CHAPTER II

LITERATURE REVIEW

2.1 Sunscreens

Sunscreen is defined as a formulation intended to be placed on the skin which containing a chemical agent known as UV filter, that have ability to interact with incident radiation by reflecting, scattering or absorbing the UV radiation. The use of sunscreen's formulation in the world was first reported in 1928 by introducing two sunscreen chemicals of benzyl salicylate and benzyl cinnamate (Kullavanijaya & Lim, 2005). In line with consumer needs nowadays, application of sunscreens not only limited to skin lotion or foundation, but they are used extensively in shampoo, eye shadow, lipstick, hair-care, aftershave and toiletries (Manova et al., 2013). According to Schalka & Reis (2011), the UV filters can be grouped into two types, inorganic (physical) and organic (chemical).

The inorganic UV filters are called physical, because their mode of action involved physical phenomena such as scattering and reflection of UV radiation. Too far, two inorganic physical UV filters that widely used are zinc oxide (ZnO) and titanium dioxide (TiO_2). Due to their large particle sizes, they tend to be opaque and form a blocking film on the skin and thus contribute to poor aesthetics effects (Serpone et al., 2007; Chisvert &

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Salvador, 2007). However, modern approaches such as nanoparticle technology and encapsulation have improved their consistency (Burnett & Wang, 2011).

The organic UV filters, on the other hand, exhibited chemical changes in molecule by absorbing the UV radiation and converting it to heat energy. They normally composed of conjugated aromatic compounds with a carbonyl group (Schalka & Reis, 2011). Examples of organic UV filters are salicylates, cinnamates, benzophenones, anthranilates, dibenzoylmethanes and *p*-aminobenzoates (Manaia et al., 2013). However, some of them may cause contact dermatitis or photosensitivity reactions (Balakrishnan & Narayanaswamy, 2011)

Thus, recently, there is increasing demand for more sophisticated UV filters from natural green sources that is effective in protecting against UV radiation with many special physiological functions including antioxidant, antimicrobial and anti-inflammatory (Evans & Johnson, 2010; Balakrishnan & Narayanaswamy, 2011). The phytochemical compounds act as the UV filter include phenolic acids such as hydroxycinnamic acid and hydroxyl benzoic acid, flavonoids and high molecular weight polyphenols (Balakrishnan & Narayanaswamy, 2011).

2.2 Ferulic Acid

Ferulic acid or 4-hydroxy-3-methoxycinnamic acid (Figure 2.1) is a phytochemical constituent that arises from metabolism of phenylalanine and tyrosine in

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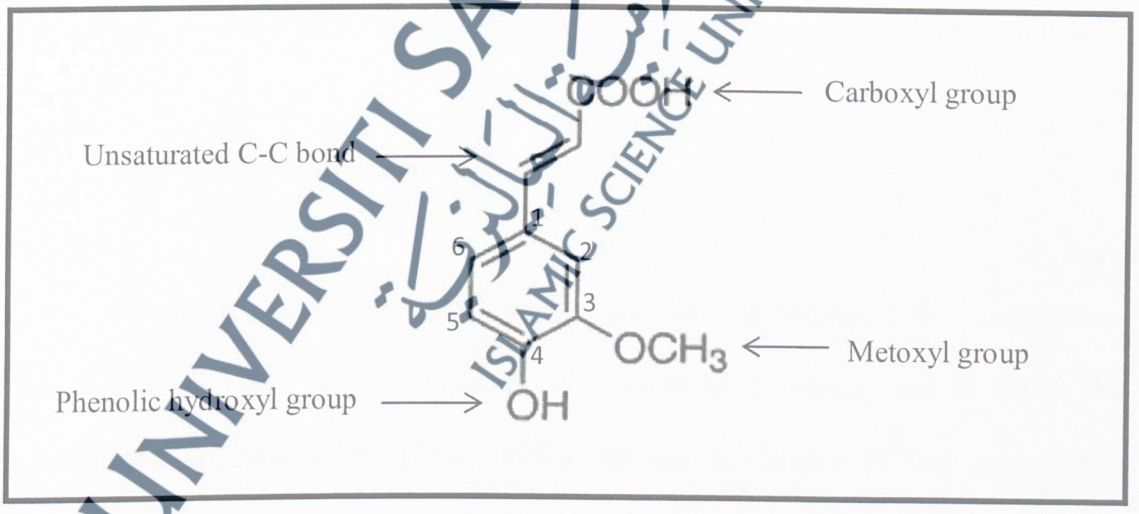
biosynthesis of plant lignin (Zhao & Moghadasian, 2008). Ferulic acid is also named as caffeic acid 3-methyl ether or coniferic acid. Structurally, ferulic acid has a phenolic nucleus and a side chain with molecular weight of 194. Ferulic acid explicitly shows three distinctive structural motifs. First, the hydroxy and metoxy groups on the benzene ring donate electrons to quench the free radicals. In addition, the carboxylic acid group with an adjacent unsaturated C-C double bond can provide additional attack sites for free radicals and thus prevent them from attacking the membrane. Further, this carboxylic acid group also acts as an anchor, by which it binds to the lipid bilayer, providing some protection against lipid peroxidation (Itagaki et al., 2009). Clearly, each element contributes toward overall properties of the compound.

FIGURE 2.1: Chemical Structure of Ferulic Acid (Zhao & Moghadasian, 2008)



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Ferulic acid was first isolated in 1866 by Hlasiwetz Barth, an Austrian, from a commercial resin of *Ferula foetida*, an umbelliferous plant. Later in 1891, ferulic acid was isolated from a pine tree species of *Pinus laricio*. Today, ferulic acid has been found in many plant species including fruits, flowers, vegetables, and predominantly in grains with 84% frequency of occurrence. There are two forms of ferulic acid in plant tissues: free (rarely) and conjugate; the sum of these two forms indicates total ferulic acid (De Man & Peeke, 1982; Graf, 1992; Zhao & Moghadasian, 2008; Kumar & Pruthi, 2014).

2.2.1 Applications of Ferulic Acid

Ferulic acid and its derivatives have been reported to have many special physiological functions including antioxidant, antimicrobial, anti-inflammatory, anti-thrombotic and anti-cancer effects. Therefore, these properties collectively led to its applications in a large aspect of industries including food, pharmaceutical and cosmetic.

i. Applications in the Food Industry

Ferulic acid is commercially prepared and used as functional food ingredients. Ferulic acid was first used in Japan in 1975 to preserve oranges and to inhibit the autoxidation of linseed oil (Graf, 1992). Strauss & Gibson (2004) successfully investigated the use of ferulic acid as a potential cross-linking agent, which offers possibility of developing gelled foods with lower calories and gelatine content. The following year, Ou et al. (2005) have demonstrated the utility of ferulic acid in preparing

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edible films from soy protein isolate. The ferulic acid not only improved mechanical properties of the films but also provides safer and more natural alternatives over petrochemical-based of synthetic films. Later, ferulic acid ester was used as supplement ingredients in sports foods to increase muscle gain, improve strength, speed recovery as well as reduce post-exercise soreness (Manore et al., 2011).

ii. Applications in the Pharmaceutical Industry

The present of ferulic acid in functional foods now widely recognized as a powerful ingredient against several human diseases. Its application in cancer treatment has been studied as early in 1990s. Researchers suggested possible applications of ferulic acid for cancer prevention in tongue (Tanaka et al., 1993), colon (Kawabata, 2000), skin (Murakami et al., 2002) and breast (Chang et al., 2006; Kampa et al., 2004). Further, Shanthakumar et al. (2012) revealed ferulic acid as an ideal adjuvant to protect normal tissues from deleterious effects of gamma-radiation due to radiotherapy in cancer treatment. Ferulic acid also showed positive results in reducing blood glucose level in induced diabetic animals (Balasubashini et al., 2004; Nomura et al., 2003), inhibiting atherosclerosis, hypertension (Suzuki et al., 2007; Dinis et al., 2002) and has therapeutic effects against Alzheimer's disease (Sgarbossa et al., 2013).

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iii. Applications in Cosmetic Industry

It is well known that increased melanin synthesis can lead to skin darkening or hyperpigmentation. Ferulic acid has been documented to has inhibitory effect on melanin production and become a potential pigmentation inhibitor. Roh et al. (2004) reported that N-feruloyserotonin, one of the active compounds of safflower seed, strongly inhibits the melanin production compare to the common melanogenesis inhibitor, arbutin. Through in-vivo study, mixed solution containing ferulic acid has been proved to provide significant protection against erythema and sunburn cell formation (Oresajo et al., 2008). Furthermore, ferulic acid also being used as stabilizer in a cosmetic containing anthocyanin-type pigment from tulip flowers against oxidative discoloration (Graf, 1992).

However due to the small polar compound of the ferulic acid, it has limited solubility in an oil phase of emulsions or anhydrous compositions (Zhou et al., 2003). This property makes ferulic acid left during sweating or swimming and thus reduces its cosmetically acceptable. Conjugation with other molecules to extend its side chain may improve its solubility in diverse medium. Furthermore, there are several documents reported that esters of ferulic acid exhibit higher antioxidant activity than the acid itself (Kikuzaki et al., 2002; Fang et al., 2006). The presence of the ester group makes the compound more lipophilic allowing a better transportation of the compound across the cell membranes, which are rich in lipids (Scapagnini et al., 2004).

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2.3 Synthesis of Ferulate Esters

Ferulate esters have been produced since decades ago via enzymatic esterification by introduction of ferulic acid to alcohols. Guyot and co-workers (1997) first reported the synthesis of ferulate esters which carried out in stirred flask and catalyzed by using Novozym 435. They found that esterification of ferulic was only possible using alcohols with a carbon number greater than or equal to 8. The finding was in accordance with other studies concerning the ability of ferulic acid to esterify in medium and long-chain alcohols using similar reaction system (Stamatis et al., 1999; 2001).

However, Kobayashi et al. (2003) reported that reaction rate of ferulate esters synthesized by using ferulic acid as substrate are generally low, because of the presence of conjugation with a carboxyl group in an acid and a branched structure in the non-carboxylic region. Later, Yoshida et al. (2006) have upgraded the reaction system by applying a plug-flow reactor system in the esterification between ferulic acid with 1-pentanol, 1-hexanol and 1-heptanol where conversions greater than 90 % were achieved. Due to the hydrophilicity, unfortunately, direct esterification of ferulic acid can badly affect the catalytic activity of enzymes and possess low stability in various solvent systems (Xin et al., 2011).

Despite such difficulties, more interests were given to produce ferulate esters by enzymatic transesterification between ethyl ferulate (naturally occurring alkyl ester of ferulic acid) and triacylglycerol (TAG). The incorporation into TAG could potentially

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result in novel molecules which having combined beneficial properties of both polyunsaturated fatty acids (from TAG) and phenolic compound (from ethyl ferulic). Repeatedly, researchers keep put much efforts on improving ferulate ester synthesis system which offer more simple reaction, maximum yield of ester produce and reach equilibrium in a reasonable amount of time to be industrially attractive. Several research papers on synthesizing ferulate ester have been reviewed and summarized as in Table 2.1:

TABLE 2.1: A Literature Survey on Ferulate Esters Synthesis

Substrates	Catalysts	Remarks	References
EF + Triolein	Novozym 435	77 % of feruloyl monoolein and feruloyl diolein was achieved using a threefold excess of neat triolein after 144 hrs	Compton et al. (2000)
EF + Triolein	Novozym 435	74 % of feruloyl monoolein and feruloyl diolein was achieved using supercritical CO_2 with 1: 1 ratio of substrates after 48 hrs	Compton & King (2001)
FA + 1-pentanol, 1-hexanol, 1-heptanol	Novozym 435	Application of a plug-flow reactor system in the esterification improves FA conversion greater than 90 % after 40 hrs	Yoshida et al. (2006)

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EF + Soybean oil	Novozym 435	Reaction with monoacylglycerol and diacylglycerol from soybean oil showed improvement in EF transesterification compare to the used of raw soybean oil	Laszlo & Compton (2006)
FA + Glycerol	Pectinase PL "Amano"	Pectinase PL "Amano" from *Aspergilles niger* is found to contain ferulic acid esterase which catalysed the esterification of ferulic acid and the immobilized enzyme can be reused at least five times without significant loss in activity	Matsuo et al. (2008)
EF and FA + Flaxeed oil	Novozym 435	A lower bioconversion yield (9 %) was obtained with EF as substrate due to the formation of ethanol as hydrolysis by-product which can inhibit the enzyme activity compare to FA as substrate (29 %)	Karboune et al. (2008)
EF + Triolein	Novozym 435	The transesterification by using a vacuum-rotary evaporation procedure affords a 59.5 % yield of ferulate ester after 72 hrs	Xin et al. (2009)
FA + Ethanol	Concentrated sulfuric acid	Ferulate esters synthesis was developed by using microwave irradiation where up to 90 % of product obtained within 3 to 5 min	Li et al. (2009)
EF + Soybean oil	Novozym 435	Solutions of EF and soybean oil formed were recirculated over fixed, packed beds to produce a mixture of feruloyl soybean oil glycerol species having a combination of one or more feruloyl moieties and one or more fatty acid moieties on the same glycerol backbone	Compton & Laszlo (2009)

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EF + Triolein	Novozym 435	A kinetic model was developed from the effect of various parameters studied such as speed agitation, catalyst load, temperature, and substrates ratio. The model can be described adequately by a ping-pong bi-bi mechanism with triolein inhibition	Xin et al. (2011)
EF + Fish oil of cod liver	Novozym 435	Effects of several parameters were studied (catalyst load, temperature, substrates ratio and reaction time) and addition of glycerol to the process increased the conversion of EF up to 92.4 % with the assistance of response surface methodology (RSM)	Yang et al. (2012)
EF + Monostearin	Novozym 435	Effects of several parameters were studied (catalyst load, temperature and reaction time) and the EF conversion reached 98 % after 23 hrs under optimization using RSM	Sun et al. (2013)
EF + Distearin	Novozym 435	Effects of several parameters were studied (catalyst load, temperature and reaction time) and the EF conversion reached 97.6 % after 24 hrs under optimization using RSM	Sun et al. (2014)
EF + Castor oil	Novozym 435	Effects of several parameters were studied (catalyst load, temperature and substrates ratio) and removal of the ethanol from the process resulted in almost 100 % of EF conversion	Sun et al. (2014)

FA indicates ferulic acid; EF indicates ethyl ferulate

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Nevertheless, there are several shortfalls exhibited from the ferulate esters syntheses before. One of them is the long reaction time to achieve high conversion (Compton et al., 2000; Compton & King, 2001; Xin et al., 2009). Shortest reaction time of three to five minutes was proposed by Li et al. (2009) using microwave irradiation. However, the use of concentrated sulfuric acid as catalyst is highly corrosive especially in the production of daily-wear cosmetic products. Due to the high stearic hindrance of TAG molecule, Laszlo & Compton (2006) have generated monoacylglycerol and diacylglycerol from soybean oil for better ferulate esters conversion, but, introducing the additional processing steps are uneconomical for large-scale production. In addition, several researchers claimed that the use of Novozym 435 brings about significant problem due to the high cost of the enzyme which makes overall process expensive and restricts its applications in industries (Fjerbaek et al., 2009; Reyes-Duarte et al., 2011; Chattopadhyay & Sen, 2013). Therefore, any modification step with render the processes economically and industrially infeasible is required.

2.4 Lipases as Biocatalyst

Among the various kinds of biocatalyst available, lipase has been widely used in chemical syntheses due to its biotechnological potential. Lipase (triacylglycerol acylhydrolase; EC 3.1.1.3) is considered as the most versatile group of enzyme found in nature with wide range of selectivity, display varied stability, commercially available and undergo simple reaction with no cofactor requirement (Hasan et al., 2006). The first commercially successful lipase was introduced by Novo Nordisk A/S in 1988 under the

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trade name of Lipolase®, which originated from the fungus Humicola lanuginosa (Jurado et al., 2007).

Lipases can be of plants origin, animals (pancreatic, hepatic and gastric) or more abundantly found in microorganisms (bacterial, fungal and yeast) (Salleh et al., 2006). Many of the lipases studied in plants shown better specificity, low cost, availability and ease of purification compare to animals and plants lipases (Barros et al., 2010). In mammals, several tissues and organs also contain lipases including heart, brain, muscle, arteries, spleen, kidney, lung, liver, adipose tissue and serum. Microbial lipases present advantages in thermostable and resistant to chemical denaturation over animal lipases which make them attractive for many industrial applications (Salleh et al., 2006).

2.4.1 Reaction of Lipases

Lipases can catalyze a large number of reactions including hydrolysis, esterification and transesterification as described below:

i. Hydrolysis

Oils and fats are compounds known as fatty esters or triglycerides. Their hydrolysis essentially involves in the presence of water to produce both carboxylic moiety (free fatty acids, mono and diglycerides) and alcohol moiety (glycerol) in an aqueous

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emulsion. The hydrolysis of oils and fats is an important industrial operation. A significant number of high-value products require fatty acids in their manufactures including coatings, adhesives, specially lubricating oils, shampoos and other products (Murty et al., 2002).

$$R_1\text{-COO-}R_2 + H_2O \rightleftharpoons R_1\text{-COO-H} + R_2\text{-OH}$$

ii. Esterification

Esterification occurs between free fatty acids with alcohol moiety in nonaqueous low-water systems. Ester and water will be generated as the reaction proceeds. Water can be removed to shift the thermodynamic equilibrium and drive the reaction towards high yields of esters. In other word, esterification is reverse of hydrolysis which is control by the water content in the reaction mixture. Various lipase esterifications of oil and fats have been published (Linder et al., 2005; Zhong et al., 2013).

$$R_1\text{-COO-H} + R_2\text{-OH} \rightleftharpoons R_1\text{-COO-}R_2 + H_2O$$

iii. Transesterification

The term transesterification refers to the exchanges of acyl group between ester and an acyl donor. When the fatty acid acts as acyl donor, the reaction is called acidolysis. Alcoholysis occurs between ester with alcohol and aminolysis is between ester

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with amine. The reaction involving an exchange of acyl donor of one ester to another ester is called interesterification. Thus, a different ester from the original esters will be produced. The study on lipase transesterification and their potential applications in industry have been established (Wu et al., 1996; Joseph et al., 2008).

Acidolysis

$$R_1\text{-COO-}R_2 + R_3\text{-COO-H} \rightleftharpoons R_3\text{-COO-}R_2 + R_1\text{-COO-H}$$

Alcoholysis

$$R_1\text{-COO-}R_2 + R_3\text{-OH} \rightleftharpoons R_1\text{-COO-}R_3 + R_2\text{-OH}$$

Aminolysis

$$R_1\text{-COO-}R_2 + R_3\text{-N-}H_2 \rightleftharpoons R_1\text{-COO-}R_3 + R_2\text{-N-}H_2$$

Interesterification

$$R_1\text{-COO-}R_2 + R_3\text{-COO-}R_4 \rightleftharpoons R_1\text{-COO-}R_4 + R_3\text{-COO-}R_2$$

Their catalytic side, composed of a serine (Ser) residue, a histidine (His) residue and a carboxylic residue [aspartic (Asp) or glutamic (Glu) acid], is usually covered by a hydrophobic oligopeptide often called a lid. The lid is closed (inactive) when exposed in water and the hydrophilic side turned towards the solvent, possibly to prevent the aggregation of the enzyme. The lid changes to open (active) upon adsorption of the

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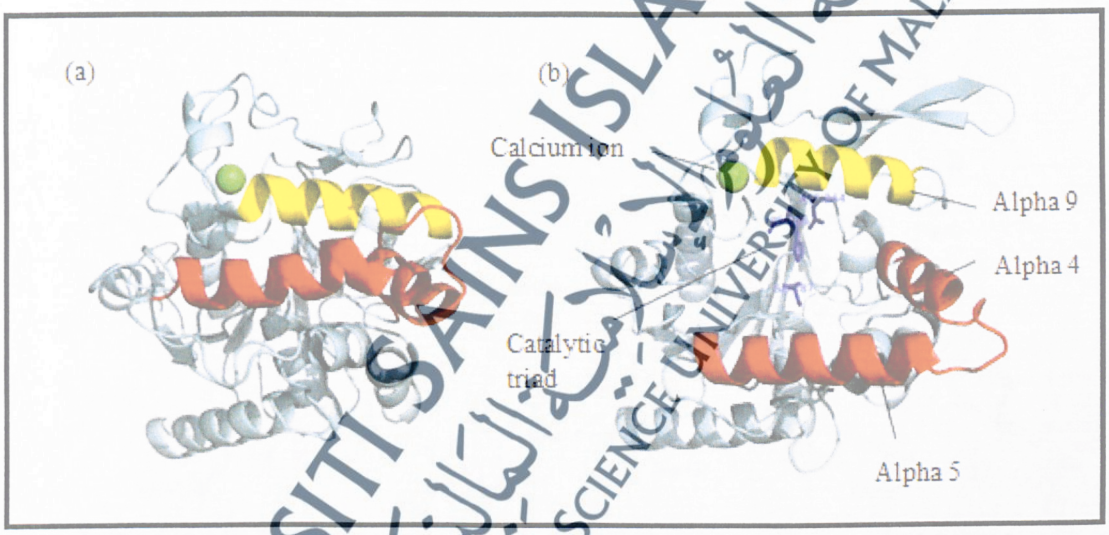
lipases onto a hydrophobic media or water/lipid interface to permit the entry and binding of the hydrophobic substrates. This phenomenon is referred to as "interfacial activation" (Overbeeke et al., 2000; Foresti et al., 2005). Housaindokht & Monhemi (2013) have investigated the large dynamical movements of the lipase lid (Figure 2.2) in different media through molecular dynamic simulation of *Burkholderia cepacia* lipase (BCL).

FIGURE 2.2: Graphical Representation of BCL in (a) Water (b) Near-Critical Propane (Housaindokht & Monhemi, 2013)



The mechanism for ester formation or hydrolysis is composed of four steps (Figure 2.3):

i. A substrate reacts with the active-site Ser, yielding a tetrahedral intermediate stabilized by the catalytic His- and Asp-residues.

ii. Alcohol is released and a covalent acyl-enzyme complex is formed.

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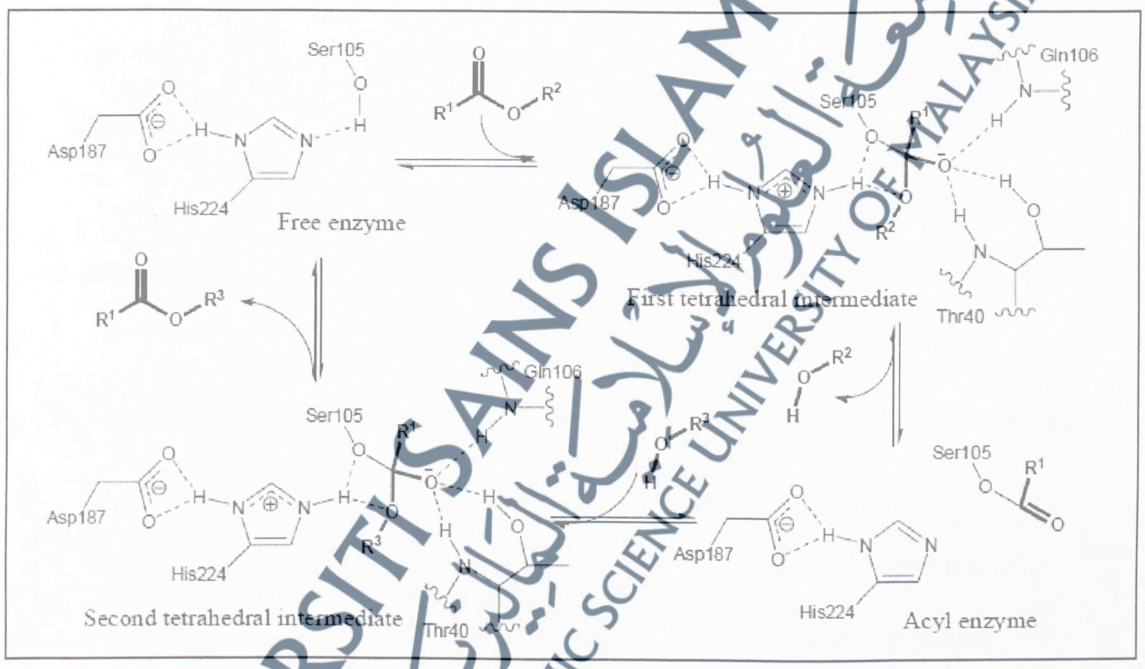
iii. Attack of nucleophile (water in hydrolysis, alcohol in (trans-) esterification) forms again a tetrahedral intermediate.

iv. Dissociation of this intermediate releases a new acid or alcohol and a new ester. The enzyme becomes free and ready for another catalytic cycle (Buchholz et al., 2005; Magnusson, 2005).

FIGURE 2.3: Reaction Mechanism of Lipase (Magnusson, 2005)



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2.4.2 Application of Lipases

The scope for the lipases application in our industry is enormous. Lipases are used in two distinct fashions; as biological catalysts to manufacture other products (such as food ingredients) and by their application (such as in making fine chemicals) (Hasan et

al., 2006). The various applications of the lipases in industry are summarized in Table 2.2.

TABLE 2.2: Industrial Applications of Microbial Lipases (Sharma et al., 2001)

Industry	Action	Application or Product
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Health foods	Transesterification	Health food
Fats and oils	Transesterification; hydrolysis	Cocoa-butter, margarine, fatty acids, glycerol
Chemicals	Enantioselectivity; synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification; hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality

2.4.3 Immobilized Lipases

Immobilized means it has been confined or localized so that it can be reused continuously. One of the main obstacles for industrial application of lipase is the high

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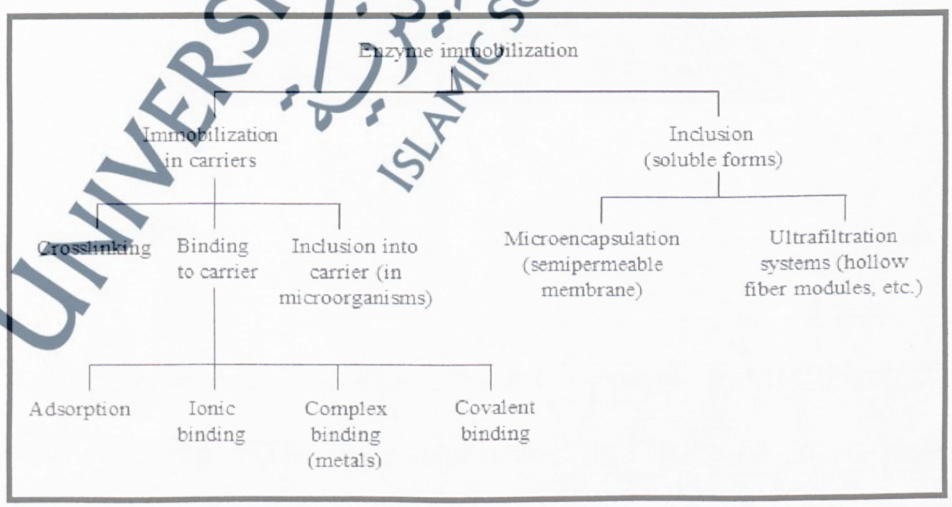
cost of the biocatalysts. Therefore, immobilization of lipases is a necessity to make them more attractive for industrial processes. The first attempt to immobilize a biocatalyst is in 1953, while in 1969 immobilized enzyme was used for the first time in industrial process (Murty et al., 2002).

The aim of immobilization is to enhance lipase properties such as thermostability and activity in non-aqueous media. Furthermore, immobilized lipase can be used repeatedly resulting greatly reduces the cost of the production. The easier recovery leads to decrease in potential for contamination of the product via residual lipases, thus avoiding the need for downstream thermal treatment (Murty et al., 2002; Knežević et al., 2004; Shafei & Allam, 2010). Immobilization of enzyme can be achieved by the use of various support materials (carriers). Buchholz and the co-workers (2005) have categorized the basic methods of enzyme immobilization into insoluble and inclusion form in a definite space as shown in Figure 2.4.

FIGURE 2.4: Enzyme Immobilization Techniques (Buchholz et al., 2005)



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2.4.4 Novozym 435

Novozym 435 (Figure 2.5) is one of the commercial immobilized enzyme, isolated from Antartic yeast *Candida antarctica* lipase B or CALB. Novozym 435 is physically adsorbed on the macroporous acrylic resin Lewatit E (Arroyo et al., 1999; Castro et al., 2000). Novozym 435 consists of 317 amino acids and has molecular weight of 33.5 kDa (Uppenberg et al., 1994). It is primarily intended for solvent-free reaction synthesis. Also known as a non-specific lipase, Novozym 435 can promote a wide range of acyl-transfer reactions between varied compounds (Yasmin et al., 2006; Duan et al., 2010; Yadav & Devendran, 2012).

FIGURE 2.5: Electron Microphotograph of Novozym 435 (Torres et al., 2008)

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2.4.5 Lipozyme RM IM

Another commercial immobilized lipase is Lipozyme RM IM (Figure 2.6) from *Rhizomucor miehei* fungi. The lipase is supported on Duolite ES 562, a weak anion-

exchange resin based on phenol-formaldehyde copolymers (Rodrigues & Fernandez-Lafuente, 2010). The enzyme is describes as a single polypeptide chain of 269 residues with molecular weight 31.6 kDa (Rodrigues & Fernandez-Lafuente, 2010). In view of regiospecificity (1, 3- specific) and economics, Lipozyme RM IM is a special interest which can catalyze reactions only on primary hydroxyl groups of triglycerides. These preferentially release fatty acids from positions 1 and 3 to give free fatty acid and di- and or mono-glycerides (Kapoor & Gupta, 2012).

FIGURE 2.6: Light Microscopic Evaluation of Lipozyme RM IM Particles (Phuah et al., 2012)

2.4.6 Dual Lipases System

Until recently, there have been few attempts to use combination of two lipases with different specificities, also known as dual lipases system. This concept has been applied in two forms; immobilization of crude lipases onto one support and application of

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commercialize immobilized lipases concurrently. Several research papers on the use of dual enzymes system have been summarized as in Table 2.3:

TABLE 2.3: A Literature Survey on Dual Enzymes System

Process	Dual Enzymes System	References
Hydrolysis of starch	Glucoamylase + pullulanase	Roy & Gupta (2004)
Modification of bulky oils	Lipase AK + Lipozyme TL IM + Lipozyme RM IM + Novozym 435	Ibrahim et al. (2008)
Production of wax esters	Novozym 435 + Lipozyme RM IM	Kuo et al. (2012)
Transesterification of biodiesel	*Penicillium cyclopium* + *Rhizomucor miehei*	Guan et al. (2010)
	Thermomyces lanuginosus + *Rhizomucor miehei*	Lee et al. (2011)
	Candida rugosa + *Rhizopus oryzae*	Rodrigues & Ayub (2011)
	Candida antarctica + *Rhizomucor miehei*	Banerjee et al. (2013)
Synthesis of bio-lubricant	Novozym 435 + Lipozyme RM IM	Malhotra et al. (2014)

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These dual enzymes system demonstrated several advantages including enhanced the conversion within time (Guan et al., 2010; Lee et al., 2011), offered advance catalytic activity (Rodrigues & Ayub, 2011) , displayed synergistic effect (Ibrahim et al., 2008) and improved thermostability (Roy & Gupta, 2004).

2.5 Olive Oil as Reaction Substrate

Olive oil is a product extracted from the fruit of *Olea europaea* L. (Oleaceae family) tree by mechanical or physical means. With a transparent, yellowish and aromatic liquid, olive oil known to be one of the healthiest source of fat around the world (Baumann & Weisberg, 2010). Practically, it is the only vegetable oil that can be consumed directly from its raw material and contains important nutritional elements with sensory properties (Baccouri et al., 2008).

In facts, olive oil has been used on the skin for thousands of years. Egyptian pharaohs used olive oil as perfume and to moisturize their skin and hair. It is said that Cleopatra was the inventor of the first anti-wrinkle cream by mixing olive oil with milk, incense and juniper berries. Romans used olive oil as massage oil for athletes and salve for soothing wounds (Viola & Viola, 2009; Baumann & Weisberg, 2010). For non-cosmetic application, olive oil is widely used in cooking, biodiesels and fertilizers (Wiesman, 2009). The widespread applications of olive oil with medicinal properties should be related to chemical compositions of the olive oil itself.

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2.5.1 Chemical Compositions of Olive Oil

Generally, chemical compositions of olive oil can be divided into two parts; major and minor components. The major component also known as saponifiable fraction, which is contributes 98 to 99 % of total oil weight. Meanwhile, the minor component in olive oil or unsaponifiable fraction represents about 0.5 % to 2 % of oil weight which includes more than 230 chemical compounds (Samaniego-Sanchez et al., 2010).

i. Major Component

In the nature of olive oil, TAGs are the main component. In chemical term, TAG is a molecule derived from three fatty acids molecules attached to a glycerol backbone. There are a few types of fatty acids can be found in olive oil. The distribution of fatty acids in olive oil TAGs is summarized in Table 2.4.

TABLE 2.4: Positional Distribution of Fatty Acids in Triglycerides of Olive Oil (Gunstone et al., 1994)

Position	Fatty acids (mol %)					
	16: 0 (Palmitic)	16:1 (Palmitoleic)	18: 0 (Stearic)	18: 1 (Oleic)	18: 2 (Linoleic)	18: 3 (Linolenic)
1	13.1	0.9	2.6	71.8	9.8	0.6
2	1.4	0.7	-	82.9	14.0	0.8
3	16.9	0.8	4.2	73.9	5.1	1.3

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Among the TAGs formed in the olive oil, the level of oleic is represented in much higher concentration compare to the other acids such as palmitic, palmitoleic, stearic, linoleic and linolenic (Baccouri et al., 2008). High content of monounsaturated fat (mainly oleic acid) is necessary for human health and has proven beneficial effect on serum cholesterol levels (Salimon & Farhan, 2012).

ii. Minor Components

a) Sterols

The main portion of the unsaponifiable fraction is represented by sterol, a chemical compound having a characteristic three dimensional arrangement of four rings. In olive oil, it is present in the free form or esterified with fatty acids (Mulinacci et al., 2005). Sterols have shown anti-inflammatory, anti-bacterial, anti-fungal, anti-ulcerative, anti-oxidant and anti-tumoral activity (Hatzakis et al., 2010).

b) Hydrocarbons

Squalene ($C_{30}H_{50}$, 2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosa-hexaene) is one of the hydrocarbon found in olive oil (Aguilera et al., 2005). High levels of squalene are also found in human skin lipids and adipose tissue (Tsimidou, 2010). Squalene shows some advantages for the skin as a quencher of singlet oxygen and protects human skin surfaces from lipid peroxidation due to exposure to UV light and other sources of oxidative damage (Huang et al., 2009). Another smaller proportion of

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hydrocarbon constituent is β-caroten, the most important provitamin A source (Samaniego-Sanchez et al., 2010).

c) Phenolic Compounds

Olive oil is unique among other vegetables oil due to high level of phenolic compounds concentration. Phenolic compounds have been identified as an important factor contribute to the extraordinary stability of the olive oil against oxidation, thus can act as potential antioxidant agent (Tura et al., 2007). Anti-inflammatory effect of these phenolic compounds can prevent certain type of diseases including atherosclerosis and cancer (Ouni et al., 2012).

2.5.2 Dermatologic Effects of Olive Oil

Because of its beneficial, the researches of olive oil's value from variety of different angles have notably increased. Nowadays, olive oil has been reported to be an effective option in treating various inflammations including xerosis (dry skin), pruritus (itchiness), seborrhea (dandruff), rosacea (skin sores), eczema or dermatitis (rashes), burns and other cutaneous damage (Badiu et al., 2010; Baumann & Wisberg, 2010; Ruiz et al., 2010). Treatment with olive oil has no side effects and does not traumatize the skin. However, the application of olive oil alone onto skin is not practicable since it is too oily to apply directly. So, addition with another valuable compound will improve its texture and performance.

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2.6 Response Surface Methodology (RSM)

A far and wide used technique for evaluating interactive effects in enzyme based processes is response surface methodology (RSM). RSM is defined as a statistical method (Appendix B) that uses quantitative data from appropriate experimental designs to determine and simultaneously solve multivariate equations and so generate mathematical model that describes the overall process (Noordin et al., 2004). According to Bezerra et al. (2008), the main objective is to achieve an optimization, in other words, to create the best possible response which influenced by several factors (independent variables). RSM was developed by Box and collaborators in the 50s and the most popular response-surface analyzers nowadays include Design-Expert, JMP and Statgraphics (Lenth, 2009).

RSM offers vast advantages over conventional studies (Vainionpaa, 1991), such as:

i. Enables study on the effect of several factors individually or in combination.

ii. Only small number of experiments necessary to conduct a research.

iii. Reduces the cost and time required for determination of the optimum process.

2.6.1 Five-Stages RSM Procedure

Based on reports by Giovanni (1983) and Bezerra et al. (2008), summarily, RSM can be successfully implemented by used of the following five stages:

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Stage 1: Screening of factors

Numerous factors may affect the response of the system studied; therefore, it is necessary to select those factors that are most important with major effects. The more prevalent factors evaluated in the enzymatic syntheses of ferulate ester were reaction temperature, reaction time, amount of enzyme, substrate concentration, amount of molecular sieve and speed of agitation (Kumar & Kanwar, 2011; Xin et al., 2011).

Stage 2: Definition levels for each factor

The range of level for each factor can be determined through experimental work. The levels are initially set fairly broad to yield more accurate representation of optimum. The factor of reaction temperature, for example, can be investigated at five levels: 40, 50, 60, 70 and 80 °C. Once set, a preliminary test with samples representing these levels are performed to ensure that the levels are appropriate.

Stage 3: Choice of experimental design and selection of test samples

Such designs can be obtained from the literature or generated using computer software (Design-Expert, JMP, Statgraphics, etc). They comprise of a selected subset of samples to be tested from the set of all possible samples that could be tested with emphasis on those closest to the central point of the factor levels. Once the samples are

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specified, experiments are performed to test these samples and obtained quantitative data for use in subsequent statistical analysis.

The simplest design which can be used in RSM is based on a linear function where the response should not present any curvature, also known as first-order model. The most common first-order models are 2^k factorial (k is the number of control factors), Plackett-Burman and simplex designs (Khuri & Mukhopadhyay, 2010). This model is assumed to be an adequate approximation of true surface in a small region of factors.

In contrast, if there is a curvature in the response surface, then a higher degree polynomial should be used, also called a second-order model. The most frequently used second-order models are central composite and the Box-Behnken designs. The central composite design (CCD) is preferred for four or five factors study at 5-levels. When less factor and levels involve, the Box-Behnken designs is more appropriate (Khuri & Mukhopadhyay, 2010). An ability to describe quadratic surfaces makes this model appropriate for analysing maximum, minimum and ridge or saddle point.

Stage 4: Mathematic-statistical treatment

The quantitative data related to each experimental point of a chosen design are analysed and interpreted then, to test either the model is fitted or not.

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Analysis of variance or ANOVA (F-test, t-test, R^2, the adjusted R^2 and lack of fit) is a more reliable way to determine the adequacy of the fitted model. The central idea of ANOVA is to compare the variation due to the treatment (change in the combination of factor levels) with the variation due to random errors inherent to the measurement of the generated response (Bezerra et al., 2008).

In addition, the relationship between factors and the response can be determined by regression analysis. A regression analysis is performed to generate coefficients, where coefficients with P-values of <0.05 are generally considered highly significant (Haaland, 1989) and therefore included in a mathematical model known as regression model. The estimated response could be easily calculated then, using this model equation. Usually the behavior of the system is unknown, so the model is checked through experimental work to fit well (Baş & Boyacı, 2007).

Stage 5: Determination of the optimal conditions

The graphical visualization of the predicted model equation can be obtained by the response surface plot and contour plot. The response surface plot is the three-dimensional dome-shaped plot showing the relationship between the response and the factors. And, two-dimensional of a contour plot is consists of lines drawn in the plane of factors. It is possible to find the optimum region through visual inspection of the surfaces (Baş & Boyacı, 2007).

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2.6.2 Advantages and Limitations of RSM

Giovanni (1983) stated that the successful use of RSM is dependent upon the following five assumptions:

i. The factors critical to the process are known.

ii. The region of interest where the factor levels influence the process is known.

iii. The factors vary continuously throughout the experimental range tested.

iv. There exists a mathematical function which relates the factors and measured response.

v. The response defined by this function is a smooth surface.

However, RSM also have several limitations such as:

i. Large variations in the factors which result in misleading conclusions.

ii. Incorrect specification and insufficient definition of the critical factors which lead to an inaccurate description of the optimum conditions.

iii. Too narrow or broad range of factor levels which prevents determination of the process optimum.

iv. The misuse of statistical principles which give biased results that in turn leads to an incorrect mathematical model for the description of the optimum.

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Therefore, today, RSM has been effectively employed for studying and optimizing numerous processes including lipase-catalyzed synthesis of various esters either through esterification (Kim & Akoh, 2007; El Boulifi et al., 2010), transesterifications (Gunawan et al., 2005; Zhao et al., 2014) or alcoholysis (Abd Rahman et al., 2011).

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