CHAPTER II

LITERATURE REVIEW

2.1 Palm stearin

Fractionation of palm oil involves crystallization at lower temperature, followed by a filtration step to separate the liquid and solid fractions (Gee, 2007). During fractionation, unsaturated fatty acids are preferentially distributed in the palm olein whereas the saturated fatty acids distributed in the palm stearin (Gee, 2007). Besides, Gee (2007) also stated that the ranges of fatty acid composition in the palm stearin are very wide due to methods and conditions used in the fractionation.

Hard stearin comes from detergent fractionation whereas dry fractionation produces intermediate and palm stearin (Gee, 2007). Because of these wide range and type of palm stearin, it gives palm stearin wider range as the useful fat stock in the food industry applications. Malaysian Palm Oil Council (MPOC) states that palm stearin is a very useful source of a fully natural hard fat component for products such as shortening and pastry and bakery margarine. Besides, palm stearin are cheaper and does not require any hydrogenation process (Hazirah et al., 2013).

Palm stearin however, have limited uses in the manufacturing of solid fat products as it confers low plasticity to the products and does not completely melt at body temperature (Pantzaris, 2000). Palm stearin have slip melting point (SMP) ranging between 44-56°C (Lai, 1998). According to Pantzaris (2000), total saturated fatty acid in palm stearin measured was 65% and the total of unsaturated fatty acid was 35%. Table 1 below shows the palm stearin characteristics from the Malaysian Palm Oil Board (MPOB), where the specifications are given in Malaysian Standard MS 815:2007.

TABLE 1: Characteristics of palm stearin

Characteristics	Observed min. to max.
Apparent density, g ml^{-1} at 50°C	0.8813 – 0.8844
Refractive index n_D 50°C	1.4482 – 1.4501
Saponification value, mg KOH g^{-1} oil	193 – 205
Unsaponifiable matter, % by weight	0.30 – 0.90
Iodine value (Wijs)	27.8 – 45.1
Slip melting point (°C)	46.6 – 53.8
Total carotenoids as (β-carotene), mg kg^{-1}	300-500

Source: Malaysian Palm Oil Board, MPOB

Modification of palm stearin actively being done to meet industrial and consumer acceptance. Research on hard stearin stated that it can be used for interesterification with oleic acid to produce 1,3-dioleoyl-2-palmitoyl-*sn*-glycerol, OPO, which useful in infant formulations, imitating the human milk (Gee, 2007). This alteration in fatty acid composition had increase and widens the usage and functions of palm stearin. Basically, the fatty acid compositions in palm stearin are as stated in Table 2.

TABLE 2: Fatty acid composition of palm stearin

Fatty acid composition	Mean	Range
Lauric acid (C12:0)	0.2	0.1-0.3
Myristic acid (C14:0)	1.4	1.1-1.7
Palmitic acid (C16:0)	59.0	49.8-68.1
Palmitoleic acid (C16:1)	0.5	<0.05-0.1
Stearic acid (C18:0)	4.8	3.9-5.6
Oleic acid (C18:1)	27.4	20.4-34.4
Linoleic acid (C18:2)	7.0	5.0-8.9
Linolenic acid (C18:3)	0.3	0.1-0.5
Arachidic (C20:0)	0.5	0.3-06

Source: Pantzaris, 2000

2.2 Oleic acid

Oleic acid is a monounsaturated fatty acid, consisting of one double bond as shown in Figure 1, the oleic acid structure. Oleic acid, also called as octadecanoic acid (C18:1). It has a low melting point (13˚C) as shows in Table 3. Because of the double bond and low melting profile, it has been favorable to have more oleic acid in palm stearin to lower the palm stearin melting point. Besides, oleic acid is the major component of unsaturated fat in palm stearin. By incorporating more oleic acid to palm stearin, it can replace the saturated portion of fatty acid in palm stearin. Because of it has only one double bond, it has less oxidation tendency as compared to other unsaturated fatty acids such as linoleic or linolenic acid.

FIGURE 1: Oleic acid structure

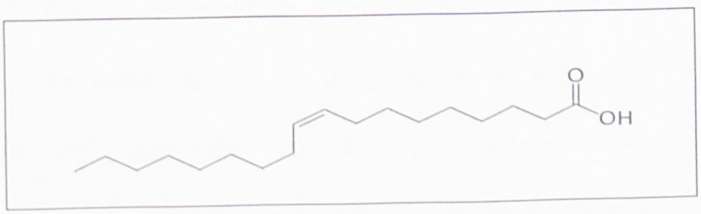

TABLE 3: Oleic acid characteristics

Characteristics	Description
Molecular formula	$C_{18}H_{34}O_2$
Molecular weight	282.5 g/mol
Density	0.891 gm/ml
Melting point	13 – 14 °C

Source : Sigma-Aldrich, 2015

It has been shown that oleic acid is effective in lowering plasma cholesterol level (lower the low-density lipoprotein (LDL) as well as total cholesterol), e.g. when it replaces palmitic acid in the human diet (Balcao and Malcata, 1998; Yankah and Akoh, 2000). In addition, Sola et al. (1997) revealed that native high-density lipoprotein (HDL) obtained after an oleic acid rich diet showed significantly less indication of lipid peroxidation, than HDL obtained after a linoleic acid rich diet. In addition, isocaloric replacement of about 5% energy from saturated fatty acids by oleic acid has been estimated to reduce coronary heart disease risk by 20-40% mainly via LDL-cholesterol reduction (Lopez-Huertas, 2010).

2.3 Lipase applications in food industry

Lipases are reported to be monomeric proteins, having molecular weight in the range of 19-60 kDa (Aravindan et al., 2007). Lipase (EC 3.1.1.3) possesses a unique feature of reacting and synthesizing various blends of oil, fats and lipid (Buchholz et al., 2012). It is known as lipid-hydrolyzing enzymes, which are increasingly used in stereoselective reactions (Gandhi, 1997; Muralidhar et al., 2002). It synthesizes esters from glycerol and long chain fatty acids when the water activity is low (Aravindan et al., 2007). Besides

that, it also catalyzes many different reactions; hydrolysis, alcoholysis, aminolysis, peroxidation, epoxidation as well as interesterifications (Rodrigues and Fernandez-Lafuente, 2010). They are soluble in water and hydrolyze insoluble substrates to more polar lipolytic products (Lasoń and Ogonowski, 2010). In fats and oil industry, lipase allows us to modify the properties of lipids by altering the location of fatty acid chain in the glyceride and replacing one or more of these with new ones (Aravindan et al., 2007).

Based on Buchholz et al. (2012) the main interest in the application of lipases in organic chemistry is due to the following reasons;

(a) lipases are highly active in a broad range of non-aqueous solvents

(b) often exhibit excellent stereoselectivity

(c) accept a broad range of esters other than triglycerides

(d) accept nucleophiles other than water (i.e., alcohols, amines).

Lipase activity can be divided into typoselectivity (ability to hydrolase a particular type of fatty acid ester), regioselectivity (ability to hydrolase carboxylic ester groups at *sn*-1 and *sn*-3 positions compared to *sn*-2 position) and stereoselectivity (ability to differentiate between two enantiomers in a racemic substrate (Muralidhar et al., 2002). Lipases may be divided into two groups according to the region-specificity exhibited with acyl glycerol substrate (Aravindan et al., 2007). Lipases from the first group show no regiospecificity and release fatty acids from all three positions of glycerols while the second group lipases release fatty acids regio-specifically from the outer 1 and 3 positions of acylglycerols (Aravindan et al., 2007). These lipases hydrolyse triacylglycerol to give free fatty acids

1,2-(2,3)-diacylglycerols and 2-monoacylglycerol (Aravindan et al., 2007). Because of its stereoselectivity and regiospecificity, lipase makes the modification and exchange of the acyl group easier.

The ability to cleave ester bonds and makes the new fatty acids can incorporate in the glycerol thus promote rearrangement of fatty acids occurs on the TAG. Figure 2 shows the cutting down and replacement of fatty acid by lipases. Thus, this advantage can lead to specifically modify any desirable fat stock to make it favorable. Besides, in this way, a relatively inexpensive and less desirable lipid can be modified to a higher value of fat (Aravindan et al., 2007).

FIGURE 2: Cutting down fatty acids at *sn*-1,3 position and replacement of new fatty acids.

Source: Xu, 2003

Lipase presents in two forms: immobilized and non-immobilized (also known as free lipase). Lipase TL IM (from *Thermomyces lanuginosus*), and lipase RM IM (from *Rhizomucor meihei*) and Novozyme 435 are the examples of immobilized lipase. Lipase AK (from *Pseudomonas sp.*), Newlase F and Lipase AY are the examples of free lipases. Free lipase easily absorb as they often present in powder form make it more soluble in the

solute during the reaction. Several microbial lipases have been produced commercially, however the high cost of lipases seems to be a major factor and may be overcome by immobilization for reuse of the enzyme where immobilized lipase is known for its fast enzyme activity, efficient, accurate and cost-effective (Aravindan et al., 2007).

2.3.1 Free lipase AK Amano

Microbial lipases are produced mostly by submerged culture but solid state fermentation can also be used (Aravindan et al., 2007). Microbiological lipases, especially those originated from bacteria are commercially significant because of low production cost, more stable and wider availability than those from plants or animals (Aravindan et al., 2007; Lasoń and Ogonowski, 2010). Most of the bacterial lipases reported so far are not specific to a particular substrate (Macrae and Hammond, 1985). There are several microorganisms that have been studied for the production of lipases such as *Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp. and *Chromatobacterium* sp (Aravindan et al., 2007).

Lipase AK Amano is an enzyme produced by fermentation of *Pseudomonas fluorescens*. Besides, microbial lipases from *Pseudomonas* sp. is known for their application in production of PUFA (polyunsaturated fatty acid) and food processing of oil manufacturing (Aravindan et al., 2007). Thus, this shown that lipase AK is remarkable for fats and oil modification process. Wang et al. (2012) studied the incorporation of capriylic acid into canola oil using free lipases and immobilized lipases. The study

resulted that Lipase AK have higher degree of incorporation compared to Newlase F and Lipase AY, which showed very low enzyme activity (Wang et al., 2012). Based on Amano Enzyme Inc., Japan, this enzyme has a high lipolytic activity and has an optimum pH of 8 and temperature at 60˚C respectively. The optimum temperature for the enzyme reaction was about 55°C and the enzyme was stable below 50°C at pH 7 for 1 h (Kojima et al., 1994). Kojima et al. (2006) stated that in acidolysis reaction of triolein with EPA (eicosapentanoic acid) and DHA (docosahexanoic acid) as acyl donor, the incorporation rate for Lipase AK increased with increasing temperature. Besides, they found in the research that the Lipase AK could discriminate between DHA and EPA on acidolysis reaction where the Lipase AK found to have highest specificity for DHA (Kojima et al., 2006).

2.3.2 Immobilized lipase *Thermomyces lanuginosus* (TL IM)

Basically, stability of the enzyme in the reaction is one of the main concerns for industrial applications. Immobilization lipase is normally more stable than their free states whereby it may use at high temperatures (Xu, 2000). Often known lipases in interesterification that have been immobilized were Lipozyme RM IM, Lipozyme TL IM, Novozymes 435. Lipozyme TL IM, immobilized within particulate mesoporous silica carriers with either hydrophilic or hydrophobic supporting surface (Sörensen et al., 2010). Based on the Novozymes biocatalyst product sheet, Lipozyme TL IM has optimum pH and temperature at pH 6-8 and 50-75˚C, respectively. Novozymes also includes that this lipase are *sn*-1,3 specific lipase, immobilize on a non-compressible silica gel carrier, highly effective

catalyst for interesterification and able to rearrange fatty acids in triglycerides as well as exhibit high degree of substrate selectivity. Immobilization lipase on a solid carrier has shown to increase enzyme stability in a non-aqueous solvent. Besides, it can facilitate recovery of product, recycling and reuse of catalyst thereby decrease the cost of enzyme in a chemical process (Novozymes®, 2014).

Based on Novozymes® (2014) point of view, immobilized enzyme does possess these advantages as follows;

a) better performance in non-aqueous solvent compare to native enzyme formulations
b) efficient recovery and separation of reaction product
c) economically viable in reuse/recycling of enzymes
d) minimizing or eliminating protein contamination of product
e) enhanced storage and operational stability from denaturation by heat, organic solvents or autolysis
f) higher catalyst productivity (kg product/kg enzyme) determining enzyme cost per kg of product
g) convenient and safer handling of the enzyme.

2.4 Modifications of fat and oils

Various modifications have been done involving palm stearin and any other fat stock to improve their health benefits. Table 4 shows several type of research on modifications of palm stearin and other fats and oils that have been studied.

TABLE 4. The previous studies on modifications of fat and oil and their parameters.

Enzyme load	Subs. Molar ratio	React. Time (h)	React. Temp (°C)	Stirring Rate (rpm)	Blends	Catalyst	Author
8%	6:1	24 and 48 h	50-60	-	Sunflower oil, mixture palmitic-stearic acid	Lipozyme RM IM	(Carrín & Crapiste, 2008)
10%	1:7	6 h	65	-	High oleic sunflower oil and fatty acid mixture	Lipozyme RM IM	(Kadivar, De Clercq, Van de Walle, & Dewettinck, 2014)
4%	70/30	30 min	70	-	milk fat with rapeseed oil	Lipozyme TL IM	(Aguedo et al., 2009)
4%	70/30	30 min	70	200	palm stearin and soybean oil	Sodium methoxide, Lipozyme TL IM	(Costales-Rodríguez et al., 2009)
10%	1:4	15	50-60	150	Canola oil and Caprylic acid	Lipozyme RM IM, Lipozyme TL IM, Novozym 435, Lipase AK, Newlase F, Lipase AY	(Wang et al., 2012)
10%	1:6	24	55	150	Perillia oils and caprylic acid	Lipozyme RM IM, Lipozyme TL IM	(Kim, Kim, Lee, Chung, & Ko, 2002)
10%	6/12/18	12,24,48	55,60,65	250	Palm olein and long chain PUFA	Novozym 435	(Nagachinta & Akoh, 2012)

Enzyme load	Subs. Molar Ratio	React. Time (h)	React. Temp (°C)	Stirring Rate (rpm)	Blends	Catalyst	Author
9%	7:3	2	60	300	Palm stearin and coconut oil	Lipozyme TL IM, Lipozyme RM IM, Lipase AK	(Ibrahim et al., 2007)
14%	1:4:1	24	60	200	Triesterin, lauric acid, oleic acid	Lipozyme RM IM	(Sellappan & Akoh, 2000)
8%	3:5:1	12	70	-	Glycerol, capric acid, oleic acid	Lipozyme RM IM	(Koh et al., 2008)
5%	1:1	12	37	150	Nonanol and caprylic acid	Novozym 435	(Kamar et al., 2011)
10%	1:3	24	40	250	Blubber oil, capric acid	Lipozyme RM IM	(Namal Senanayake & Shahidi, 2002)
10%	1:2	72	60	200	Triesterin, lauric acid, oleic acid	Lipozyme RM IM	(Yankah & Akoh, 2000)
10%	3:1	24	48	250	Triesterin , various FA	Lipozyme ML IM, Free lipase , Novozym 435	(Hamam & Shahidi, 2007)
10%	1:1-1:10	24	55	150	Perilla oil, caprylic acid	Lipozyme RM IM, Lipozyme TL IM	(Kim et al., 2002)
-	-	12	50-60	250	Sunflower oil, palmitic-stearic acid mixture	Lipozyme RM IM	(Palla et al., 2012)

Palm stearin modification through enzymatic interesterification has been carried out in the presence of liquid oils such as coconut oil (Ibrahim et al., 2007), rice bran oil (Reshma et al., 2008), soybean oil (Costales-Rodríguez et al., 2009) and linseed oil (Farfán et al., 2013). By performing this kind of reactions, interesterified palm stearin or its interesterified blends with other oils or fats serve as good raw materials for several food applications (Aini and Miskandar, 2007).

Interesterfiication reaction have been done not only for modifying palm stearin but also for the synthesis of cocoa butter equivalent which have been done by Kadivar et al. (2014) from acidolysis of high oleic sunflower oil with fatty acid mixture as listed in Table 4. The effect of optimized reaction conditions towards the percentage of saturate-oleoyl-saturate (SOS) and saturate-saturate-oleoyl (SSO) TAG have been studied previously. The result found that the highest yield (59.1%, SOS) and lowest acyl migration (2.9%, SSO) was obtained from 10% of enzyme load, 1:7 substrate molar ratio, 65°C reaction temperature and 6h of reaction time.

Besides as seen in Table 4, Carrín and Crapiste (2008) was successfully produced a structured lipid (SL) via lipase-catalyzed acidolysis of sunflower oil with a mixture of palmitic-stearic acids. The incorporation of palmitic-stearic acids was higher at temperature 50-60°C resulting the changes in melting point of SL (Carrín and Crapiste, 2008). Moreover, modifications of lipid also being done on perilla oils (which have traditionally been consumed by the oriental countries such as Korea, Japan, and China). Perilla oils were acidolyzed with caprylic acid by Kim et al. (2002). Modification of

perilla oil with caprylic acid (a medium chain fatty acid, MCFA) can impart desirable benefits because the MCFA metabolize mainly via portal vein thus provide quick energy, have high oxidative stability, low viscosity and melting points and have high solubility in water (Kim et al., 2002). Kim et al. (2002) findings showed that modification of perilla oil TAG to contain more MCFA has improved the perilla oils' physiological properties and oxidative stability.

Based on the Table 4, there are few corresponding parameters that to be observed and utilized to meet the research goals. Enzyme load as well as enzyme concentration towards the total solution reaction is believed to be one important parameter. Seager and Slabaugh (2010) stated that when the enzyme load increased, the expected yield believed to increase according to the reaction rate theory, thus the availability of more enzyme molecules to catalyze a reaction leads to formation of more yields and a higher reaction rate.

The enzyme load range from previous findings of interesterification was stated in Table 4. The enzyme load range from the least one, 4% (Aguedo et al., 2009; Costales-Rodríguez et al., 2009) up to highest 14% (Sellappan and Akoh, 2000). Based on Table 4 the range of enzyme load is between 8% and 12% was commonly used to give high product yield after the reaction. Thus, the range for the acidolysis of palm stearin parameters measured fall between the corresponding ranges in the experimental design in response surface methodology.

Besides enzyme load, substrate concentration significantly influence the reaction rate but at certain concentration, however, the rate levels out and remain constant because the enzyme may saturate with the substrate and cannot work any faster in that condition (Seager and Slabaugh, 2010). As from Table 4, it can be seen that the most of the total substrate supplied based on their molar ratio. This would be easier in the calculation as it follows the molecular weight to find the exact ratio to make the perfect blends. The balance molar ratio of palm stearin towards oleic acid is 1:3 where the number of moles of palm stearin and oleic acid approaching equal number.

2.4.1 Enzymatic interesterification and acidolysis reaction

Interesterification is one of the four modification process to alter the physico-chemical characteristics of fats and oils, including blending, fractionation and hydrogenation (Idris and Dian, 2005). Interesterification, defined as the exchange of acyl groups between ester and an acid (acidolysis), an ester and an alcohol (alcoholysis) or between two esters (transesterification) (Rodrigues and Fernandez-Lafuente, 2010). There are two types of interesterification which are chemical interesterification and enzymatic interesterification. A chemical, such as sodium methoxide, is used as a catalyst in chemical interesterification which produces complete positional randomization of the acyl group of the triacylglycerols (Idris and Dian, 2005).

This enzymatic interesterification is more specific, require less severe reaction conditions and produce fewer by-products than chemicals interesterifications (Idris and Dian, 2005).

Enzymatic interesterification, in addition, may lead to the desired physical properties in oil or blend, containing large proportion of stearin, as fatty acid rearrangement often causing a lower melting point (Zainal and Yusoff, 1999; Soares et al., 2009). Through enzymatic interesterification, it is possible to incorporate the desired acyl group into a specific position of TAG, whereas chemical catalysis does not possess this regiospecificity (Balcao et al., 1998; Kim et al., 2002). Hence, this has been the added value as the regiospecificity comes from the lipase enzyme behavior, who cut the TAG at the *sn*-1,3 position.

Enzymatic acidolysis has been widely applied into the modification of lipid. The acidolysis process is defined as an exchange of acyl group between an esters and an acid (Rodrigues and Fernandez-Lafuente, 2010). Acidolysis reaction of saturated fatty acid (the one that contribute to high melting points and solid structure) is replaced with short chain fatty acid or unsaturated fatty acids and catalyze by lipase enzyme. Acidolysis reaction have been done on various fats and oil including tuna oil and caprylic acid (CA), sunflower oil and palmitic-stearic acid mixture, tristearin with lauric and oleic acid, perilla oil with caprylic acid (Hita et al., 2009; Palla et al., 2012; Sellappan & Akoh, 2000; Kim et al., 2002).

2.4.2 Dual lipase system

Through lipase-catalyzed modification, one can alter the fatty acid composition and physicochemical, nutritional and functional properties of fats and oils to meet particular

applications (Sellappan and Akoh, 2000). However, this reaction usually being performed in single lipase system (either it immobilized lipase or free lipase) as seen in Table 4, much of previous studies focus on single lipase system reaction with various lipases as one of the parameters to be observed and little attention have been stressed on dual lipase system.

In a study done by Ibrahim et al. (2007), enzymatic interesterification of palm stearin with coconut oil was conducted by applying dual lipase system. The results indicated that a synergistic effect occurred for many lipase combinations but largely depending on the lipase species mixed and their ratios (Ibrahim et al., 2007). Thus, these efforts provided us with useful information for the better understanding on the interaction among lipases on catalyzing the reaction of various lipids for modification besides promoting some new favorable fats in the market shelves ahead.

As mentioned earlier, the immobilized lipases are more efficient biocatalyst as compared to free lipase. However, in a dual lipase reaction system, as reported by (Ibrahim et al., 2007), the support of the immobilized lipase can possibly act as a carrier to adsorb co-existing free enzyme. In short, besides acting as biocatalyst, the immobilized lipase also plays the role of 'immobilizing' the co-existing lipase in free form which facilitates the efficient interaction between enzyme and substrate (Ibrahim et al., 2007).

2.5 Optimization via Response Surface Methodology (RSM)

Optimization study is important in determining the best conditions of the reactions and improving the reactions performance. This work was done by using Response Surface Methodology (RSM). This technique simplifies 4 level experiment designs by giving a series of experiment randomly according to the parameters range to be observed. The RSM designed by central composite rotatable design (CCRD) and evaluated the interactive effects, explored the relationship between several undependable variables and one or more response variables and then obtained optimum condition for the reactions (Huang et al., 2010; Koh et al., 2008).

RSM has successfully optimized the synthesis of cocoa butter equivalent from high oleic sunflower oil as seen in Table 4. The quadratic model sufficiently describes the acidolysis reaction. The highest yield (59.1% saturate-oleoyl-saturate) and the lowest acyl migration (2.9% saturate-saturate-oleoyl) was obtained at 10% enzyme load, 1% water content, 1:7 substrate molar ratio, 65°C reaction temperature and 6 h reaction time (Kadivar et al., 2014).

Nunes et al. (2011) also successfully studied the efficiency of RSM in optimization process of interesterification of milk fat and canola oil. A central composite rotatable design (CCRD) in RSM was used to optimize the reaction considering the effects of different mass fractions of binary blends of milk fat and canola oil and reaction temperature. Empiric models for interesterification degree (ID) and consistency (at 10°C)

obtained allowed establishing the best interesterification conditions: blends with 65% of milk fat and 35% of canola oil, temperature at 45°C. They stated that under these conditions, the product obtained has desirable milk-fat flavor and better spreadability under refrigerated conditions.