LIPASE MEDIATED HYDROLYSIS OF CRUDE PALM OIL IN ENZYMATIC MEMBRANE REACTOR AND RECOVERY OF CAROTENES AND TOCOPHEROL

by

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UNIVERSITI SAINS MALAYSIA

2012

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This thesis is submitted in fulfillment of the requirement for the degree of Doctor of Philosophy

July 2012

ACKNOWLEDGEMENTS

Alhamdulillah, great thanks to The Great Almighty ALLAH SWT for granted me the knowledge, strength and determination to accomplish my PhD research work. First of all, I would like to express my deepest gratitude to my beloved mother, Pn. Rokiah Hussin, for her endless love, support and blessing. I would also like to express my thanks to my big brother En. Mohd Amin Serri, her wife Pn Zainah and also to my adorable niece Luqman Hakim for their love, cares and understanding throughout my studies.

My deepest appreciation goes to my dedicated supervisor Prof. Azlina Harun@Kamaruddin for her patient guidance, advices and encouragement through my graduate program. Also my gratitude goes to my co-supervisor, Dr. Mohamad Hekarl Uzir for his valuable and constructive comments throughout my study. Besides, I am very much indebted to Ministry of Science, Technology and Innovation (MOSTI) for providing the financial support National Science Fellowship (NSF) and Yayasan Felda for providing research grant for this project. To all the technicians and staffs from School of Chemical Engineering, millions thanks for their cooperation and helping hands during the completion of my research work especially to En. Faiza, En. Shamsul Hidayat, Pn. Latiffah and Pn. Aniza.

Lastly, to all my friends Dr. Sunarti, Husaini, Fadzil, Azie, Eka, Zira, Rahmah, Pesar, Emma, Syidot, Dr. Wan Zaharatul Ashikin, Dr. Nur Satinati, Syuhanis, Izzah, Yunus, Kak Ida, bowling friends and others, thank you so much for your motivation, sincere help, concern, moral support and kindness. Thanks for the everlasting friendship and sweet memories. To all people who helped me directly or indirectly in this research work, your contribution given shall not be forgotten. Only ALLAH can repay all of you. Thank you.

Noor Aziah Serri

Jun 2012

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LIST OF SYMBOLS

a	Fiber internal radius	mm
A_0	Initial enzyme activity	
A_r	Residual enzyme acitivity	
BF_3	Boron trifluoride	
b_i	Coefficient for the linear effect	
b_{ii}	Coefficient for quadratic effect	
b_{ij}	Coefficient for the interaction effect	
b_o	Constant coefficient	
B_o	Bodenstein number	
$Ca(OH)_2$	Calcium oxide, precipitation agent	
c_b or [FA]	Concentration of product	mol L^{-1}
C_i	Mass of protein absorbed on the membrane	mg L^{-1}
C_p	Protein concentration of permeate	mg L^{-1}
C_r	Protein concentration of retentate	mg L^{-1}
c_{s0}	Concentration of CPO at time = 0	mol L^{-1}
D_e	Diffusivity	cm^2 s^{-1}
E	Enzyme	
E_0	Initial enzyme acitivty	mol L^{-1} min^{-1}
Ea	Acitivation energy constant	J. mol^{-1}
E_d	Denaturation energy for lipase	J mol^{-1}
EFA	Enzyme-inhibitor complex	
ETg	Enzyme-substrate complex	
ETgI	Enzyme-substrate inhibitor complex	
F	Volumetric flow rate	ml min^{-1}
h	Planck constant	J
k_B	Boltzman constant	J K^{-1}
k_{cat} or v	Reaction rate	mol L^{-1} hr^{-1}
k_d	Deactivation constant	s^{-1}
kDa	Kilodalton	
k_{do}	Constant	s^{-1}
K_I,K_{FA}	Inhibition constant	mol L^{-1}

K_M/K_{Mapp}	Michaelis Menten constant/apparent	mol L^{-1}
L	Effective length of fiber	cm
Log P	Partition coefficient of a given compound in octanol and water two phase system	
m	Mass of protein	mg
n	Sample size	
N	Number of fiber	
R	Universal gas constant	J mol^{-1} K^{-1}
R	Dimensionless radial coordinate	
r	Fiber radius	mm
R^2	Coefficient of determination	
S/Tg	Substrate/Triglyceride	
T	Temperature	K
$t_{1/2}$	Enzyme half-life	hr
V_i	Initial protein volume	L
V_{Max}/V_{Maxapp}	Maximum rate of reaction/apparent	mol L^{-1} hr^{-1}
V_p	Volume of permeate	L
V_r	Volume of retentate	L
x_i, x_j	Factors (independent variables)	
Y	Response (yield of fatty acids)	
ΔG	Gibbs energy	J mol^{-1}
ΔH	Enthalpy of activation	J mol^{-1}
ΔS	Entropy of activation	J mol^{-1} K^{-1}
[E]	Concentration of enzyme	mg L^{-1}
[Tg]	Concentration of substrate/Triglycerides	mol L^{-1}
<	Smaller than	
>	Larger than	
ξ_P	Dimensionless product inhibition	
α_p	Porosity of membrane	
φ	Molar fraction of product and substrate	
Φ^2	Thiele modulus	
Θ	Dimensionless Michaelis constant	
Ψ	Lipase activity coefficient	

LIST OF ABBREVIATIONS

ADP	Adenosine -5'-diphosphate
ANOVA	Analysis of variance
ATP	Adenosine-5'-triphosphate
4-AAP	4-aminoantipyrine
C.rugosa	*Candida rugosa*
CCD	Central composite design
CCRD	Central composite rotatable design
CPKO	Crude palm kernel oil
CPO	Crude palm oil
CSTR	Continuous stirred tank reactor
DAP	Hydroxyacetone phosphate
DOE	Design of experiment
3D	Three dimensional
EMR	Enzymatic membrane reactor
ESPA	Sodium *N*-ethyl-*N*-(3-sulfoproply) m-anisidine
FAME	Fatty acid methyl esters
FFB's	Free fruit bunch
FID	Flame ionization detector
g	Gram
G-1-P	Glycerol-1-phosphate
GC	Gas chromatography
GK	Glycerol kinase
GPO	Glycerol phosphate oxidase
H_2O_2	Hydrogen peroxide
hr	Hour
kg	Kilogram
L/l	Litre
LU	Lipase unit
M	Molar
mg	Miligram
MPa	Mega pascal

MPOC	Malaysian Palm oil council
^{o}C	Degree Celsius
PAN	Polyacrylonitrile
PBR	Packed bed reactor
PEEK-WC	Polyetheretherketone
PFAD	Palm fatty acid distillate
PKO	Palm kernel oil
POD	Peroxidase
ppm	Part per million
RBD	Refined, bleached and deodorized
rpm	Rotation per minute
RSM	Response surface methodology
TMP	Transmembrane pressure
UF	Ultrafiltration
α	Alpha
β	Beta
γ	Gamma
Δ	Delta

HIDROLISIS MINYAK SAWIT MENTAH MENGGUNAKAN PERANTARAAN LIPASE REAKTOR MEMBRAN BERENZIM DAN PEROLEHAN KAROTENA DAN TOKOFEROL

ABSTRAK

Penghasilan asid lemak and gliserol daripada minyak merupakan proses penting terutama dalam industri oleokimia. Kini, para penyelidik memilih enzim dalam proses hidrolisis kerana penjimatan tenaga dan meminimumkan penyusutan produk akibat haba. Kelebihan penggunaan enzim dalam proses hidrolisis termasuk; penggunaan bioteknologi yang hanya memerlukan suhu sederhana, langkah operasi yang mudah dan kos yang rendah termasuk penggunaan tenaga. Kajian terkini menjurus kepada hidrolisis trigliserida untuk menghasilkan asid lemak bebas dan gliserol daripada minyak sawit mentah (CPO) bermangkinkan *Candida rugosa* lipase secara kelompok dan reaktor membran berenzim (EMR). Perolehan semula karotena dan tokoferol juga dikaji pada masa yang sama.

Pengoptimuman proses hidrolisis secara kelompok telah menggunakan rekabentuk ujikaji yang lebih tertumpu kepada kaedah sambutan permukaan (RSM) untuk mendapatkan tindak balas hidrolisis yang optima. Pengaruh boleh ubah dalam proses yang diambil kira termasuk beban katalis, A (0.30 – 0.80 g), kepekatan minyak, B (0.15 – 0.35 g/ml), suhu tindak balas, C (40^{o}C - 50^{o}C) and pH larutan penimbal D, (6.5-7.5). Nilai optimum yang diperolehi untuk hidrolisis CPO berenzim adalah: 0.43 gram enzim, 0.15 g/ml minyak pada suhu 45^{o}C dan larutan penimbal pH 7.0. Capaian dijangkakan untuk penghasilan asid lemak boleh mencapai 90.95% dengan nilai sebenar sebanyak 90.67% (5.59×10^{-5} mol jam^{-1} g^{-1} enzim). Tenaga pengaktifan dan penyahtabii untuk lipase telah dinilai melalui plot

Arrhernius memberi bacaan masing-masing 23.4 kJ/mol (R^2 = 0.92) dan 42.5 kJ/mol (R^2 = 0.94). Penilaian untuk pemalar penyahtabii, k_d menunjukkan kenaikan daripada 0.086 - 0.235 $hari^{-1}$ dan nilai separuh-hayat ($t_{1/2}$) iaitu 71.45 - 192.54 jam dengan peningkatan suhu daripada 60^oC - 45^oC.

Nilai optimum untuk operasi berterusan menggunakan reaktor membran berenzim adalah menggunakan 3 g/l kepekatan lipase awalan semasa proses sekatgerak, dengan kepekatan minyak 0.2 g/ml, 40 ml/min kadar aliran fasa organik, 30 ml/min kadar aliran fasa akuas, 40^oC suhu tindak balas dan tekanan transmembran sebanyak 6 psi menghasilkan capaian menghampiri 50% (5.47 x 10^{-3} mol jam^{-1} g^{-1} enzim)dengan nilai sebenar 346mmol/ml asid lemak. Percubaan untuk memperoleh semula karotena dan tokoferol daripada fasa organic unit EMR telah dilakukan melalui proses pemendakan secara berkelompok menggunakan $Ca(OH)_2$ sebagai agen pemendakan.

Tindak balas hidrolisis CPO bermangkin lipase mematuhi sistem keseimbangan pantas bersama perencatan asid lemak (asid palmitic / asid oleic). Nilai kadar tindak balas maksima (V_{Max}) dan pemalar Michaelis (K_M) untuk lipase bebas ialah V_{Max} = 0.194 mol L^{-1} h^{-1} dan K_M = 1.452 mol L^{-1}. Nilai untuk lipase tersekatgerak dalam sistem EMR diperolehi adalah V_{Maxapp} = 0.036 mol L^{-1} h^{-1} dan K_{Mapp} = 0.912 mol L^{-1}. Satu model matematik berjaya dibangunkan dan pengaruh faktor nombor Bodenstein (B_o), pemalar Michaelis tak bermatra (Θ) dan modulus Thiele (Φ^2) telah dibincangkan.

LIPASE MEDIATED HYDROLYSIS OF CRUDE PALM OIL IN ENZYMATIC MEMBRANE REACTOR AND RECOVERY OF CAROTENES AND TOCOPHEROL

ABSTRACT

Production of fatty acid and glycerol from oils are important especially in oleochemical industries. Nowadays, researchers prefer to use enzyme to conduct hydrolysis in order to reduce energy consumption and minimize thermal degradation of the products. The advantages of the enzyme hydrolysis technique include; the use of bio-route technology that only requires a mild temperature, simple operational procedure and low cost as well as energy consumption. The present investigation focuses on hydrolysis of triglyceride to produce free fatty acids and glycerol from crude palm oil (CPO) using *Candida rugosa* lipase in batch and enzymatic membrane reactor (EMR). At the same time, the recovery of carotenes and tocopherol was also studied.

The optimization in hydrolysis of CPO for batch process was carried out using Design of Experiment that focuses on response surface methodology (RSM) to optimize the hydrolysis reaction. The process variables which were taken into account include; enzyme loading, A (0.30 – 0.80 g), oil loading, B (0.15 – 0.35 g/ml), reaction temperature, C (40^{o}C - 50^{o}C) and pH of buffer solution D, (6.5-7.5). The optimum conditions found for the enzymatic hydrolysis of CPO under investigation are: 0.43 grams of enzyme, 0.15 g/ml of oil with temperature of 45^{o}C and buffer solutions at pH 7.0. The yield predicted for fatty acids produced can reach up to 90.95% and the actual value was found to be 90.67% (5.59×10^{-5} mol hr^{-1} g^{-1} enzyme). Lipase activation and denaturation energy were predicted using

Arrhernius plot and gave a value of 23.4 kJ/mol (R^2 = 0.92) and 42.5 kJ/mol (R^2 = 0.94), respectively. Prediction of denaturation constant, k_d was found increasing from 0.086 - 0.235 day^{-1} and half-life ($t_{1/2}$) of 71.45 - 192.54 hr with the increasing temperature from 60^{o}C - 45^{o}C.

A setup of enzymatic membrane reactor have been design and fabricated. In continuous operation using enzymatic membrane reactor an optimum conditions of initial lipase concentration during immobilization using 3 g/l, with oil concentration of 0.2 g/ml, organic phase flow rate of 40 ml/min, aqueous phase flow rate of 30 ml/min, reaction temperature 40^{o}C and transmembrane pressure of 6 psi have resulted a yield of almost 50% and actual of 346mmol/ml of fatty acids (5.47 x 10^{-3} mol hr^{-1} g^{-1} enzyme). An attempt of recovering carotenes and tocopherol from organic phase of EMR unit was done by precipitation process using $Ca(OH)_2$ as precipitation agent in batch process.

The study of lipase-catalyzed hydrolysis reaction of CPO obeys the rapid equilibrium system with inhibition of fatty acid (palmitic acid/oleic acid). The maximum reaction rate (V_{Max}) and Michaelis constant (K_M) values for free lipase were V_{Max} = 0.194 mol L^{-1} h^{-1} and K_M = 1.452 mol L^{-1}, respectively. For immobilized lipase in EMR system the values were found to be V_{Maxapp} = 0.036 mol L^{-1} h^{-1} and K_{Mapp} = 0.912 molL^{-1}. In addition, a mathematical model was successfully developed and discussed taken into account the effect of Bodenstein number (B_o), dimensionless Michaelis constant (Θ) and Thiele Modulus (Φ^2).

CHAPTER 1

INTRODUCTION

1.1 Large-Scale Fatty Acids Production

The production of fatty acids generally involves two separate operations; hydrolysis of fat or oil to produce a mixture of fatty acids and glycerol, followed by separation of the two products, separation and purification of fatty acid mixtures into two or more fatty acid products by simple or fractional distillations. Further processing of the compounds is required in order to obtain customer-tailored products. Conventionally, fatty acids are industrially produced from splitting of fats at high temperature and pressure, sometimes in the presence of chemical catalyst such as the Twitchell process in which the oils are heated by steam spargers and closed coils in open vessels (Anozie and Dzobo, 2006). Pugazhenti and Kumar (2004) reported that the conventional process for fats and oil hydrolysis (Colgate Emery process) required pressure of about 4.82 MPa and temperature of 250^{o}C or higher. Thus, these methods are energy intensive and not environment-friendly as chemicals used are hazardous and toxic to human and environment. Furthermore, under these extreme conditions, polymerization of fat would also take place and unwanted by products could be formed. Destruction of minor valuable product cannot be prevented under these conditions.

1.2 Hydrolysis Products from Palm Oil

1.2.1 Fatty Acids

In general fatty acids are aliphatic carboxylic acid with varying hydrocarbon lengths at one end of the chain joined to terminal carboxyl (-COOH) group at the other end. The general formula is R-$(CH_2)_n$-COOH. Fatty acids composed of a mixture of saturated and unsaturated fatty acids with chain lengths varying from 12 to 22 carbon atoms (Chen and Chuang, 2002). They are predominantly unbranched and react with glycerol to form lipids (fat-soluble components of living cells) in plants, animals, and microorganisms. The typical fatty acid composition of palm oil from Malaysia consists of myristic (14:0), palmitic, stearic, oleic (unsaturated) and linoleic (18:2, polyunsaturated) (Sambanthamurthi *et al.,* 2000). The saturated fatty acids have no double bonds, while oleic acid is an unsaturated fatty acid has one double bond and polyunsaturated fatty acids such as linolenic acids. It is reported that palm oil has equal amount of saturated and unsaturated fatty acids (Sambanthamurthi *et al.,* 2000).

Lauric acid (also called dodecanoic acid) is the main acid in which hold about 45 to 50 % in coconut oil and 45 to 55% in palm kernel oil. Nutmeg butter is rich in myristic acid (also called tetradecanoic acid) which constitutes 60 to 75% of the fatty acid content. Palmitic acid (also called hexadecylic acid) constitutes between 20% and 30% of most animal fats and is also an important constituent of most vegetable fats (35 – 45% of palm oil). Stearic acid (also called octadecanoic acid) is nature's most common long-chain fatty acids, derived from animal and vegetable fats. It is widely used as a lubricant and as an additive in industrial preparations. It is used in the manufacturing of metallic stearates, pharmaceuticals, soaps, cosmetics, and food

packaging. It is also used as a softener, accelerator activator and dispersing agent in rubbers. Oleic acid (systematic chemical name is cis-octadec-9-enoic acid) is the most abundant of the unsaturated fatty acids in nature. Mostly, fatty acids are feedstock in productions of oleochemical such as fatty alcohols, fatty amines and fatty esters. In addition fatty acids are raw materials for building the membranes of every cell in our body, including bones, nerves and brain. The micronutrients keep our body cells healthy and functioning properly (Fatty acids, 2010).

1.2.2 Glycerol

Glycerol is abundant in nature, since it is the structural component of many lipids. The general properties of this compound are that it is colourless, odourless, a viscous liquid and very soluble in water because of the existence of the three hydrophilic hydroxyl groups. Glycerol (1,2,3-propanetriol) or also known as glycerine is the principal by-product obtained during transesterification of vegetable oils and animal fats (Solomon *et al*., 1995; Barbirato *et al*., 1997a,b, 1998; Colin *et al.,* 2001; da Silva *et al.,* 2009). It can be produced either by microbial fermentation, chemical synthesis from petrochemical feedstock or recovered of by-product from soap manufacturing. Normally, glycerol is released as a by-product during the hydrolysis of fats (Da Silva *et al.,* 2009). It is hygroscopic; i.e., it absorbs water from the air; which property makes it valuable as a moisturizer in cosmetics. Glycerol has a sweet taste and insoluble in hydrocarbon. It boils at 290°C at atmospheric pressure and melts at 17.9°C. Its specific gravity is 1.262 at 25°C referred to water at 25°C, and its molecular weight is 92.09. It has a very low mammalian toxicity.

Glycerol is present in many applications, for instance; in cosmetic, paint, food, tobacco, pharmaceutical, pulp and paper, leather and textile industries. It is also is used as a feedstock for the production of various chemicals (Wang *et al.*, 2001). New applications are being evaluated in the food industry, the polyglycerol and polyurethane industry, the field of wood stabilizers and production of small molecules, such as dihydroxyacetone, glyceric and hydroxypyruvic acids and glycerol carbonate (Da Silva *et al.*, 2009). Glycerol has also been considered as a feedstock for new industrial fermentations in the future in the production of antibiotics and in medicine (Wang *et al.*, 2001).

1.2.3 Phytochemicals

Palm oil contains about 1% of minor components such as carotenoids, vitamin E and sterols (Basiron and Weng, 2004). Carotenoids are natural chemical compounds that give crude palm oil its orangey-red colour. Unrefined palm oil and crude palm oil are nature's richest source of carotenoids as compared to the other vegetable oils; 15 times more than carrots, and 30 times more than tomatoes. The most active and important form of carotenoids found in palm oil is carotene (beta-carotene). Beta-carotene can be converted to Vitamin A which plays an important role in the visual process (Edem, 2002). Vitamin E is a powerful anti-oxidant, capable of reducing the harmful types of oxygen molecules (free radicals) in the body. It helps to protect human from certain chronic diseases, while delaying the body's ageing process (Edem, 2002). In hydrolysis reaction, phytochemical is not involved in the reaction but it can be destroyed by the method and conditions used in the process. Therefore, it is important to select the appropriate technology for hydrolysis process to ensure the valuable minor product is not destroyed.

1.3 Lipase and its Application

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and oils. Lipase hydrolyzes lipids, the ester bonds in triglycerides, to form fatty acids and glycerol. Interfacial activation of lipases occurs at the lipid–water interface, a phenomenon that can be traced back to the unique structural characteristics of this class of enzymes (Reetz, 2002). Because of their wide range of applications, lipases remain as a subject of intensive study.

In addition to their biological function in bacteria, fungi, plants and higher animals, lipases have received a great deal of attention as biocatalysts in numerous industrial processes including areas such as oils and fats, detergents, baking, cheese making, hard-surface cleaning as well as leather and paper processing (Schmidt and Verger, 1998; Jaeger *et al.*, 1999; Villeneuve *et al.*, 2000; Reetz, 2002). Moreover, lipases are the mostly used enzymes in synthetic organic chemistry, catalyzing the hydrolysis of carboxylic acid esters or the reverse reaction in organic solvents depicted by Equations 1.1 and 1.2.

$$RCO_2H + R'OH \xleftrightarrow{\text{lipase}} RCO_2R' + H_2O \qquad \text{(esterification)} \qquad (1.1)$$

$$RCO_2R' + R''OH \xleftrightarrow{\text{lipase}} RCO_2R'' + R'OH \quad \text{(transesterification)} \quad (1.2)$$

The development of lipase based technologies for the synthesis of novel compounds is rapidly expanding the application of these enzymes has drastically increased (Liese *et al.*, 2000). Their advantages of the enzyme-catalyzed reaction versus the classical chemical catalysts are that they exhibit improved substrate specificity and operate in milder reaction conditions. Moreover, the fact that they

retain their activities in organic solvents and their catalytic promiscuity extend their range of application (Villeneuve, 2007). Due to their abilities to hydrolyze fats, lipases are widely used as additives in oil and fat-based industries and also in the production of household detergents. In food industry, lipases are used to modify the properties of lipids by altering the location of fatty acid chains in the glyceride and replacing one or more of the fatty acids with the new ones. This way, a relatively inexpensive and less desirable lipid can be modified to a higher value fat (Colman and Macrea, 1980; Pabai *et al.*, 1995a,b; Undurraga *et al.*, 2001; Sharma *et al.*, 2001).

1.4 Enzymatic Membrane Reactor

1.4.1 Membrane

Membrane can be defined as a thin pliable sheet of material that is permeable to substances in liquid solution. There are many types of membrane configuration such as a flat-sheet, assembled in a plate-and-frame module (Fig. 1.1a) or a spiral wound module (Fig. 1.1b), or tubular-like, assembled in tube-and-shell modules (Fig. 1.1c); it can also have a symmetric (Fig. 1.1d) or an asymmetric (Fig. 1.1e) structure.

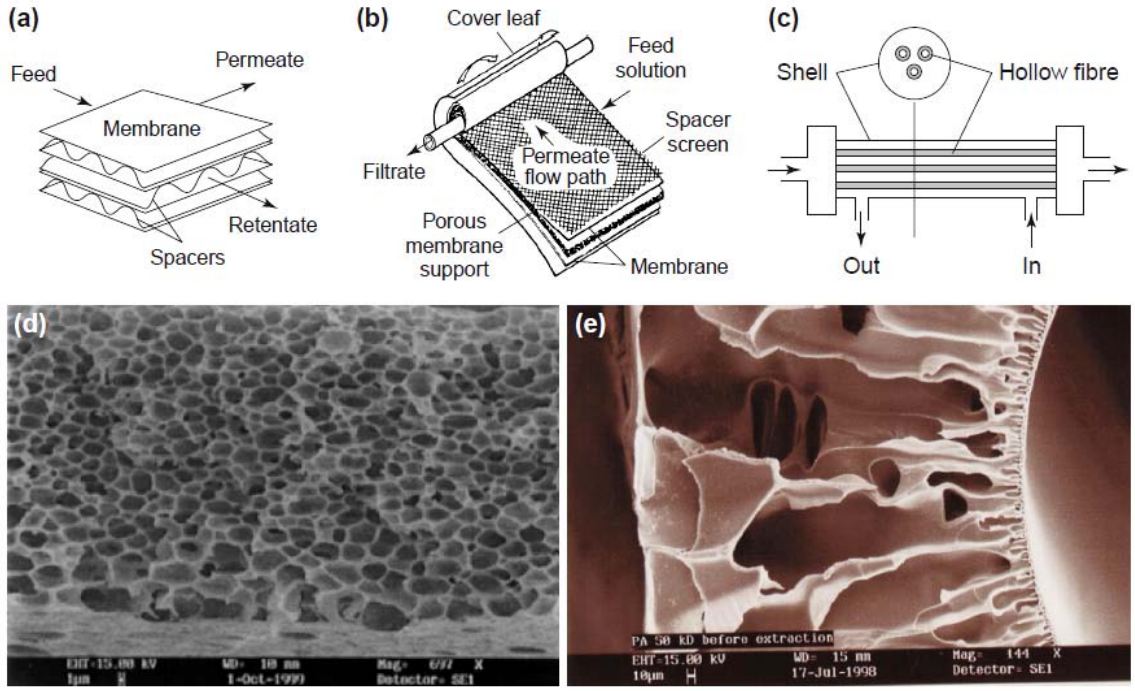

Figure 1.1: The different types of membrane and membrane modules: flat-sheet membranes assembled in (a) plate and frame, and (b) spiral wound modules; (c) a hollow fibre membrane assembled in a tube-and-shell module; (d) a symmetric membrane: a cross section of a flat membrane made of polyetheretherketone (PEEK-WC); and (e) an asymmetric membrane: a cross section of a capillary membrane made of polyamide. (Giorno and Drioli, 2000)

1.4.2 Enzymatic Reaction

The usage of enzyme in many chemical reactions is one the best solutions to overcome environmental pollution and diminishing of natural sources of raw materials in order to maximize productions. Although columns and other traditional type of reactors have been extensively use in the chemical industry for decades, an important disadvantage is the interdependence of two fluid-phases to be contacted, which sometime resulted in difficulties such as; emulsions, foaming, unloading and flooding. In order to overcome these disadvantages; the membrane reactor offers substantially more interfacial area than the conventional approaches with non-dispersive contact using a microporous membrane (Gabelman and Hwang, 1999; Giorno *et al.*, 2006). Therefore, by combining membrane technology with biocatalyst

will further improve the usage of expensive enzymes and solvent and thus, reducing the cost of production.

1.4.3 Membrane Bioreactor

In a membrane bioreactor, enzymes is confined in a well-defined region of space by means of a selective membrane or immobilized by adsorption or entrapment within the membrane matrix itself. In additions, the possibility of simultaneously carry out a desired biological reaction and product separation in one device is the best motivation for choosing enzymatic membrane reactors. Among those membrane configurations shown in Figure 1.1, hollow fiber membrane is more favorably used for membrane reactors due to its high surface-to-volume ratio that permits high biocatalyst density in a small reactor volume (Trusek-Holownia, 2005). Although there are relatively few disadvantages of using membrane such as membrane fouling and pressure drop constraints however, the numerous advantages that will be explained further in the next chapter eventually attracted the attention of many parties from both academia and industry for a diverse range of applications.

1.5 Problem Statement

The current industrial hydrolysis of oils and fats employed alkaline high pressure steam splitting also known as Twitchell process (Gan *et al.,* 1998). Additionally, Colgate Emery process caused polymerization of fat and byproduct which gave an extremely dark fatty acids and discolored aqueous glycerol solution (Pugazhenti and Kumar, 2004). These methods involve high energy utilization and yield a product that required a costly purification step. This has then turned the researchers' attentions to enzymatic hydrolysis as it is carried out under mild conditions, allowing

energy saving and producing better quality products. Enzyme hydrolysis of oil seems to be a promising alternative to a classic, high temperature and high pressure technology used in industries. Oil hydrolysis by lipase has been paid great attentions as a reaction that saves energy, does not create waste materials and is also available for food processing industry. Lipases are now available at a reasonable cost (Hasan *et al.,* 2006). Further reduction in cost of the enzyme is expected due to genetic manipulation of the microbe in producing the enzymes. This would have made the enzymatic hydrolysis of oils and fats an important reaction for industrial hydrolysis industries.

Conventionally, fatty acids and glycerol produced from energy intensive fat splitting process is separated using distillation method to obtain pure product. As an alternative, with membrane bioreactor, hydrolysis reaction and separation process can be undertaken simultaneously and therefore, reduced some downstream unit operations compared to other type of reactors. The advantages of enzymatic membrane reactor include; simplified product recovery, the ability to recycle the enzyme, possibility to run under continuous-mode operation and improved stability. In addition, the present study includes theoretical modeling to observe the controlling transport in the reaction mechanism inside the membrane reactor.

Colgate Emery process or chemical process using Twitchell reagent can only be conducted at a very high temperature and pressure. In this work crude palm oil (CPO) is used as raw material for this hydrolysis process. CPO consist of 500-700 mg/L carotenoids and some traces of phytochemical such as tocopherol and tocotrienols (Edem, 2002) which are eventually destroyed if conventional process for

production of fatty acids is being used. Moreover, the recovery of phytochemicals which are present in CPO is not possible due to the heat sensitive nature of phytochemicals compounds (Nakajima, *et al.,* 2000). Thus, enzymatic approached is the best solution to resolve this issue because of its simple and milder operation conditions.

There are many reports published on the topic of oil and fat hydrolysis using lipases but none are available specifically on the hydrolysis of CPO. The present study chooses CPO as starting materials because cost of CPO is much lower than using refined oil that has undergone stages of refinery and can easily be available in the store nearby. Although refined oil is clean from debris and other materials and produce high yield of fatty acids, but the main aim is that the technology suggested is a bio-route, an environmentally friendly process (minimize waste produce) and the raw material used is low in cost compared to refined oil. This study on the production of fatty acids and glycerol via enzymatic reaction in membrane reactor also attempt to overcome the disadvantage of Colgate Emery and Twitchell method which involves high temperature and pressure reactors commonly used in industries. In addition, work is carried out to recover the phytochemical in CPO via precipitation process. This will ensure that all minor valuable products in CPO are not wasted but recovered from the process.

1.6 Objectives

The overall objective of this research is to investigate the parameters effecting hydrolysis reaction of crude palm oil in batch and enzymatic membrane reactor including the recovery of carotenes and tocopherol. The specific objectives are;

i. To study the effect of process parameters in batch configuration, optimization, kinetics and thermodynamics of lipase-catalyzed hydrolysis of CPO using free lipase.

ii. To design and fabricate an enzymatic membrane reactor (EMR) system suitable for hydrolysis of crude palm oil (CPO) using lipase-catalyzed reaction.

iii. To optimize the process parameters to improve the performance of EMR for hydrolysis reaction of CPO simultaneously with separation of fatty acids and glycerol and the kinetics of immobilized enzyme.

iv. To study the process parameters affecting the precipitation process for phytochemical recovery.

v. To develop and simulate a mathematical model taking into accounts the enzymatic reaction and mass transfer in the EMR unit and compared with experimental values.

1.7 Scope of Research

1.7.1 Design and fabrication of EMR

The current study starts with designing an enzymatic membrane reactor (EMR) system suitable for hydrolysis of crude palm oil (CPO). The system was checked by running with water and iso-octane before further studies can be carried out on the hydrolysis reaction of CPO.

1.7.2 Lipase-mediated hydrolysis of CPO

Hydrolysis of crude palm oil using *Candida rugosa* lipase for the production of fatty acids and glycerol was studied. Understanding the basic performance of free lipase is very important before it can be subjected to any improvement of lipase properties in the membrane system. Therefore, preliminary study in batch for various parameters on the behavior of *Candida rugosa* lipase was conducted before pursuing into the EMR system. In the screening stage, effect of different lipases, various organic solvent, aqueous-oil phase ratio, agitation speed and reaction time were investigated. Then, by using Design of Experiment (DOE) method, optimization for several crucial variables such as enzyme loading, oil concentration, temperature and pH was carried out and observed. The result was then used as a basis to carry out study in the enzymatic membrane reactor.

Enzymatic membrane reactor is used to enhance the performance of *C.rugosa* lipase in catalyzing the hydrolysis of oil. The main aim is to achieve the highest yield of fatty acids with minimum requirement of lipase usage and a milder working operation. The effect of enzyme loading for immobilization, oil concentration, flow rate for organic and aqueous phase, temperature, transmembrane pressure (TMP)

were studied in order to measure the extent of achievement of the developed *C.rugosa* immobilized enzymatic membrane reactor.

1.7.3 Phytochemical recovery

A precipitation process has been investigated to further purify the organic medium/ product stream from the EMR unit operation in order to evaluate the feasible amount of phytochemicals that can be recovered after the hydrolysis reaction using EMR system. In this part, fatty acids will be separated from other materials to ensure the product produced is high in purity and at the same time phytochemical can be collected. The studies of several affecting parameters (precipitation agent loading and temperature, agitation speed) were conducted to determine the optimum operating conditions for the phytochemical recovery in batch process.

1.7.4 Modeling of Hydrolysis Reaction in the EMR

The proposed model describes interfacial mass transfer of the enzymatic hydrolysis reaction. The models were coupled through mass balances at the respective confined region of study, i.e in the membrane matrix support. The number of parameters used in this mathematical model is reduced by dimensionless analysis. The attention was focused on the influence of significant dimensionless parameters, related to the system operating conditions, in order to predict the controlling transport on the reaction mechanism and the parameters that may optimize the reactor performance. The overall process study for batch and EMR is summarized in the flow chart as in Figure 1.2.

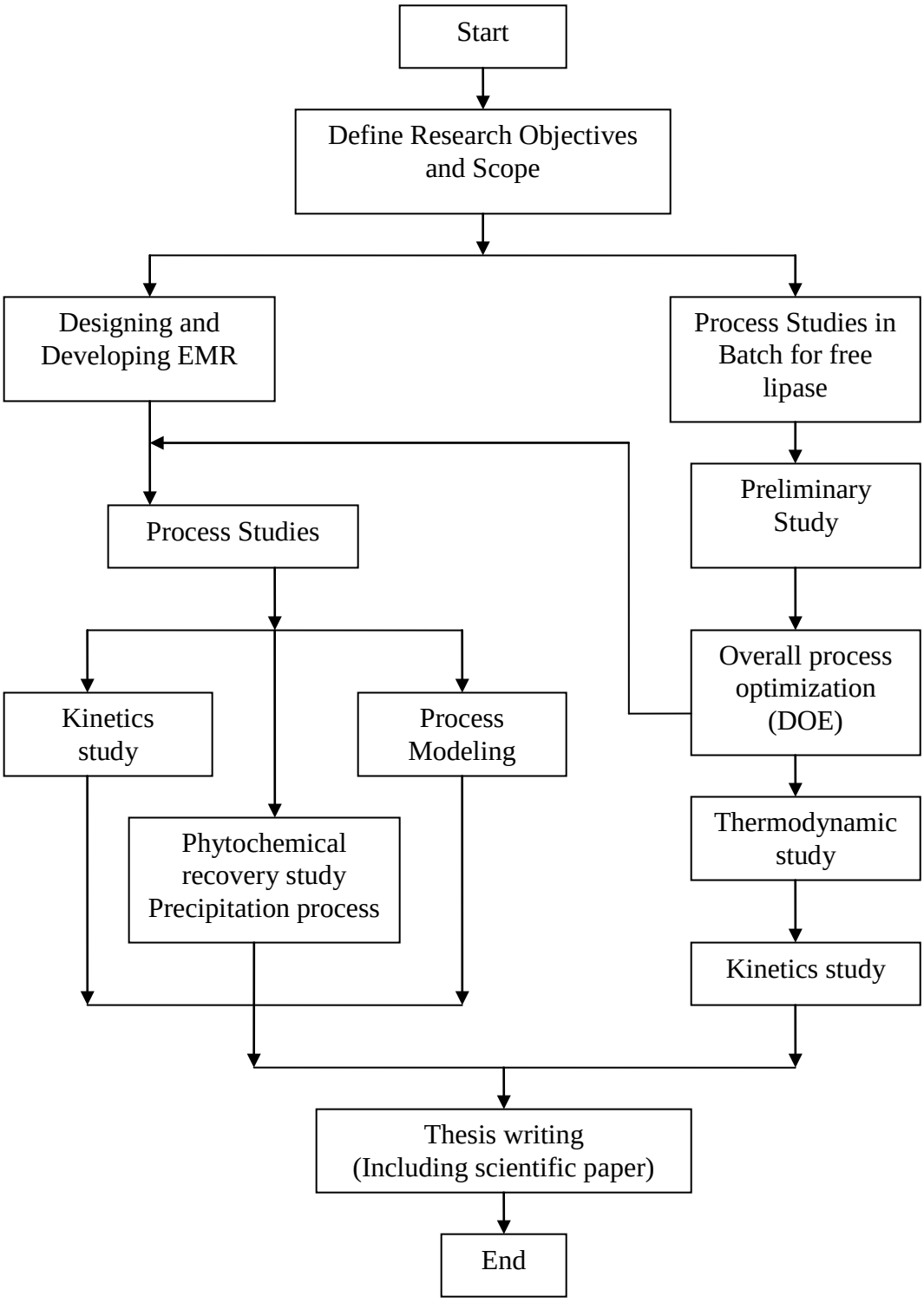

Figure 1.2: Research methodology flow chart.

1.8 Organization of Thesis

This thesis is divided into six chapters as follows:

Chapter 1 gives a brief introduction about palm oil and its availability to be one source of raw material in hydrolysis of fats especially in Malaysia. Hydrolysis products such as fatty acids and glycerol, overview of lipase and its current applications in industrial process and viability of enzymatic membrane reactor for current study are also highlighted. This chapter focuses on the problem statement and the objectives of the project.

Chapter 2 gives the information of palm oil processing and its products, properties of CPO and value added products such as tocopherols and carotenes and methods applied in the present days for the industrial production of fatty acids. It is followed by a discussion on potential using enzymatic reaction and the advantages of using immobilized lipase in hydrolysis process. Reviews on variables affecting hydrolysis reaction, statistical method for optimization, thermodynamics, kinetic, advantages of enzymatic membrane reactor and potential for recovery of phytonutrients are also recovered.

Chapter 3 describes the methods and analysis required for the hydrolysis process. It also gives details on the chemical requirements and equipment used throughout the whole process of this study. The overall experimental flowchart is also presented and discussed. The subsequent topics explain clearly the methodology of this research project, preliminary study using free lipase in batch process, optimization, thermodynamic and kinetics study, immobilized lipase in enzymatic membrane

reactor and phytochemical recovery. Finally, applied analytical methods and set up are also included in this chapter.

Chapter 4 presents the enzyme kinetics mechanism for hydrolysis of CPO. Explanation on the model formulations to predict the behavior of lipase-catalyzed hydrolysis of CPO in hollow fiber membrane reactor system is also included.

Chapter 5 presents the result obtained from experimental runs and discusses on every effect of parameters on the synthesis of fatty acids and glycerol using free and immobilized lipase and finally the recovery of tocopherols and carotenes. The end of this section discusses the model verification of the predicted model against actual experimental conditions.

Chapter 6 concludes the research project. Recommendations for future work related to this research project are also given.

CHAPTER 2

LITERATURE REVIEW

2.1 Crude Palm Oil

The oil palm is tropical perennial plant and grows well in lowland with humid places and therefore it can be cultivated easily in Malaysia (Ong *et al.,* 2011). Currently, 4.49 million hectares of land in Malaysia is under oil palm cultivation; producing 17.73 million tonnes of palm oil and 2.13 tonnes of palm kernel oil. Malaysia is one the largest producers and exporters of palm oil in the world, accounting for 11% of the world's oils and fats production and 27% of export trade of oils and fats. The oil palm is the most efficient oil-bearing crop in the world, requiring only 0.26 hectares of land to produce one tonne of oil while soybean, sunflower and rapeseed require 2.22, 2.0 and 1.52 hectares, respectively, to produce the same (The Oil palm tree, 2010). Oil palm tree will start bearing fruits after 30 months of field planting and will continue to be productive for the next 20 to 30 years of its life span of 200 years (Ong *et al.*, 2011). Thus, this will ensure a consistent supply of oils.

There are two main products produced by the oil palm fruit and they are crude palm oil (CPO) which is obtained from mesocarp and crude palm kernel oil (CPKO) from endosperm (kernel) (Ong *et al.,* 2011). CPO is deep orangey red in colour due to the high content of natural carotenes (500-700 mg/L) (Edem, 2002). Crude palm oil is one of the rich sources of carotenoids and vitamin E, which confers natural stability against oxidative deterioration. Palm oil consists mainly of glycerides made up of a range of fatty acids. Table 2.1 shows the fatty acid

composition in crude palm oil (CPO) produced by the Palm Oil Research Institute of Malaysia (PORIM) (Crabbe *et al.*, 2001).

Table 2.1: Quality characteristics of crude palm oil (CPO) (Crabbe *et al.*, 2001)

Parameters	**PORIM* specification**
Moisture (% w/w)	ND
Acid value (mg KOH/g)	ND
Fatty acid composition	
Lauric	0–0.4 %
Myristic	0.6–1.6 %
Palmitic	41–47%
Palmitoleic	0–0.6 %
Stearic	3.7–5.6 %
Oleic	38.2–43.5 %
Linoleic	6.6–11.9 %
Linolenic	0–0.5 %
Arachidic	0–0.8%
Mean molecular weight (g)	
Unsaturated fatty acids	44.8–57.3 %
Saturated fatty acids	45.3–55.4 %

ND- not determined *PORIM – Palm Oil Research Institute of Malaysia, 2010

Table 2.2: Composition of carotenes in CPO (Ng and Tan, 1998)

Carotene	**Composition**
Phytoene	1.27
Cis-β-carotene	0.68
Phytonefluene	0.06
β-Carotene	56.02
α-Carotene	35.16
Cis- α-Carotene	2.49
ζ-Carotene	0.69
γ-Carotene	0.33
δ-Carotene	0.83
Neurosporene	0.29
β-Zeacarotene	0.74
α-Zeacarotene	0.23
Lycopene	1.30

Table 2.3: Tocopherols and tocotrienols in CPO (Ooi, 1999)

Composition	**Concentration (ppm)**
α-tocopherol	279
γ-tocopherol	61
α-tocotrienol	274
γ-tocotrienol	398
δ-tocotrienol	69

Other than that, there are small amount of impurities in CPO. The average composition of carotenes in CPO is shown in Table 2.2. The concentration of carotenes in CPO can range from 400 to 3500 ppm depending on the species of oil palm (Ooi, 1999). The concentration of Vitamin found in CPO is shown in Table 2.3.

2.1.1 Valuable Nutrients of Crude Palm Oil

Palm oil also supplies important fat-soluble micronutrients like carotenoids including pro-vitamin A, vitamins D, E and K as well as very rich in calories. One gram of palm oil supplies 9 kcal of energy, which is 2½ times more than one gram of protein (4 kcal) or carbohydrates (4 kcal). The total carotenoids content in CPO are quite high as clearly depicted in Table 2.2. Vitamin A is an effective antioxidant that helps strengthening the body's immune system and reduces the risk of cancer, heart disease and cataract. Lack of vitamin A can lead to blindness and a variety of serious medical conditions (Health and Nutrition, 2010).

In addition, crude palm oil is also rich in vitamin E (tocopherols and tocotrienols) which is about 559 to 1000 ppm (Edem, 2002). Tocotrienols are members of the vitamin E family comprising of tocotrienols and tocopherols. Tocotrienols differ from the tocopherols in that they contain three double bonds in the side-chain (Figure 2.1). Tocotrienols isoprenoid side chain has three double bonds as compared to tocopherols saturated side-chain. In total, there are four type’s tocopherols namely alpha, beta, gamma and delta and four corresponding tocotrienols isomers (What are tocotrienols, 2010).

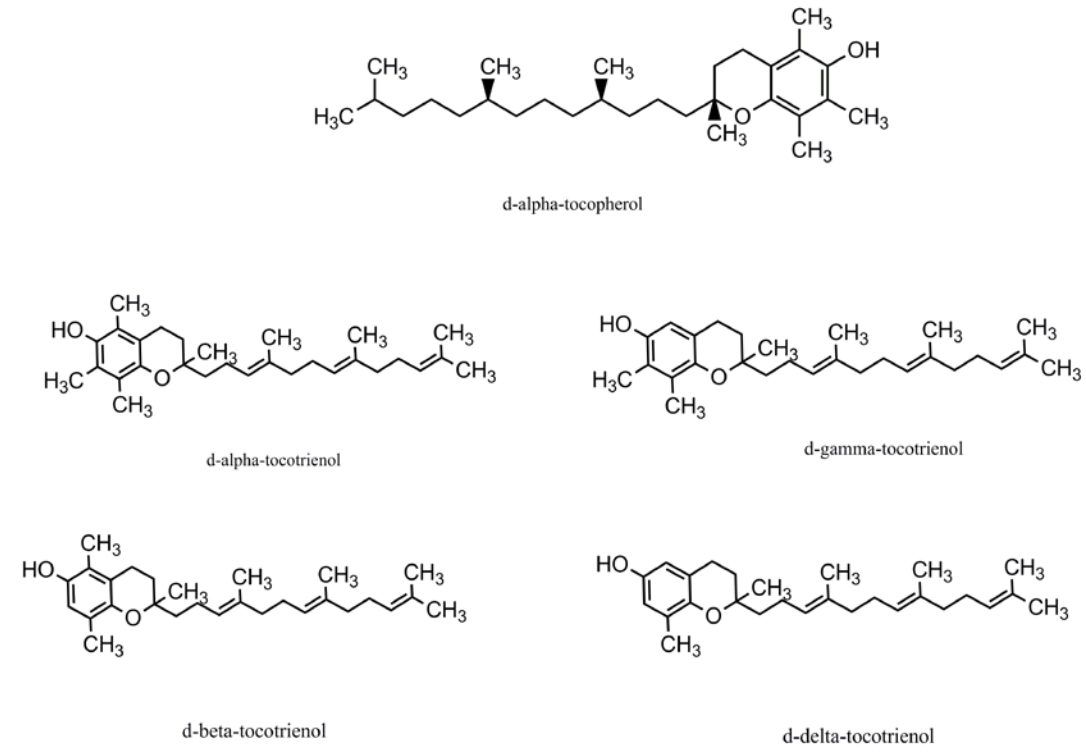

Figure 2.1: Molecular structures of tocopherol and tocotrienol isomers (Edem, 2002)

In fact, no other vegetable oils have as much vitamin E compared to palm oil. The tocotrienols have been reported to be natural inhibitors of cholesterol synthesis (Edem, 2002). Tocotrienols are surprisingly not found in any other vegetable oils such as; soy bean oil, canola oil, rape seed oil and sunflower oil. The compounds can be found naturally, but in much lesser quantities in rice barn, barley, wheat gem and oats. The vitamin E content in CPO ranges between 600 - 1000 parts per million (ppm) with a mixture of tocopherols (30%) and tocotrienols (70%) (Basiron and Weng, 2004). The major tocotrienols contain in palm oil are α-tocotrienols (22%), γ-tocotrienol (46%) and δ-tocotrienol (12%) (Edem, 2002).

2.2 Enzymatic Approach and Potential

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is an enzyme with many industrial applications in hydrolysis, alcoholysis, acidolysis, amidolysis, and inter-esterification (Pandey *et al.,* 1999; Li and Wu 2009). It is used in various fields such as food technology, detergents, beverages, cosmetics, biomedical and chemical industries (Li and Wu, 2009). Biocatalysts especially lipases are particularly useful for certain applications, specifically in terms of energy consumption, safety, pollution prevention and the high quality of products formed. However, the use of biocatalysts in industrial scale is yet to be fully established (Giorno and Drioli, 2000).

Rapid development of enzyme technology has brought considerable attentions to the application of lipase in fat and oil industries (Halling *et al.,* 1996; Chang *et al.,* 1999). Major industrial applications of enzymes are summarized in Table 1.1. Enzymatic reaction using lipase offers a lot of advantages over conventional chemical reaction. Lipases can be used effectively and economically under mild conditions (Sharon *et al.,* 1998). This is an important characteristic because extreme conditions could cause polymerisation of fat, forming by-product which cause difficulties during separation (Al-Zuhair *et al.,* 2003). Hence, the use of lipase could reduce the need to remove the by-products through further separation method such as the distillation process, which is an energy intensive process. In addition, recovery of valuable pyhtochemical in crude oil is possible because via enzymatic approach; hydrolysis process can be carried out at a considerably low temperature.

Table 2.4: Industrial applications of enzymes (Giorno and Drioli, 2000)

Type of industry	Enzyme	Application
Detergent	Protease	To remove organic stains
	Lipases	To remove greasy stains
	Amylase	To remove residues of starchy foods
	Cellulases	To restore a smooth surface to the fiber and restore the garment to its colours.
Food	Proteases and lipase	To intensify flavor and accelerate the aging process
	Lactases	To produce low-lactose milk and related products for special dietary requirements
Wine	β-Glucanases	To help the clarification process
	Cellulase	To aid the breakdown of cell walls
	Cellulase and pectinase	To improve clarification and storage stability
Fruit juices	Pectinases	To improve fruit-juice extraction and reduce juice viscosity
	Cellulase	To improve juice yield and colour of juice
Oil and fats	Lipases	The industrial hydrolysis of fats and oils or the production of fatty acids, glyceri, polyunsaturated fatty acids used to produce pharmaceuticals, flavours, fragrances and cosmetics.
Alcohol	α-Amylases	Liquefaction of starch or fragmentation of gelatinized starch
	Amiloglucosidase	Saccharification or complete degradation of starch and dextrins into glucose.
Starch and sugar	α-Amylases	Enzymatic conversion of starch to fructose: liquefaction, saccharification and isomerization Liquefaction of starch
	Glucoamylase and pullulanase	Saccharification
	Glucose isomerase	Isomerization of glucose.
Animal feed	β-Glucanases	The reduction of β-glucans
Brewing industry	β-Glucanase	The reduction of β-glucans and pentosans
Fine chemical	Lipases, amidases and nitrilases	Enantiomeric intermediates for drugs and agrochemicals Hydrolysis of esters, amides, nitriles or esterification reactions.
Leather	Lipases	To remove fats in the de-greasing process
Textiles	Amylases and cellulases	To produce fibers from less-valuable raw materials.
Pulp and paper	Xylanases	Used as bleaching catalyst during pre-treatment for the manufacture of bleached pulp for paper

Lipase also can be used as catalyst in both organic and aqueous phases and maintains its activity in organic solvents (Kazlauskas, 1994; Li and Wu 2009); however, its low stability, limits its potential applications in industrial hydrolytic

reactions (Sharma *et al.,* 2001; Villeneuve *et al.,* 2000). In addition, the application of lipase is still in its infancy due to its high cost (Kittikun *et al.,* 2000; Kaewthong *et al.,* 2005). This problem can be overcome by employing lipase in immobilized form, where the enzyme can be reutilized easily. Besides, immobilization of enzyme enables processes to be operated continuously. Furthermore, it can also increase the thermal stability enzymes. Several methods of enzyme immobilization have been reported such as adsorption, ionic binding, covalent binding, cross-linking, entrapment and encapsulation (Murty *et al.,* 2002, Li and Wu 2009). Murty *et al.,* (2002), reported that the use of immobilized lipase in several types of reactor configurations such as packed bed reactor (PBR), continuous stirred tank reactors (CSTR's), fluidized bed reactors, batch reactors and membrane reactors have been studied by researchers for the hydrolysis various types of oil rather than in free form. Therefore, it is believed that immobilized lipase has the potential in the present reaction process and need to be study comprehensively to maximize the catalytic activity of lipase especially in production of fatty acids.

2.3 Hydrolysis Process and Parameter Affecting the Reaction System

The factors, catalyst loading, substrate concentration, temperature, and pH value have significant effects on oil hydrolysis. Therefore, a brief description on each parameter is discussed in order to understand the catalytic activity of lipases in the hydrolysis of oil.

2.3.1 Enzyme Loading

An optimum amount of enzyme for use in a reaction is a crucial parameter especially those involving lipase. Too little enzyme would cause hydrolysis with low

conversion. Whereas, too much of enzyme would definitely give high conversion but with higher cost of production incurred. Certain enzymes are very expensive and they are only used in sufficient quantity based on type of reaction. The enzyme concentration is measured in LU (lipase unit). The effect of enzyme concentration to the degree of palm oil hydrolysis was normally studied in order to obtain the optimum amount. The main concern encountered by enzymatic hydrolysis is the fatty acids release during the process, which competes with the enzyme for the active-site and thus, leads to the reduction of the rate of hydrolysis. Hence, by adding more enzymes the hydrolysis rate will not be improved, as the rate of hydrolysis is dependent on the amount of product formed at a fixed interfacial area under specific operating conditions (Puthli *et al.,* 2006).

2.3.2 Oil Loading.

Another important parameter in hydrolysis of oil is the level of substrate concentration. Researchers reported that the inhibition of substrate was observed when oil concentration reaches a particular value (Noor *et al.,* 2003). In order to increase the rate of hydrolysis, researchers attempt by dissolving the fat in a water immiscible organic solvent and dispersed the solvent in the aqueous phase containing the enzyme (Hasan *et al.,* 2006). Okada and Morrissey (2007) also observed that the rate of hydrolysis of sardine oil by lipase from *C.rugosa* varied linearly with oil concentration. Many investigations only focus on the oil-aqueous ratio instead of the oil concentration. Lipase does not only able to catalyze hydrolysis reaction but also esterification too (reversible reaction). In the presence of low amount of water, catalytic activity may shift the thermodynamic equilibrium into preferring the esterification route. Therefore, higher quantity of water is

required for hydrolysis reaction to take place. Besides, lipase's activity in hydrolysis is known to increase with increasing water content. The phenomenon can be explained by oil-water interfacial activation of lipase and the content of water in the oil-phase. The active site of lipase is enclosed with a α-helix (named lid) of which the outer layer is hydrophilic and the inner is hydrophobic. When lipase is absorbed on the oil-water interface, the active site of lipase would be contacted with the oil-phase. This observation indicates that the reaction equilibrium is formed in the oil phase (Kobayashi *et al.,* 2008).

2.3.3 Temperature

Biocatalyst possessed several desirable qualities, but their thermal stability in many desired process formulations has hindered their applications. All enzymatic reactions are very sensitive to operating temperature and are only performed at their best at a particular temperature. When a protein applied in the presence of extreme temperature, irreversible denaturation of protein/enzyme will occur (Polizzi *et al.,* 2007). The thermal inactivation of enzymes explains such a behavior. Immobilized systems always favor higher optimum operating temperature compared to the free lipase systems. Enzymes are intrinsically labile, but temperature provides opposite effects on its stability, and reactivity becomes an important variable in any processes that includes enzymes as biocatalysts. The average temperature for hydrolysis of oils via various lipases was found to be at the range of 30^{o}C to 50^{o}C as shown in Table 2.5.