beta_{ij}$ interaction coefficients for the interaction of variable i and j, and $X_i$ are independent variables and $\varepsilon$ is the random error.

**3.10 Calorie Value Determinations of Structured Lipids**

The TAG compositions of structured lipids obtained from each run are shown in Table 1. Heat energy values of fatty acids were estimated by using the equation (3.2) proposed by (Taguchi et al., 2001).

$$-\Delta H_c(fatty\ acid) = 0.653n - 0.166d - 0.421 \quad (3.2)$$

where $-\Delta H_c$ is the heat of combustion in MJ/mol, n is the number of carbon atoms/molecule of fatty acid, and d is the number of double bonds/FA.

Heats of combustion of each produced TAGs were also determined separately in MJ/ grams of fatty acids in 100 g TAG using the approach of Livesey (1984) according to the equation (3.3)

$$-\Delta H_c(triacylglycerol) = 1.66 \sum_{FA_1}^{FA_\infty} \frac{B'}{3D} + \sum_{FA_1}^{FA_\infty} \frac{B'}{D} I \quad (3.3)$$

in which B' is g fatty acid /100 g TAG total fatty acid, D is molecular weight of the corresponding fatty acid and I is -ΔHc of the corresponding fatty acid. The constant 1.66 is heat of combustion (MJ/mol) for glycerol. -ΔHc*(triacylglycerol)* is the potential metabolizable energy of the TAG. To give -ΔHc*(triacylglycerol)* in MJ/ g TAG, -ΔHc*(triacylglycerol)* due to equation (3) was divided by the molecular weight of the triacylglyceride/ grams of fatty acids in 100 g TAG.

Caloric values of SLs produced from each run were calculated according to their TAG composition by using equation (3.4)

$$-\Delta H_{c,\,SL} = X_{coc}{*}\Delta H_{c,\,coc} + X_{coo}{*}\Delta H_{c,\,coo} + X_{poc}{*}\Delta H_{c,\,poc} + X_{ooo}{*}\Delta H_{c,\,ooo} + X_{poo}{*}\Delta H_{c,\,poc} + X_{pop}{*}\Delta H_{c,\,pop} \quad (3.4)$$

here, $-\Delta H_{c,\,SL}$ is the caloric value of produced structured lipids in kJ/ g structured lipid, X represents the fraction of TAG/ g structured lipid, and $\Delta H_c$ is the heat of combustion of TAG in kJ/g TAG and $-\Delta H_{c,\,SL}$ is the energy value of produced structured lipids in kJ/ g structured lipid.

## CHAPTER 4

## RESULTS AND DISCUSSION

### 4.1 Lipase Screening

In the acidolysis of triolein with caprylic (CA) and palmitic (PA) acids using *sn*-1,3 specific lipase there are six possibilities of triacylglycerol (TAG) components, namely, COC, 1,3-capryloyl-2-oleoyl-glycerol; COO, 1-capryloyl-2,3-dioleoyl-glycerol; POC, 1-palmitoyl-2-oleoyl-3-capryloyl; OOO, triolein; POO, 1-palmitoyl-2,3-dioleoyl-glycerol and POP, 1,3-dipalmitoyl-2-oleoyl-glycerol (Figure 4.1). COC, POC and POP are the most desired triacylglycerols to produce a reduced calorie spreadable structured lipid (SL) from triolein using caprylic and palmitic acids. COC is the most effective TAG on reducing calorie, POP is the most effective one on increasing melting point and POC combines the unique characteristics of caprylic and palmitic acids in one TAG.

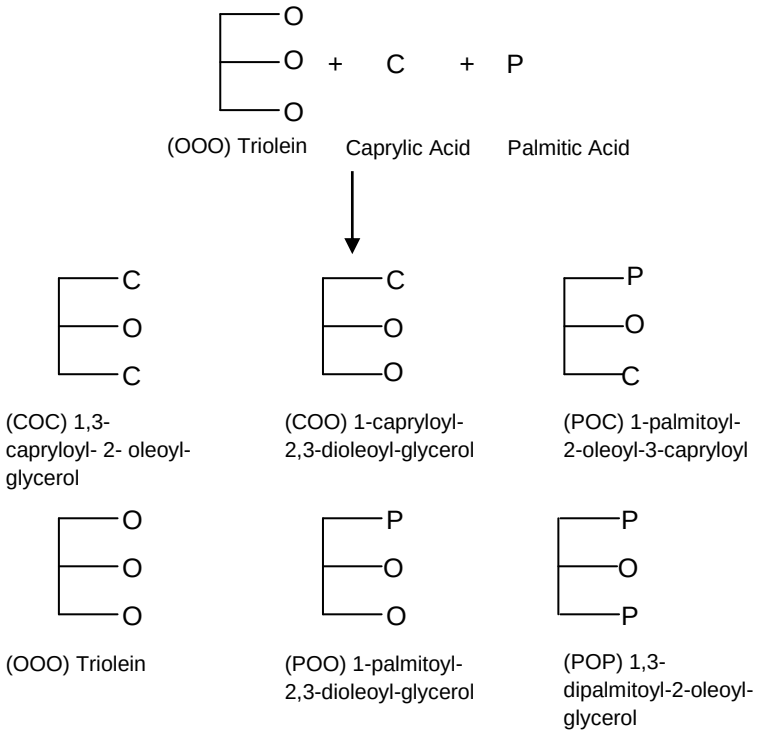



Figure 4.1. Six possible products of the interesterification of triolein

Four enzymes from different sources were analyzed for their ability to produce desired triacylglycerols. Figure 4.2 shows the effect of different lipases on percent yield of structured TAGs. Lipase from *Pseudomonas fluorescence* showed the lowest catalytic activity and gave

lowest yields of desired TAGs. *Mucor miehei* catalyzed acidolysis reaction promised the highest incorporation of caprylic and palmitic acids into triolein at *sn*-1,3 positions and resulted in the less percent residual triolein. Our results were in the aggrement with Hamam and Shahid (2007) who suggested that the lipase from *Rhizomucor miehei* (formerly, *M. miehei*) might be considered the most active catalyst in the acidolysis reactions of triolein and a mixture of equimole amounts of n-3 FA.

The percent of residual triolein was lower in lipase from *M. miehei* than *Candida antarctica* although the percent incorporation at *sn*-1,3 positions were slightly higher in the case of *C. antarctica* as it was seen in Figure 4.2. Huang and Akoh (1995) produced a SL via transesterification of caprylic acid ethyl ester and triolein using eight lipases. Among the enzymes tested, immobilized IM 60 from *Rhizomucor miehei* converted most of the triolein into SL dicaprylolein (41.70%) and monocaprylolein (46.0%). However, lipase SP 435 from *C. antarctica* catalyzed the conversion of triolein into dicaprylolein (62.0%) and monocaprylolein (33.5%) at 55°C. These results are in parallel with our results. However, due to total yield of produced triacylglcerols and economic considerations, *M. miehei* was selected as the catalyst for further acidolysis reactions among the analyzed lipases. In further studies, the acidolysis reactions were carried out using lipase from *M. miehei.*

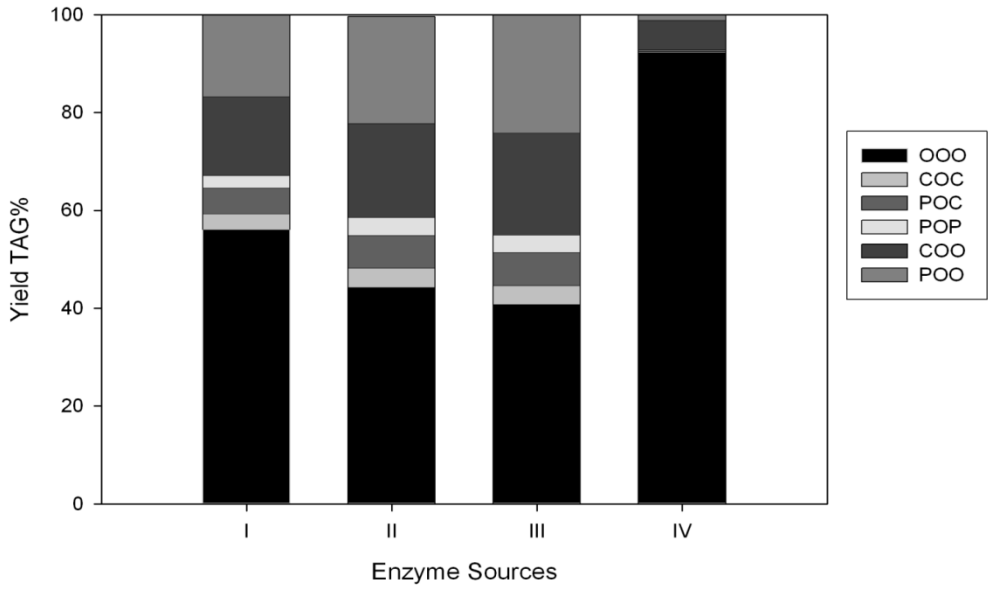



Figure 4.2. Effect of different lipases on the production of structured TAGs and residual triolein. (I) *R. oryzae,* (II) *C. antarctica*, (III) *M.miehei*, (IV) *P. fluorescence*

Tsuzuki (2005) examined ten kinds of lipase as biocatalyst in the incorporation of short chain fatty acids into triolein. He concluded that the progress rate of lipases from *Aspergillus oryzae*, *Pseudomonas cepecia, Rhizopus niveus*, *Rhizopus arhizus* and *Mucor miehei* depended on the length of a fatty acid chain used and *A. oryzae* lipase was selected as the most powerful biocatalyst for the acidolysis reaction. Carrin and Crapiste (2008) studied

three immobilized lipases using fractional factorial methodology. They performed acidolysis reaction of sunflower oil with a mixture of palmitic-stearic acids in a batch reactor. They found the highest incorporation occurred in the following order: *Rhizomucor miehei*, *Thermomyces lanuginose* and *Candida antarctica*. They found *C. antarctica* had the lowest activity opposite to our result. This can be related to changeable regiospecificity of *C. antarctica* depending on used reactants. Novo (1992) reported that the positional specificity of *C. antarctica* depends on the reactants. In some reactions, *C. antactica* shows a 1,3-positional specificity, whereas in other reactions, the lipase functions as a nonpositional specific lipase (Seriburi and Akoh, 1998b). In this study, lipase from *C. antarctica* behaved as an *sn*-1,3 specific lipase.

### 4.2 Determination of Mole Ratio of CA:PA for Experimental Design

A preliminary study was conducted prior to the RSM work to understand the effect of different CA:PA mole ratio on the *sn*-1,3 specific lipase catalyzed acidolysis reaction. The molar ratio of CA:PA was changed from 1:1 to 1:4 and from 2:1 to 4:1. Table 4.1 shows the yields of TAGs produced after acidolysis when different caprylic acid:palmitic acid mole ratios were used. In the acidolysis reactions varying substrate mole ratio between 2:1 and 4:1, high amounts of COC (32-34%) were observed as seen in Table 4.1. However, no yield of POP was observed in this range of substrate mole ratio. Vice versa in the acidolysis reactions varying substrate mole ratio between 1:3 and 1:4, although high yields of POP were observed, yields of COC were so low (1.84-1.99%). These compositions would not be suitable to produce a reduced calorie spreadable structured lipid from triolein because of lower incorporations of caprylic but higher incorporations of palmitic acid and vice versa. It was obviously clear that increasing the amount of one of the fatty acids gave rise to the incorporation of this fatty acid while decreasing the incorporation of the other fatty acid.

Table 4.1 Effect of CA:PA mole ratio on triacylglycerol formation

| Triolein:CA:PA | COC% | COO% | POC% | OOO% | POO% | POP% |
|---|---|---|---|---|---|---|
| **1:1:1** | 3.80 | 20.76 | 6.85 | 40.79 | 24.25 | 3.55 |
| **1:2:1** | 32.05 | 39.08 | 9.24 | 12.28 | 7.33 | - |
| **1:3:1** | 32.84 | 34.45 | 14.14 | 11.70 | 6.85 | - |
| **1:4:1** | 34.54 | 36.87 | 11.10 | 11.73 | 5.84 | - |
| **1:1:2** | 1.87 | 12.55 | 4.65 | 50.80 | 25.38 | 4.73 |
| **1:1:3** | 1.84 | 10.88 | 9.74 | 21.84 | 38.57 | 17.10 |
| **1:1:4** | 1.99 | 9.25 | 10.67 | 17.07 | 38.66 | 22.34 |

Based on these results, 1:1 was selected as the substrate mole ratio between CA and PA and this ratio was remained constant in the experimental design since desired TAGs (COC, POC and POP) could be seen in moderate amounts in the final product. When the substrate mole ratio was 1:1, percentages of COC, POC, and POP were observed as 3.80, 6.85 and

3.55, respectively. The effect of Triolein:CA:PA mole ratio studied in the experimental design.

### 4.3 Diagnostic Checking of Fitted Models

RSM was implemented to model the six responses, weight percent of COC, COO, POC, POO, and POP and residual weight percent of OOO. The data of responses obtained from reactions under different conditions as defined in central composite rotatable design (CCRD) are shown in Table 4.2. The models predicted for COC, COO, POC, OOO, POO, and POP% were significant at the 99% confidence level and the lack of fits were not significant ($P<0.05$) with high coefficients of determinations ($R^2$) between 0.92 and 0.96. The high values of coefficient of determination, significance of model and non-significant lack of fit indicate that the model is a good fit. The best fitting quadratic models were determined by regression and backward elimination by means of elimination of insignificant factors and interactions in the models.

Table 4.2 TAG% composition of the SLs obtained by acidolysis of triolein with CA and PA under the conditions generated by RSM

| Run | COC % | COO% | POC% | OOO% | POO% | POP% | Energy (kJ/g) |
|---|---|---|---|---|---|---|---|
| **1** | 1.70 | 25.15 | 1.78 | 46.43 | 24.13 | 0.81 | 39.17 |
| **2** | 2.43 | 24.50 | 5.45 | 40.05 | 24.79 | 2.78 | 39.07 |
| **3** | 4.85 | 24.69 | 9.03 | 32.23 | 25.80 | 3.40 | 38.91 |
| **4** | 5.87 | 23.98 | 10.06 | 30.58 | 24.61 | 4.90 | 38.86 |
| **5** | 2.24 | 22.62 | 4.26 | 46.19 | 23.49 | 1.20 | 39.14 |
| **6** | 3.05 | 22.65 | 4.47 | 43.97 | 22.99 | 2.87 | 39.10 |
| **7** | 4.73 | 21.86 | 10.06 | 31.61 | 26.36 | 5.38 | 38.92 |
| **8** | 6.65 | 24.20 | 14.57 | 24.75 | 23.25 | 6.58 | 38.74 |
| **9** | 14.50 | 27.27 | 14.03 | 29.42 | 12.08 | 2.70 | 38.48 |
| **10** | 23.10 | 28.25 | 18.80 | 10.33 | 14.83 | 4.69 | 38.07 |
| **11** | 19.89 | 28.91 | 16.56 | 20.08 | 11.96 | 2.60 | 38.23 |
| **12** | 29.10 | 27.55 | 25.15 | 2.61 | 10.47 | 4.38 | 37.74 |
| **13** | 22.11 | 32.73 | 10.64 | 18.80 | 12.37 | 3.35 | 38.22 |
| **14** | 29.10 | 31.09 | 18.42 | 4.42 | 12.17 | 4.80 | 37.84 |
| **15** | 28.23 | 29.65 | 20.11 | 6.70 | 11.03 | 4.28 | 37.86 |
| **16** | 33.83 | 25.88 | 25.27 | 1.85 | 6.79 | 6.38 | 37.62 |
| **17** | 2.03 | 28.66 | 5.01 | 49.23 | 13.18 | 1.89 | 39.06 |
| **18** | 25.02 | 28.19 | 20.13 | 10.76 | 10.7 | 5.20 | 37.99 |
| **19** | 23.31 | 35.20 | 12.01 | 15.75 | 11.93 | 1.80 | 38.12 |
| **20** | 23.44 | 36.30 | 17.73 | 6.01 | 11.76 | 4.76 | 37.98 |
| **21** | 12.10 | 35.41 | 13.19 | 14.70 | 21.94 | 2.66 | 38.45 |
| **22** | 16.60 | 34.27 | 13.14 | 9.36 | 22.08 | 4.55 | 38.31 |
| **23** | 1.02 | 4.93 | 1.85 | 62.11 | 28.11 | 1.98 | 39.45 |
| **24** | 30.78 | 14.91 | 28.17 | 13.27 | 7.67 | 5.20 | 37.82 |
| **25** | 28.30 | 29.51 | 20.10 | 6.1 | 12.68 | 3.31 | 37.87 |
| **26** | 33.20 | 28.72 | 24.89 | 0.2 | 8.94 | 4.45 | 37.78 |
| **27** | 25.60 | 30.86 | 24.70 | 4.57 | 10.96 | 3.31 | 37.85 |
| **28** | 26.70 | 29.58 | 26.00 | 0.72 | 12.57 | 4.45 | 37.81 |
| **29** | 27.20 | 30.17 | 25.30 | 2.48 | 11.75 | 3.10 | 37.79 |
| **30** | 29.80 | 30.85 | 24.50 | 0.9 | 10.56 | 3.39 | 37.71 |

C: caprylic acid, O: Oleic acid, P: Palmitic acid

The linear, quadratic, and interactive coefficients of independent paramaters are given below:

$$COC\% = 28.47 + 3.40*En + 1.50*Te + 1.53*Ti + 9.53*Sr + 1.67*En*Sr + 1.51*Ti*Sr - 4.12*En^2 - 1.65*Te^2 - 3.91*Ti^2 - 3.52*Sr^2 \quad (4.1)$$

$$COO\% = 29.9 - 0.24*En - 0.22*Te - 0.07*Ti + 2.57*Sr + 0.90*Ti*Sr - 0.53*En^2 + 1.30*Te^2 - 1.07*Ti^2 - 5.16*Sr^2 \quad (4.2)$$

$$POC\% = 24.2 + 2.75*En + 2.68*Te + 0.29*Ti + 5.91*Sr + 1.06*En*Sr - 3.06*En^2 - 2.29*Te^2 - 2.91*Ti^2 - 2.45*Sr^2 \quad (4.3)$$

$$OOO\% = 2.49 - 6.24*En - 4.53*Te - 1.84*Ti - 12.4*Sr - 2.42*En*Sr + 7.16*En^2 + 2.38*Te^2 + 2.67*Ti^2 + 9.09*Sr^2 \quad (4.4)$$

$$POO\% = 12.2 - 0.51*En - 0.29*Te - 0.41*Ti - 6.03*Sr + 1.67*En*Sr - 0.80*En*Ti + 0.99*Ti*Sr + 2.90*Ti^2 - 1.87*Sr^2 \quad (4.5)$$

$$POP\% = 3.7 + 0.84*En + 0.86*Te + 0.51*Ti + 0.49*Sr + 0.38*Te*Ti - 0.66*Te*Sr \quad (4.6)$$

### 4.4 Effects of Reaction Parameters: Statistical Evaluation of the Reaction Parameters and Response Surface Plotting

The effects of the reaction parameters on the enzymatic production of structured triacylglycerols and residual triolein were evaluated by statistical analysis and response surface plotting. By this way, it was possible to analyse the linear and quadratic effects of reaction parameters and interactions amongst them for each responses. Effects of the parameters were interpreted relating the coefficients of the parameters and corresponding P values. Coefficients and P values were calculated by Design Expert 7.0 software for each responses. A P value below 0.05 indicates a confidence interval of 95%. In this thesis, it was analyzed such that effects had a value of 'P>F' less than 0.05 effects are significant and vice versa effects had a value of 'P>F' higher than 0.05 effects are nonsignificant. Coefficients and P values for each responses were presented in Tables 4.2-7.

Response surface plots were obtained using Design Expert 7.0 software. In Figures 4.3-8 response surface plots were represented for each triacylglycerols. The relationships between independent and dependent variables are shown in the three-dimensional representation as response surfaces (Wanasundara and Shahidi,1999). Response surface plots composed of three axis. The levels of parameters are shown on x and y axis and the yield of related triacylglcerol is shown on z axis. Other two corresponding parameters which were not shown on the plots were kept constant at their center levels.

#### 4.4.1 Effect of Substrate Mole Ratio on the Production of TAGs

Substrate mole ratio had the highest coefficient for COC and POC (Table 4.2-3). Thus, it can be said that substrate mole ratio was the most effective factor in the production of COC and POC ($P<0.0001$). Positive sign of this coefficient means increasing the substrate mole ratio caused increase in COC and POC. This result was also supported by response surface

plots. In Figure 4.3a,e,f and 4.4a,e,f, it is seen that increase in substrate mole ratio increases COC and POC until a maximum point of 1:2.5:2.5. Bektas et al. (2008) found that the similar results in their study. Zhou et al. (2001) and Lumor and Akoh (2005) also found the substrate mole ratio was the most important factor affecting incorporation of fatty acid into oil. Beside the main effect of substrate mole ratio, it had also an interaction effect with another factor, enzyme load. In Figure 3-4a, it is seen that increase in the percentage yield at higher enzyme loads is higher than the increase at lower enzyme loads with increasing substrate mole ratio. This may be due to the saturation of of enzyme with substrate at lower enzyme loads and so some of the substrates cannot find any active sites to which to bind. However, the term En*Sr were not statistically significant as it was seen in Table 4.2-3.

Similar to that of COC and POC, substrate mole ratio had also a positive coefficient for POP and it was significantly important (P<0.0001) (Table 4.4). However, it had the lowest coefficient amongst the other parameters. This means it had the least effect on the production of POP. In parallel to this ANOVA result, in Figure 4.5a-e-f, it can be observed that increasing the substrate mole ratio caused increase in POP. However, in this case there is a slight change in the production of POP unlike to COC and POC in which a large change occurred.

Table 4.3 Analysis of variance table and estimated coefficients for COC%

| Source | Coefficients | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|---|
| **Model** | | 3603.48 | 10 | 360.34 | 30.62 | < 0.0001 | significant |
| **Intercept** | 28.47 | | | | | | |
| **Linear** | | | | | | | |
| **EnzymeLoad** | 3.40 | 277.44 | 1 | 277.44 | 23.57 | 0.0001[1] | |
| **Temp** | 1.50 | 53.76 | 1 | 53.76 | 4.56 | 0.0458[1] | |
| **Time** | 1.53 | 56.30 | 1 | 56.30 | 4.78 | 0.0414[1] | |
| **Sr** | 9.53 | 2177.41 | 1 | 2177.41 | 185.03 | < 0.0001[1] | |
| **Interaction** | | | | | | | |
| **En*Sr** | 1.67 | 44.42 | 1 | 44.42 | 3.77 | 0.0670[2] | |
| **Ti*Sr** | 1.51 | 36.36 | 1 | 36.36 | 3.08 | 0.0949[2] | |
| **Quadratic** | | | | | | | |
| **$En^2$** | -4.12 | 464.54 | 1 | 464.54 | 39.47 | < 0.0001[1] | |
| **$Te^2$** | -1.65 | 74.93 | 1 | 74.93 | 6.36 | 0.0207[1] | |
| **$Ti^2$** | -3.91 | 419.15 | 1 | 419.15 | 35.62 | < 0.0001[1] | |
| **$Sr^2$** | -3.52 | 340.17 | 1 | 340.17 | 28.90 | < 0.0001[1] | |
| **Residual** | | 223.57 | 19 | 11.76 | | | |
| **Lack of Fit** | | 186.42 | 14 | 13.31 | 1.79 | 0.2696 | not significant |
| **Pure Error** | | 37.15 | 5 | 7.43 | | | |
| **Cor Total** | | 3827.06 | 29 | | | | |

$R^2$= 0.94, adj $R^2$=0.91, pred $R^2$= 0.77
Te: temperature, Enz: enzyme load, Sr: substrate mole ratio
[1] Significant at 'Prob>F' less than 0.05; [2] not significant at 'Prob>F' higher than 0.05

Table 4.4 Analysis of variance table and estimated coefficients for POC%

| Source | Coefficients | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|---|
| **Model** | | 1794.60 | 9 | 199.40 | 45.14 | < 0.0001 | significant |
| **Intercept** | 24.2 | | | | | | |
| **Linear** | | | | | | | |
| **En** | 2.75 | 181.28 | 1 | 181.28 | 41.04 | < 0.0001[1] | |
| **Te** | 2.68 | 172.80 | 1 | 172.80 | 39.12 | < 0.0001[1] | |
| **Ti** | 0.29 | 1.94 | 1 | 1.94 | 0.44 | 0.5141[2] | |
| **Sr** | 5.91 | 839.45 | 1 | 839.45 | 190.05 | < 0.0001[1] | |
| **Interaction** | | | | | | | |
| **En*Sr** | 1.06 | 17.80 | 1 | 17.80 | 4.031 | 0.0584[2] | |
| **Quadratic** | | | | | | | |
| **$En^2$** | -3.06 | 257.39 | 1 | 257.39 | 58.27 | < 0.0001[1] | |
| **$Te^2$** | -2.49 | 169.83 | 1 | 169.83 | 38.44 | < 0.0001[1] | |
| **$Ti^2$** | -2.91 | 233.00 | 1 | 233.00 | 52.75 | < 0.0001[1] | |
| **$Sr^2$** | -2.45 | 165.08 | 1 | 165.08 | 37.37 | < 0.0001[1] | |
| **Residual** | | 88.34 | 20 | 4.41 | | | |
| **Lack of Fit** | | 66.27 | 15 | 4.41 | 1.00 | 0.5484 | not significant |
| **Pure Error** | | 22.06 | 5 | 4.41 | | | |
| **Cor Total** | | 1882.94 | 29 | | | | |

$R^2$= 0.95, adj $R^2$=0.93, pred $R^2$= 0.85
Te: temperature, Enz: enzyme load, Sr: substrate mole ratio
[1] Significant at 'Prob>F' less than 0.05; [2] not significant at 'Prob>F' higher than 0.05

Table 4.5 Analysis of variance table and estimated coefficients for POP%

| Source | Coefficients | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|---|
| **Model** | | 56.13 | 6 | 9.35 | 49.96 | < 0.0001 | significant |
| **Intercept** | 3.7 | | | | | | |
| **Linear** | | | | | | | |
| **En** | 0.84 | 17.13 | 1 | 17.13 | 91.51 | < 0.0001[1] | |
| **Te** | 0.86 | 17.71 | 1 | 17.71 | 94.61 | < 0.0001[1] | |
| **Ti** | 0.51 | 6.36 | 1 | 6.36 | 33.99 | < 0.0001[1] | |
| **Sr** | 0.49 | 5.70 | 1 | 5.70 | 30.46 | < 0.0001[1] | |
| **Interaction** | | | | | | | |
| **Te*Ti** | 0.38 | 2.32 | 1 | 2.32 | 12.41 | 0.0018[1] | |
| **Te*Sr** | -0.66 | 6.89 | 1 | 6.89 | 36.79 | < 0.0001[1] | |
| **Residual** | | 4.30 | 23 | 0.18 | | | |
| **Lack of Fit** | | 2.42 | 18 | 0.13 | 0.35 | 0.9508 | not significant |
| **Pure Error** | | 1.87 | 5 | 0.37 | | | |
| **Cor Total** | | 60.44 | 29 | | | | |

$R^2$= 0.92, adj $R^2$=0.91, pred $R^2$= 0.89
Te: temperature, Enz: enzyme load, Sr: substrate mole ratio
[1] Significant at 'Prob>F' less than 0.05; [2] not significant at 'Prob>F' higher than 0.05

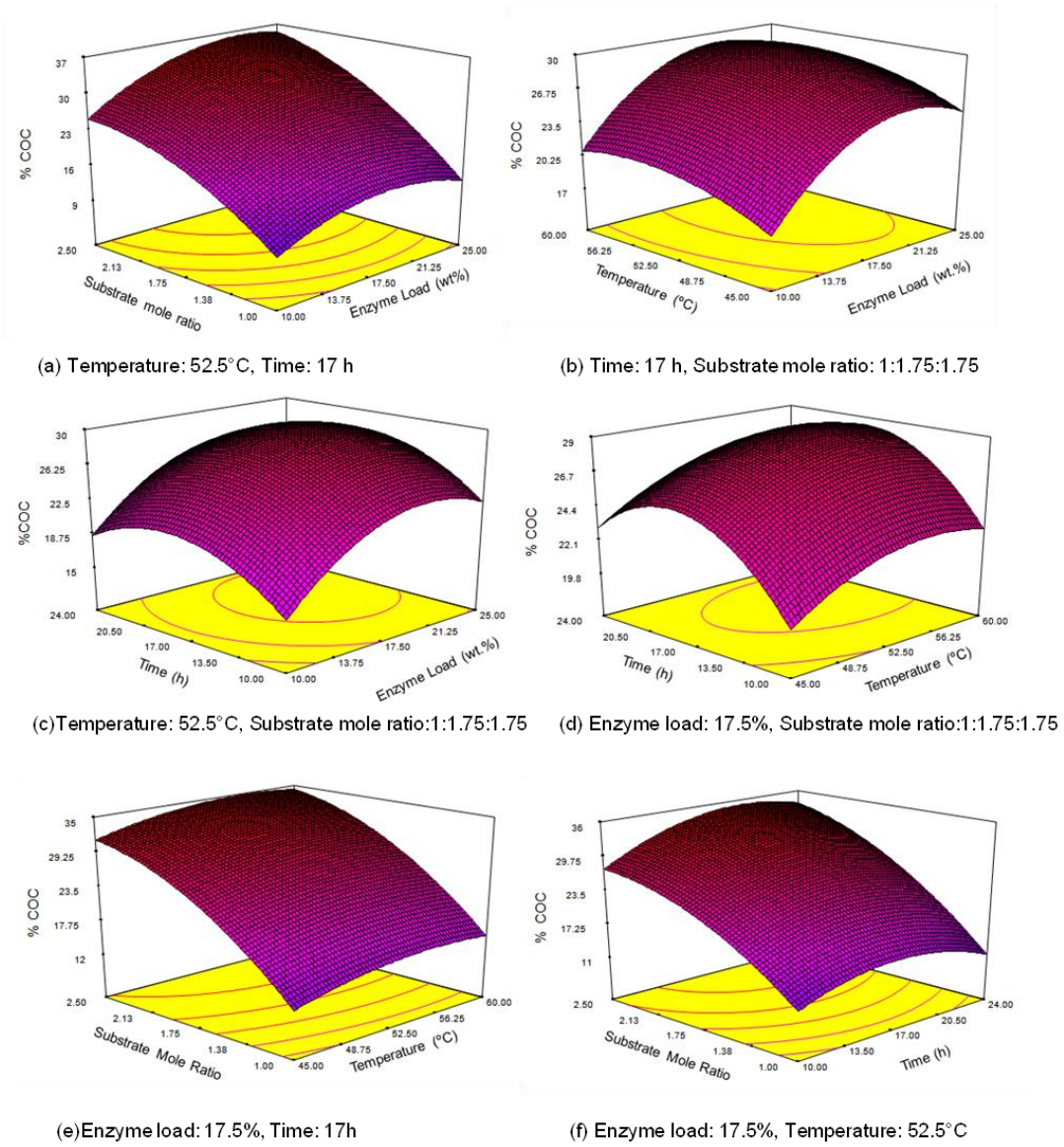
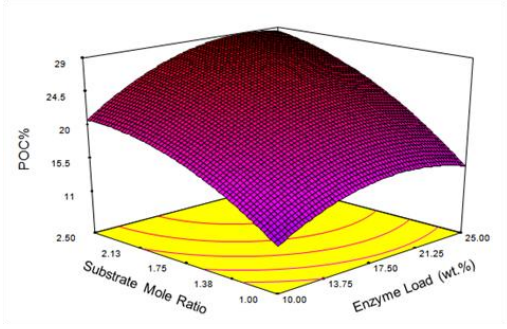


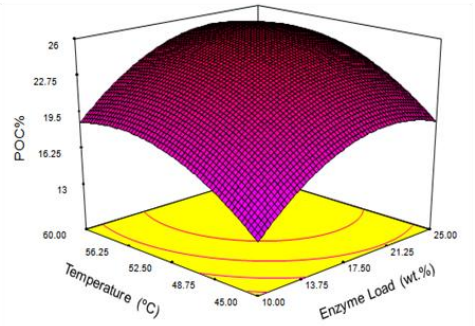

Figure 4.3. Response surface plots for COC%: (a) substrate mole ratio versus time; (b) temperature versus time; (c) time versus enzyme load; (d) substrate mole ratio versus enzyme load; (e) time versus temperature; (f) time versus enzyme load

In the production of COO and POO, substrate mole ratio was the only statistically significant factor ($P<0.0001$) (Table 4.5-6). It had a positive coefficient for COO while it had a negative coefficient for POO. This means increasing the substrate mole ratio affects the production of COO positively while affecting POO negatively.

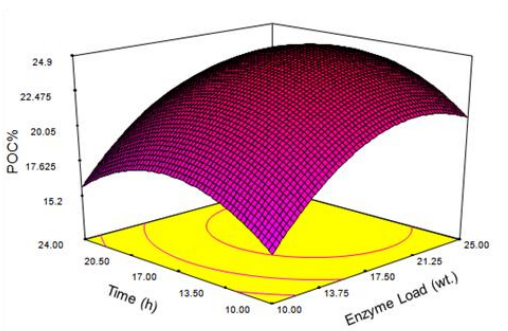
In the case of OOO, substrate mole ratio was also the predominant parameter amongst the other parameters with the highest coefficient (Table 4.8). As it is expected, there is a contrary relationship between OOO and substrate mole ratio. Also, in Figure 4.8a,e,f, it is seen that increasing substrate mole ratio decreased the residual OOO when the other parameters were fixed at a constant value.



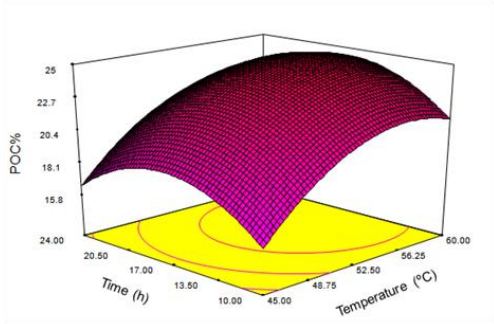

(a) Temperature: 52.5°C, Time: 17 h



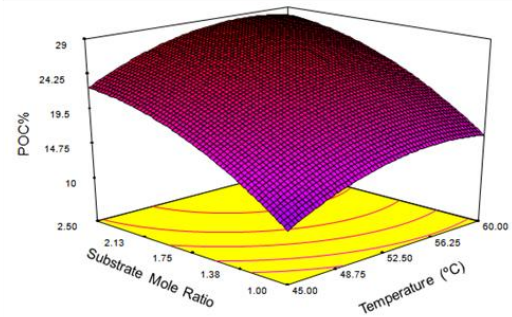

(b) Time: 17 h, Substrate mole ratio: 1:1.75:1.75



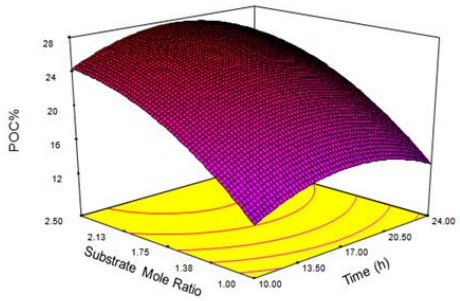

(c)Temperature: 52.5°C, Substrate mole ratio:1:1.75:1.75




(d) Enzyme load: 17.5%, Substrate mole ratio:1:1.75:1.75




(e)Enzyme load: 17.5%, Time: 17h




(f) Enzyme load: 17.5%, Temperature: 52.5°C

Figure 4.4. Response surface plots for POC%: (a) substrate mole ratio versus time; (b) temperature versus time; (c) time versus enzyme load; (d) substrate mole ratio versus enzyme load; (e) time versus temperature; (f) time versus enzyme load

Figure 4.8f shows the change of OOO with the substrate mole ratio and time when the other parameters were kept constant. Increase in the substrate mole ratio caused a marginal decrease in the residual weight of OOO. However, after 1:2:2, the value of triolein did not changed. This is probably the results of the enzyme saturation with substrate. The further increase in substrate mole ratio might probably cause acidification of enzyme.

### 4.4.2 Effect of Enzyme Load on the Production of TAGs

Enzyme load followed the substrate mole ratio in the decreasing effectiveness order for COC and POC when the coefficients of enzyme load were considered (Table 4.2-3). Enzyme load was more effective than time and temperature ($P<0.0001$) and it had a positive coefficient. The response surface plots (Figure 4.3-4b) demonstrated the statistical analysis results.

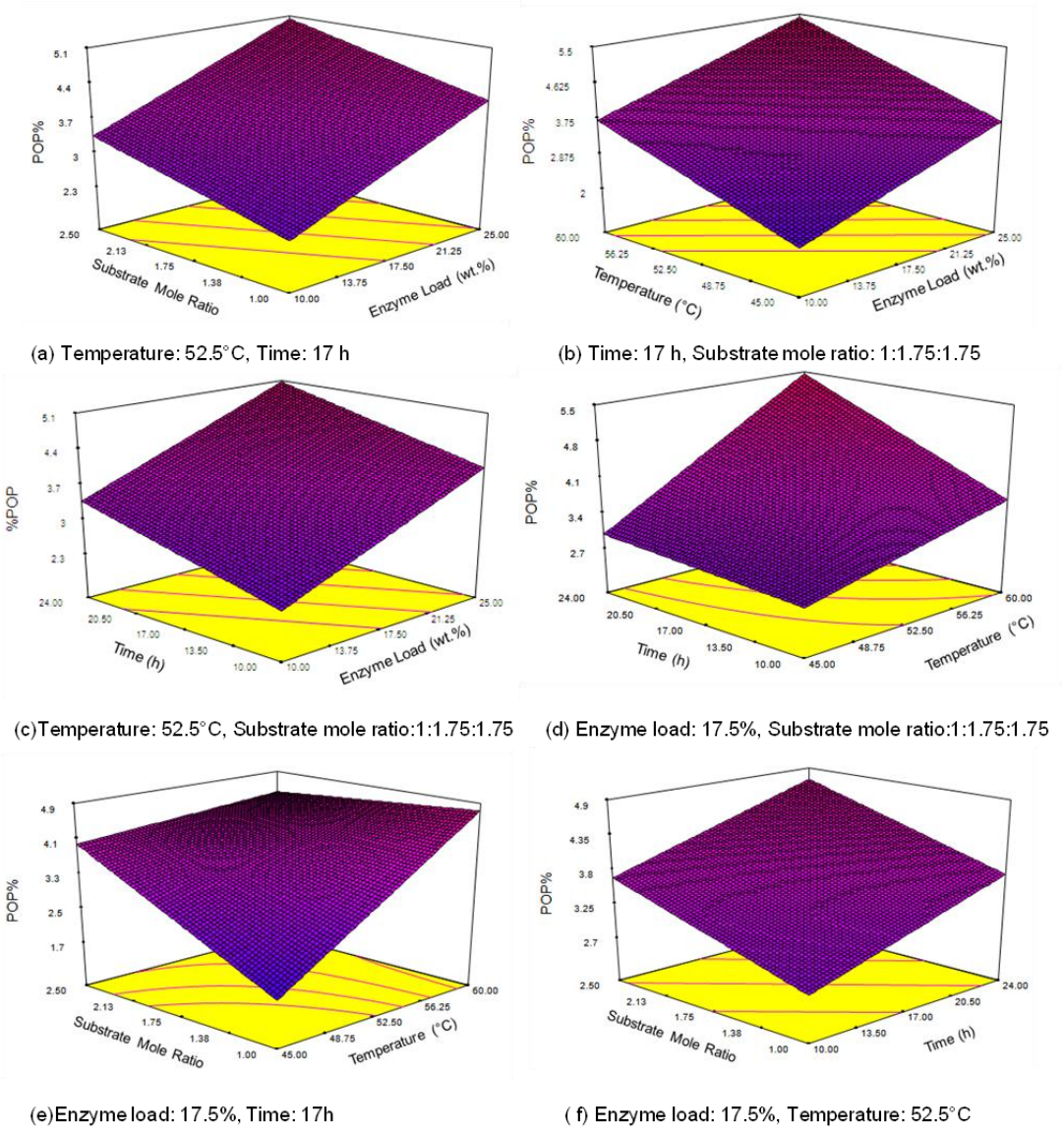



Figure 4.5. Response surface plots for POP%: (a) substrate mole ratio versus time; (b) temperature versus time; (c) time versus enzyme load; (d) substrate mole ratio versus enzyme load; (e) time versus temperature; (f) time versus enzyme load

In Figure 4.3-4b, it is seen that increasing the enzyme load resulted in an increase in COC and POC until an optimum point when the other parameters were kept constant. The optimum point of enzyme load is 20 wt% for COC and POC. After that point, increasing the enzyme load did not change the incorporation and so the yields reached equilibrium at 20 wt% enzyme load. This result supported the study of Akoh and Yee (1997) in which they concluded no major improvements were observed at greater enzyme loads.

Enzyme load was also one of the most important factors for POP ($P<0.0001$) (Table 4.5). It was slightly less effective than temperature while more effective than substrate mole ratio and time. As seen in Figure 4.5b, increasing the enzyme load caused increase in the production of POP linearly when the other parameters were kept constant. POP reached its maximum point at enzyme load 25 wt%.

Table 4.6. Analysis of variance table and estimated coefficients for COO%

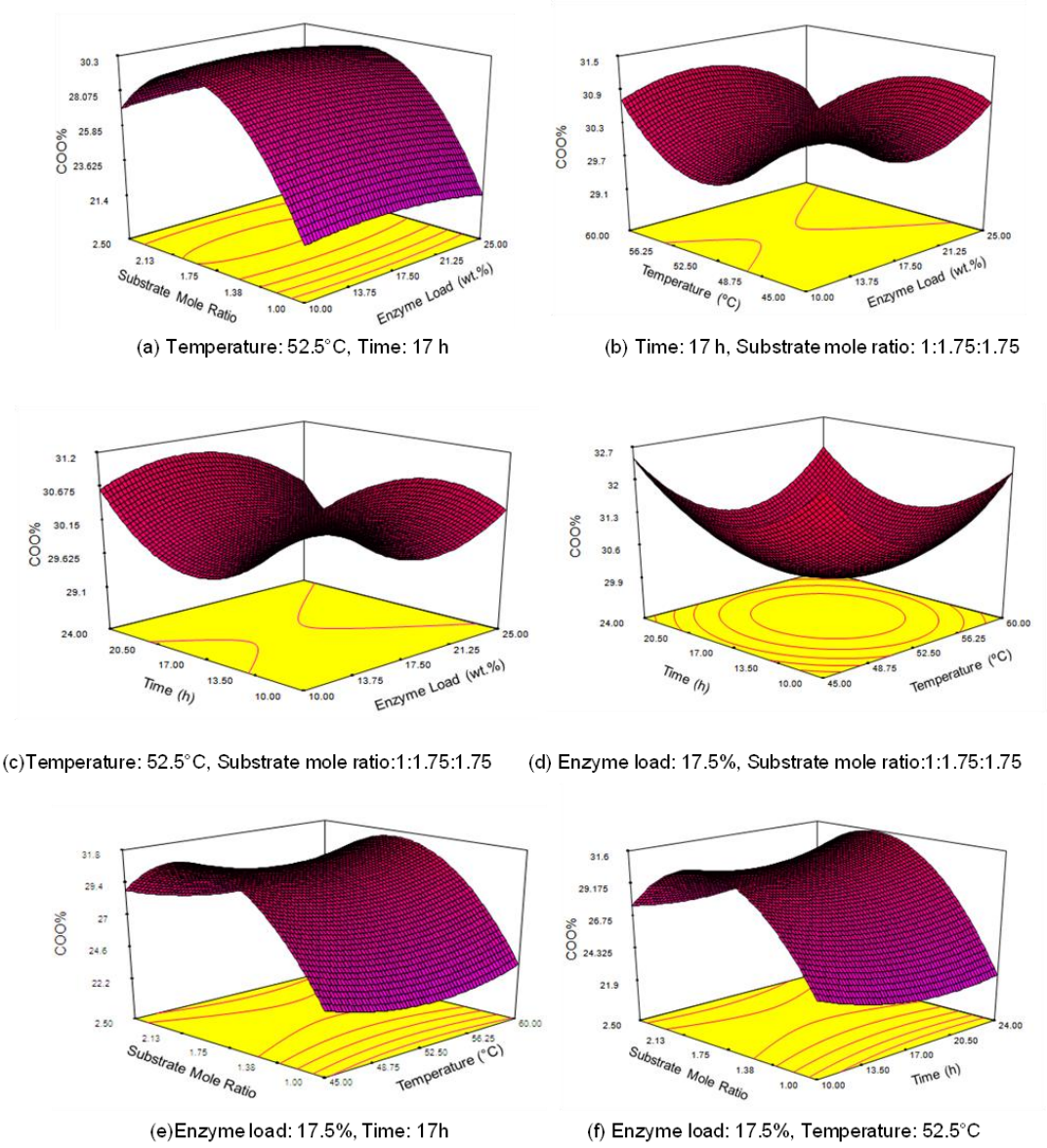
| Source | Coefficients | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|---|
| **Model** | | 1090.24 | 9 | 121.13 | 64.33 | < 0.0001 | significant |
| **Intercept** | 29.9 | | | | | | |
| **Linear** | | | | | | | |
| **En** | -0.24 | 1.36 | 1 | 1.36 | 0.72 | 0.4049[2] | |
| **Te** | -0.22 | 1.18 | 1 | 1.18 | 0.63 | 0.4363[2] | |
| **Ti** | -0.07 | 0.15 | 1 | 0.15 | 0.07 | 0.7804[2] | |
| **Sr** | 2.57 | 158.31 | 1 | 158.31 | 84.07 | < 0.0001[1] | |
| **Interaction** | | | | | | | |
| **Ti*Sr** | 0.90 | 12.88 | 1 | 12.88 | 6.84 | 0.0165[1] | |
| **Quadratic** | | | | | | | |
| **$En^2$** | -0.53 | 7.83 | 1 | 7.83 | 4.16 | 0.0548[2] | |
| **$Te^2$** | 1.30 | 46.11 | 1 | 46.11 | 24.49 | < 0.0001[1] | |
| **$Ti^2$** | 1.07 | 31.35 | 1 | 31.35 | 16.65 | 0.0006[1] | |
| **$Sr^2$** | -5.16 | 730.53 | 1 | 730.53 | 387.95 | < 0.0001[1] | |
| **Residual** | | 37.66 | 20 | 1.88 | | | |
| **Lack of Fit** | | 34.13 | 15 | 2.27 | 3.22 | 0.1009 | not significant |
| **Pure Error** | | 3.52 | 5 | 0.70 | | | |
| **Cor Total** | | 1127.91 | 29 | | | | |

$R^2$= 0.96, adj $R^2$=0.95, pred $R^2$= 0.91
Te: temperature, Enz: enzyme load, Sr: substrate mole ratio
[1] Significant at 'Prob>F' less than 0.05; [2] not significant at 'Prob>F' higher than 0.05

Table 4.7. Analysis of variance table and estimated coefficients for POO%

| Source | Coefficient | Sum of Squares | df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|---|
| **Model** | | 1218,60 | 8 | 152.32 | 49.03 | < 0.0001 | significant |
| **Intercept** | -12.2 | | | | | | |
| **Linear** | | | | | | | |
| **En** | -0.51 | 6,28 | 1 | 6.28 | 2.02 | 0.1696 | |
| **Te** | -0.29 | 1,99 | 1 | 1.99 | 0.64 | 0.4318 | |
| **Ti** | -0.41 | 4,11 | 1 | 4.11 | 1.32 | 0.2626 | |
| **Sr** | -6.03 | 871,21 | 1 | 871.21 | 280.47 | < 0.0001 | |
| **Interaction** | | | | | | | |
| **En*Te** | -0.80 | 10,14 | 1 | 10.14 | 3.26 | 0.0851 | |
| **Te*Sr** | -0.99 | 15,64 | 1 | 15.64 | 5.03 | 0.0357 | |
| **Quadratic** | | | | | | | |
| **$Ti^2$** | 2.90 | 239,88 | 1 | 239.88 | 77.22 | < 0.0001 | |
| **$Sr^2$** | 1.87 | 99,90 | 1 | 99.90 | 32.16 | < 0.0001 | |
| **Residual** | | 65,23 | 21 | 3.10 | | | |
| **Lack of Fit** | | 55,29 | 16 | 3.45 | 1.73 | 0.2814 | not significant |
| **Pure Error** | | 9,93 | 5 | 1.98 | | | |
| **Cor Total** | | 1283,83 | 29 | | | | |

$R^2$= 0.94, adj $R^2$=0.92, pred $R^2$= 0.84
Te: temperature, Enz: enzyme load, Sr: substrate mole ratio
[1] Significant at 'Prob>F' less than 0.05; [2] not significant at 'Prob>F' higher than 0.05




(a) Temperature: 52.5°C, Time: 17 h

(b) Time: 17 h, Substrate mole ratio: 1:1.75:1.75

(c)Temperature: 52.5°C, Substrate mole ratio:1:1.75:1.75

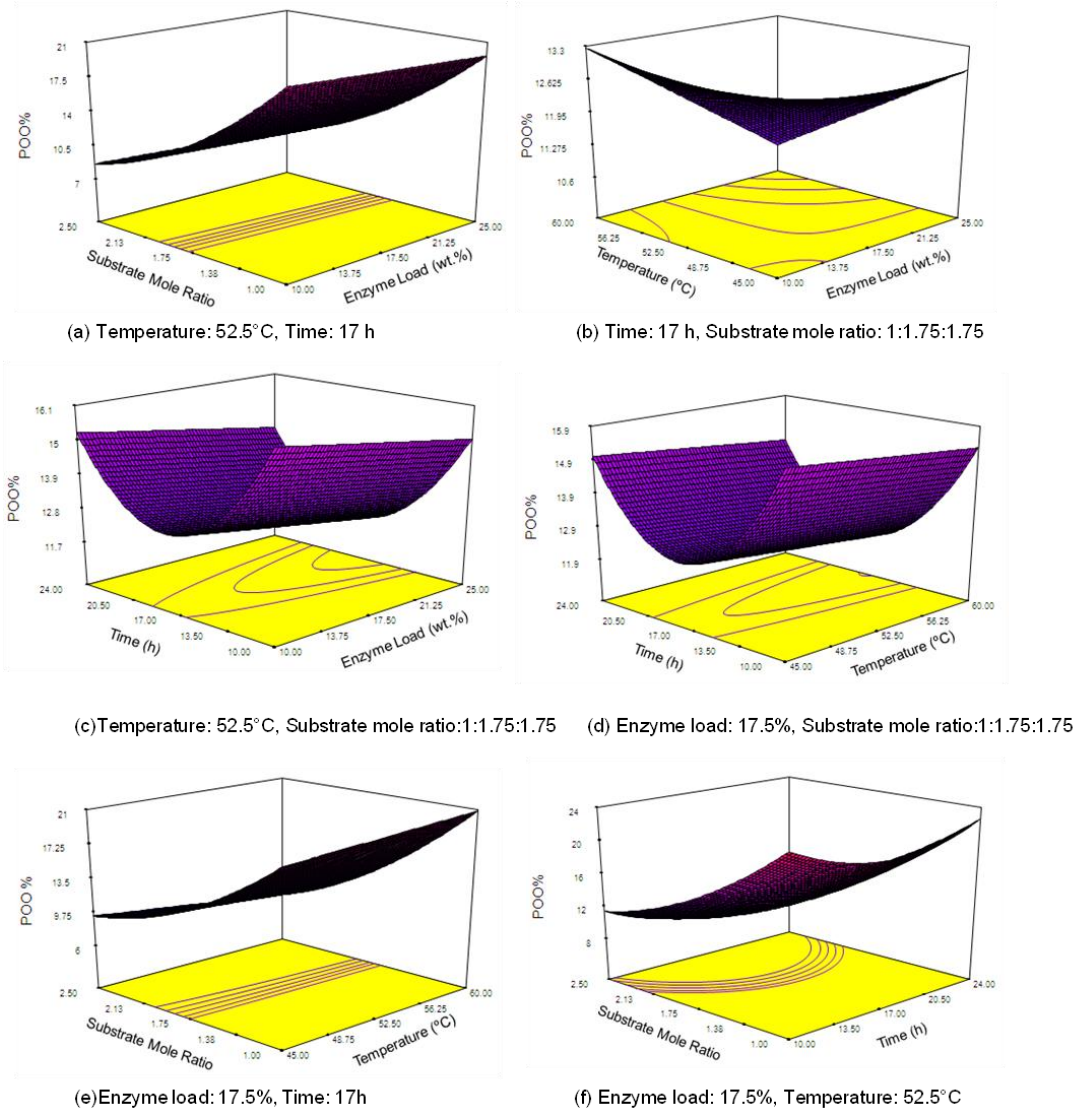
(d) Enzyme load: 17.5%, Substrate mole ratio:1:1.75:1.75

(e)Enzyme load: 17.5%, Time: 17h

(f) Enzyme load: 17.5%, Temperature: 52.5°C

Figure 4.6. Response surface plots for COO%: (a) substrate mole ratio versus time; (b) temperature versus time; (c) time versus enzyme load; (d) substrate mole ratio versus enzyme load; (e) time versus temperature; (f) time versus enzyme load

In the ANOVA results of POP, the interaction term En*Sr was eliminated. However, in Figure 4.5a it can be clearly observed that there was an interaction between substrate mole ratio and enzyme load. When the amount of substrate mole ratio was fixed, the incorporation of palmitic acid at *sn*-1,3 positions was higher in the case of higher enzyme loads than that of lower enzyme loads. Similarly, when the amount of enzyme load was fixed, the incorporation of palmitic acid at *sn*-1,3 positions was higher in the case of higher substrate mole ratio than that of lower substrate mole ratio. Thus, it can be concluded that substrate mole ratio and enzyme load interacted each other positively in the production of POP. This interpretation can be also done for the cases of COC and POC (Figure 4.3-4a).




(a) Temperature: 52.5°C, Time: 17 h

(b) Time: 17 h, Substrate mole ratio: 1:1.75:1.75

(c)Temperature: 52.5°C, Substrate mole ratio:1:1.75:1.75

(d) Enzyme load: 17.5%, Substrate mole ratio:1:1.75:1.75

(e)Enzyme load: 17.5%, Time: 17h

(f) Enzyme load: 17.5%, Temperature: 52.5°C

Figure 4.7. Response surface plots for POO%: (a) substrate mole ratio versus time; (b) temperature versus time; (c) time versus enzyme load; (d) substrate mole ratio versus enzyme load; (e) time versus temperature; (f) time versus enzyme load

Effect of enzyme load on COO and POO exhibited a similar trend to COC and POC. Enzyme load was followed substrate mole ratio in the decreasing effectiveness order. However, enzyme load was not statistically significant for COO and POO ($P>0.05$) and its effect was negative in this case (Table 4.6-7).

For the residual OOO, enzyme load was a significantly important parameter due to the ANOVA results ($P<0.0001$) (Table 4.8). Effectiveness order of enzyme load for OOO showed a similar trend to other TAGs, COC; POC; COO and POO. Enzyme load was less effective than substrate mole ratio while more effective than time and temperature.

Table 4.8 Analysis of variance table and estimated coefficients for OOO%

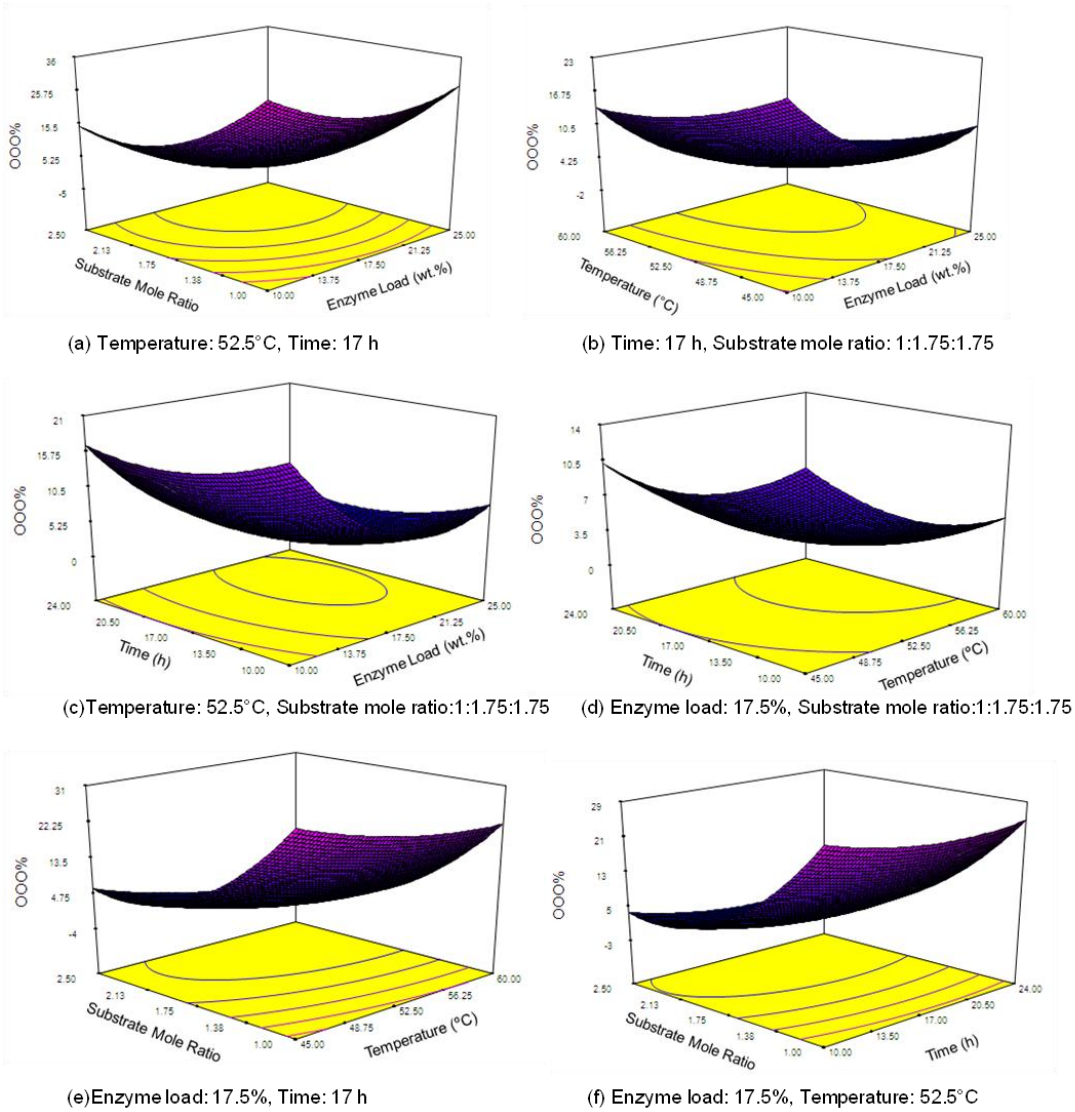
| Source | Coefficient | Sum of Squares | Df | Mean Square | F Value | p-value Prob > F | |
|---|---|---|---|---|---|---|---|
| **Model** | | 8576.03 | 9 | 952.89 | 46.16 | < 0.0001 | significant |
| **Intercept** | 2.49 | | | | | | |
| **Linear** | | | | | | | |
| **En** | -6.24 | 935.50 | 1 | 935.50 | 45.31 | < 0.0001 | |
| **Te** | -4.53 | 492.13 | 1 | 492.13 | 23.84 | < 0.0001 | |
| **Ti** | -1.84 | 81.10 | 1 | 81.10 | 3.92 | 0.0614 | |
| **Sr** | -12.4 | 3732.02 | 1 | 3732.02 | 180.79 | < 0.0001 | |
| **Interaction** | | | | | | | |
| **En*Sr** | -2.42 | 93.50 | 1 | 93.50 | 4.52 | 0.0459 | |
| **Quadratic** | | | | | | | |
| **$En^2$** | 7.16 | 1407.28 | 1 | 1407.28 | 68.17 | < 0.0001 | |
| **$Te^2$** | 2.38 | 155.91 | 1 | 155.91 | 7.55 | 0.0124 | |
| **$Ti^2$** | 2.67 | 195.77 | 1 | 195.77 | 9.48 | 0.0059 | |
| **$Sr^2$** | 9.09 | 2264.70 | 1 | 2264.70 | 109.71 | < 0.0001 | |
| **Residual** | | 412.85 | 20 | 20.64 | | | |
| **Lack of Fit** | | 384.58 | 15 | 25.63 | 4.53 | 0.0519 | not significant |
| **Pure Error** | | 28.26 | 5 | 5.65 | | | |
| **Cor Total** | | 8988.88 | 29 | | | | |

$R^2$= 0.95, adj $R^2$=0.93, pred $R^2$= 0.85
Te: temperature, Enz: enzyme load, Sr: substrate mole ratio
[1] Significant at 'Prob>F' less than 0.05; [2] not significant at 'Prob>F' higher than 0.05

### 4.4.3 Effect of Temperature on the Production of TAGs

Temperature was significantly important for COC and POC (P<0.05 and P<0.0001, respectively) as seen in Table 4.3-4. It had positive coefficient for both of the responses and affected their production positively. Response surface plot of time versus temperature showed a damn shaped curve for COC and POC (Figure 4.3-4d). Increasing the temperature gave rise to the production of COC and POC. However, this rise in COC was not as higher as the rise in POC.

Temperature was found to be the most effective parameter (P<0.0001) with its highest coefficient for the production of POP (Table 4.5). Positive coefficient of this parameter indicates that temperature affected production of POP synergistically. This effect reflected to response surface plots. When all other parameters were kept constant, further increase in temperature caused a linear increase in the production of POP (Figure 4.5b). This increase were more obvious than that of COC and POC. This can be related with the higher reactivity of PA and the ability of incorporation of PA at *sn*-1,3 positions at higher temperatures. Temperature had also interactive effects as well as main effect. Interaction terms of Te*Ti and Te*Sr were statistically significant (P<0.05 and P<0.0001, respectively). In the plot of time versus temperature, it is clearly seen that incorporation of palmitic acid at *sn*-1,3 positions were higher at longer reaction time than shorter reaction time (Figure 4.5d).




(a) Temperature: 52.5°C, Time: 17 h

(b) Time: 17 h, Substrate mole ratio: 1:1.75:1.75

(c)Temperature: 52.5°C, Substrate mole ratio:1:1.75:1.75

(d) Enzyme load: 17.5%, Substrate mole ratio:1:1.75:1.75

(e)Enzyme load: 17.5%, Time: 17 h

(f) Enzyme load: 17.5%, Temperature: 52.5°C

Figure 4.8. Response surface plots for OOO%: (a) substrate mole ratio versus time; (b) temperature versus time; (c) time versus enzyme load; (d) substrate mole ratio versus enzyme load; (e) time versus temperature; (f) time versus enzyme load

The optimum temperatures for maximum yields of desired TAGs were 56°C for COC and POC and 60°C for POP. Temperatures below these points caused lower caprylic and palmitic acid incorporation. The optimum temperature recommended by the manufacturer for *Rhizomucor miehei* lipase activity is between 30 and 70°C (Cheong et al., 2007). Sellappan and Akoh (2001) reported that the optimum temperature for the incorporation of caprylic acid was 55°C and they explained the sustained incorporation of stearic acid at all temperatures was caused by the higher selectivity of IM60 toward stearic acid. In this study, as a long chain fatty acid, palmitic acid showed the same trend of stearic acid with *sn*-1,3 specific lipase from *Mucor miehei* (newly, *Rhizomucor miehei*). This observation also revealed that Lipozyme RM IM has higher selectivity towards long chain fatty acids at critical conditions. A similar observation was also reported in the work of Fu and Parkin (2004).

The coefficients of temperature for COO and POO were given in Table 4.6 and 4.7,

respectively. Negative coefficients of temperature were a sign of increasing temperature decreased the production of COO and POO. This was related with the increase in incorporation at *sn*-1,3 positions with increasing temperature. Thus, an increase in COC, POC and POP were observed while the amount of COO and POO decreased in this sudy. Te*Sr was the only significantly important interaction term for the yield POO ($P<0.05$). At higher temperatures and higher substrate mole ratios, low percentages of POO were observed (Figure 4.5e). This can be also related with the higher reactivity of PA and the ability of incorporation of PA at *sn*-1,3 positions at higher temperatures. Thus, production of POP increased while POO decreased.

OOO was affected significantly ($P<0.0001$) from temperature in a negative way (Table 4.8). In Figure 4.8d, it is clearly seen that when the other parameters were fixed at a constant value, increasing temperature caused decrease in the residual OOO. In the plot of substrate mole ratio versus temperature, at lower substrate mole ratio, change in temperature did not change the percentage of residual OOO (Figure 4.8d). However, at higher substrate mole ratio temperature was so effective and caused decrease in OOO with increasing temperature.

### 4.4.4 Effect of Time on the Production of TAGs

Results of ANOVA indicated that time was also effective on the production of desired TAGs. Increasing time caused increase in the yields of COC, POC and POP. This increase was statistically significant for COC ($P<0.05$) and POP ($P<0.0001$) but not significant for POC ($P>0.05$) (Table 4.2-4). As seen in Figure 4.3-4e percent yields reached maximum at 17 h and thereafter there was a slight decrease in COC and POP%. These results were in the agreement with the study of Arifin et al. (2010) in which it was reported that esterification reaction of glycerol with capric and stearic acids reached equilibrium at 16 h. Yankah and Akoh (2000) reported that caprylic acid incorporation was time dependent and attained a maximum after 12 h incubation, after which no further increase was obtained, suggesting that equilibrium had been reached.

The trend of time for POP obtained from ANOVA results was reflected to response surface plots. Further increase in time caused a linear increase for POP. This trend was seen obviously at higher temperatures and enzyme loads (Figure 4.5c-d). Thus, it can be said that the reaction could not be completed at lower reaction time and need more time to be completed. It is seen in Figure 4.5d, at lower temperatures time did not change the production of POP. This is related with the unsuitability of lower temperatures for the incorporation of palmitic acid into triolein. So, it can be said that when the other conditions were suitable, the optimum time for POP was 24 h.

Time had negative coefficient for COO and POO as well as enzyme load and temperature. These negative effects were not statistically significant for COO and POO ($P>0.05$). Beside the main effect, the interaction term of Ti*Sr was significant for COO ($P<0.05$) as seen in

Table 4.5. Figure 4.6f which represents the interaction of substrate mole ratio and time showed a damn curve surface. The percentage of COO was minimized at 17 h when the COC and POC were maximized.

Time had a negative and statistically nonsignificant coefficient for OOO (Table 4.8). However, its P value is not so higher than 0.05. Thus, we could not say that it was entirely nonsignificant. In Figure 4.8c,d,f, it is seen that the percentage of residual triolein was minimized nearly at 17 h. It could be said that increase in time gave rise to the percent composition of desired TAGs and reduced the amount of COO, POO and OOO.

It is seen that some linear, interaction or quadratic terms were not eliminated by backward elimination to maintain the hierarchy of the model although they were statistically nonsignificant. Presence of nonsignificant terms in ANOVA tables was the reason of this elimination principle. En*Sr interaction was significant only in model for OOO%. Interaction terms of Te*Ti and Te*Sr for POP were highly significant ($P<0.0001$). Another interaction term of Ti*Sr was eliminated in the models for COC, POC, OOO and POP% by backward elimination quadratic model while it affected COO and POO significantly. Quadratic terms of the parameters were found significant for COC and POC. Since the second order variable of the parameters had significant negative effect on the responses, COC and POC (Table 4.2-3), it can be deduced that an optimal value of the responses must exist. However, the quadratic terms of parameter were eliminated since they are statistically nonsignificant for POP%.

Koh et al., 2008; Bektas et al., 2008; Zhou et al., 2001 also reported that most of the interaction between variables were found to be insignificant at 99% confidence level. Second order variables and interactions from/between these factors were insignificant. Therefore, it may be expected that the influence of factor on the responses is in a linear relationship within the chosen ranges (Zhou et al., 2001). The same relationship of the parameters was probably present in this study.

### 4.5 Thermal Characteristics

Some structured lipids were selected from the experimental design based on their triacylglycerol composition and these SLs were synthesized in gram scale. These structured lipids were analyzed for melting profile and solid fat content and compared with fat extracts of commercially available margarines. Only the results of SL obtained from run12 which showed the most similarities to properties of margarine fat were given. The alterations in physical properties like solid fat content and melting isotherms of triolein, structured lipids and margarine fat extracts were monitored by differential scanning calorimetry (DSC).

#### 4.5.1 Melting Characteristics

The complexity of the thermal profiles of vegetable oils is essentially due to the great variety of TAGs as principal constitutes. Therefore, oils do not have specific melting temperature,

rather, they melt over a temperature range, as long as a dynamic method is used to measure the melting process, and often exhibit multiple endotherms. This change may not be visible to the eye, but it can be studied by physical means such as in a DSC instrument. The application of the DSC method for studying the melting behaviour of vegetable oils has proved very useful (Tan and Che Man, 2000).

Onset and melting peak temperatures for margarine fats and structured lipids are listed in Table 4.9. Melting point of pure triolein was found as -12°C and 6.78°C with two endothermic peaks in the agreement with Ilyasoglu and Ozcelik (2011) who were observed the melting point of triolein at ~5°C (Figure 4.9). However, Hagemann and Tallent (1972) observed that triolein melted at -12°C and -5°C and Seriburi and Akoh (1998a) reported that triolein melted at -15.2°C and -2.4°C with two peaks.

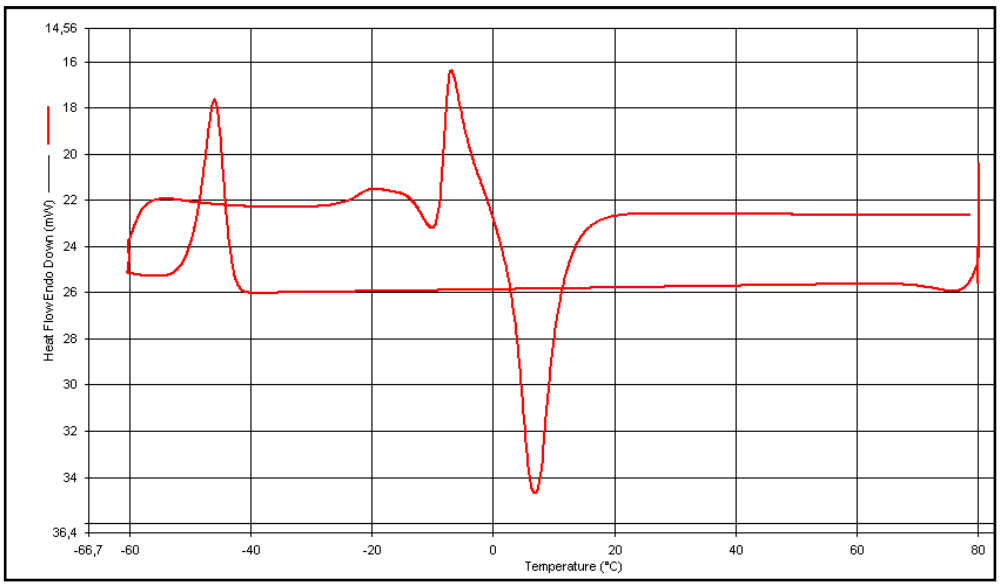



Figure 4.9. DSC melting thermogram of triolein

SL obtained from run 12 gave two endothermic peaks with peak temperatures at -13.5 and 42.09°C, respectively. The first peak region depicted the caprylic acid incorporated TAG and triolein fraction while the second peak region probably defined the palmitic acid incorporated TAG fraction.

The onset temperature of first peak for SL (-27.73°C ) was very similar to that of different margarine fat extracts A (-26.73°C), C (-25.99°C), and D (-23.99°C) and its melting peak temperature (42.09°C) was very close to fat extracts A (39.39°C) and D (39.54°C) (Table 4.9).

### 4.5.2 Solid Fat Content

SFC of the interesterified products were determined from the area under their melting curves. These calculations were computed by the software from Perkin Elmer 6 DSC (Norwalk, CN, USA) instrument.

Table 4.9 Melting profile of structured lipid, triolein and fat extracts of commercial margarines

| Sample | Peak | $T_{onset}$ (°C) | $T_{peak}$ (°C) |
|---|---|---|---|
| BU | I | 15.30 | 20.31 |
| | II | 31.23 | 36.99 |
| A | I | -26.73 | -19.30 |
| | II | 16.21 | 39.39 |
| B | I | -20.47 | -13.89 |
| | II | 13.66 | 36.47 |
| C | I | -25.99 | -20.13 |
| | II | 13.59 | 33.49 |
| D | I | -23.94 | -15.44 |
| | II | 17.59 | 39.54 |
| E | I | -25.88 | -19.25 |
| | II | 12.95 | 34.62 |
| F | I | -18.48 | -11.59 |
| | II | 6.95 | 24.12 |
| Triolein | | -12.00 | 6.72 |
| SL (run 12) | I | -27.73 | -13.50 |
| | II | 28.95 | 42.09 |

BU, fat extract of butter; A, B, C, D: fat extracts of soft margarines; E, F: fat extracts of hard margarines; SL (run 12): structured lipid obtained from run12 from experimental design

SFC is the percentage of the lipid that is solid at various temperatures, and it is responsible for many properties of the margarine, such as general appearance, ease of packing, organoleptic characteristics (flavor release, coolness and thickness), ease of spreading and oil exudation. Lida and Ali (1998) were reported that a SFC not greater than 32% at 10°C is essential for good spreadability at refrigeration temperature and SFC not less than 10% at 20°C is essential for product's stability and resistance to oil exudation at room temperature. Goli et al. (2004) suggested that to eliminate a waxy mouthfeel, a margarine should have less than 3.5% solids at temperatures greater than 33°C or should melt at or below body temperature.

Table 4.10 shows the solid fat content profiles for margarine fats, pure triolein and SLs from run 12. Changes in TAG composition of the triolein after acidolysis were resulted in changes in SFC.

SFC of triolein was higher than SL (run12) at lower temperatures (-20-4°C) but very lower at higher temperatures (10-35°C). Incorporated palmitic acids were the possible reason of improved solid fat content. Our results indicated that the SFC of SL (run12) showed a similar trend with soft margarine fats, especially D, at various temperatures. SFC of SL at 35°C was higher than all margarines except E. This means the produced structured lipid is melted slower than the other margarines. SL (run12) had 40.69% SFC at 0°C, 30.63% at 10°C, and 27.73% SFC at 20°C, which designates a suitable SFC profile for good plasticity based on the suggestions of Lida and Ali (1998). Thus, the structured lipid obtained from run12 has an extended plastic range and hence good spreadability at room temperature.

Table 4.10 Solid fat content values of structured lipid, triolein and fat extracts of commercial margarines

| Samples | SFC (%) at °C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | -20 | -10 | 0 | 4 | 10 | 20 | 25 | 30 | 35 |
| **BU** | 100 | 99.82 | 95.47 | 91.64 | 82.65 | 54.95 | 34.17 | 20.74 | 9.85 |
| **A** | 78.48 | 48.53 | 32.27 | 29.02 | 25.82 | 22.34 | 17.96 | 13.33 | 8.52 |
| **B** | 94.94 | 55.93 | 31.20 | 19.18 | 24.88 | 19.32 | 14.73 | 10.11 | 6.27 |
| **C** | 81.86 | 41.14 | 27.86 | 26.17 | 24.69 | 20.34 | 16.12 | 11.42 | 6.27 |
| **D** | 91.41 | 64.75 | 44.61 | 39.53 | 33.88 | 26.50 | 21.11 | 15.73 | 11.23 |
| **E** | 100 | 94.83 | 82.53 | 78.78 | 69.13 | 53.31 | 41.26 | 28.20 | 18.75 |
| **F** | 100 | 95.72 | 89.71 | 88.58 | 77.01 | 52.68 | 34.47 | 18.75 | 9.09 |
| **Triolein** | 100 | 94.98 | 77.29 | 94.08 | 21.93 | 0.12 | 0 | 0 | 0 |
| **SL (run 12)** | 87.66 | 63.49 | 40.69 | 35.71 | 30.63 | 27.73 | 25.87 | 22.35 | 16.99 |

BU: fat extract of butter; A, B, C, D: fat extracts of soft margarines; E, F: fat extracts of hard margarines; SL (run 12): structured lipid obtained from run12 from experimental design

### 4.6 Caloric Values of Structured Lipids

The systematic investigation of the gross energy content of food and of the availability of that energy can be credited to Rubner in Germany and to Atwater in the United States. Using a bomb calorimeter, Rubner measured the heats of combustion of many different proteins, fats, and carbohydrates found in individual foods. He thus determined the energy density of dietary fat to be 9.3 kcal/g (~39 kJ/g) on the basis of the mean combustion values for olive oil (9.384 kcal/g), animal fat (9.372 kcal/g), and butterfat (9.179 kcal/g). The energy density of dietary carbohydrate (specifically of starch and sugar in a mixed diet) was determined to be 4.1 kcal/g (~17 kj/g) on the basis of the average combustion values for glucose (3.692 kcal/g), lactose (3.877 kcal/g), sucrose (3.959 kcal/g), and starch (4.116 kcal/g), which were weighted for their average contribution to a mixed diet (Buchholz et al., 2004).

Taguchi et al. (2001) determined the energy values of the DAG and TAG by theoretical calculations and by bomb calorimetry and they reported that the combustion energies measured by bomb calorimeter were in good agreement with the theoretical values. Caloric values of the structured lipids were calculated basen on this calculation method in this study.

The energy values of TAGs were determined as COC (36.28 kJ/g), COO (38.30 kJ/g), POC (37.69 kJ/g), OOO (39.65 kJ/g), POO (39.46 kJ/g) and POP (39.21 kJ/g). Then caloric values of structured lipids from each run were calculated based on their TAG composition (Table 4.2). In our study, the acidolysis reaction was deduced with a tolerable decrease in caloric value of triolein. SL from run 12 which was the most similar SL to the margarine fats was resulted in a decrease from 39.65 kJ/g to 37.74 kJ/g which represents approximately 5% decrease in the caloric value compared to triolein. Table 4.2 shows the caloric values of SLs and their TAG composition. All of the produced SLs had a lower caloric value than triolein. The largest decrease (37.62kJ/g) was observed at run16 with a TAG composition of 33.83% COC, 25,27% POC and 6.38% POP.

### 4.7 Optimization

In this study, it was aimed to produce a reduced calorie structured lipid with similar physical characteristics to those of margarine fat extracts. For this purpose, the optimal conditions for the lipase catalyzed acidolysis reaction of triolein with caprylic and palmitic acids were predicted using Design Expert Software 7.0. Optimization was based on melting point, solid fat content, and caloric value requirements. SFC and melting point of margarine fat extracts served as target criteria to make the properties of structured lipid similar to those of margarine fat extracts. In this respect, the target response intervals were defined considering the TAG compositions which satisfied the desired properties chiefly. The necessary response intervals were set as COC (25-30%); POC (25-30%) and POP (3-4%) into the software. Our observations showed that the TAG composition did not change after 15 wt% enzyme load. Thus, enzme load was kept at 15% due to the cost considerations in industrial point. After setting the ranges, the recommended optimal conditions were enzyme load 15 wt%, reaction temperature 58ºC, substrate mole ratio (1:2.1:2.1), and time 15 h to obtain a reduced calorie spreadable structured lipid with a desirability value of 1.00.

## CONCLUSION

In this study, it was aimed to perform acidolysis reaction of triolein with caprylic and palmitic acids to synthesize a reduced calorie spreadable structured lipid using *sn*-1,3 specific lipase in n-hexane. Firstly, four different kinds of immobilized lipase were screened in terms of their ability to incorporate fatty acids at *sn*-1,3 positions of triolein. Among the lipases examined immobilized *sn*-1,3 specific lipase from *Mucor miehei* was found the most effective one. So, it was used in further studies as an *sn*-1,3 specific lipase.

A five level four factorial central composite design composed of 30 experiments consisting of 16 axial points, 8 star points, 6 center points was adopted. The main parameters were selected as time, temperature, enzyme load and substrate mole ratio. The use of response surface models allowed the evaluation of the effects of experimental factors on individual responses (COC, COO, POC, OOO, POO, and POP), evidencing that different optimum settings are predicted depending on the response variable considered and helped of a relatively small number of experiments, thus reducing the time and cost of the study. The best fitting quadratic models were determined by regression and backward elimination (modified model) and this model was used to analyze the effects of the main parameters and their relationships. The experimental results were in good agreement with the predicted mathematical model.

Investigations of the thermal charecteristics of produced SLs and calorie value determinations have presented that the study succeeded. The analysis showed that melting profile and solid fat content of produced structured lipid were comparable to those of commercial soft margarine fat extracts. The caloric value of produced SL was reduced approximately 5% compared to triolein.

Optimization of the acidolysis reaction of triolein with two fatty acids was successfully performed by response surface methodology for the production of structured lipid with desired properties. A structured lipid with desired properties can be obtained at 15 wt% enzyme load, reaction temperature of 58°C, reaction time at 15 h, and substrate mole ratio (Triolein:CA:PA) of 1:2.1:2.1 with a TAG composition of COC (29.68%), POC (25.47%) and POP (3.80%).

The results of this model system showed that it is possible to produce a reduced calorie spreadable fat from vegetable oils rich in triolein.

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