PRETREATMENT OF OIL PALM FROND BY *Phanerochaete crysosporium* CK01 AND ENZYMATIC HYDROLYSIS FOR ETHANOL PRODUCTION BY *Saccharomyces cerevisiae* HC10

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by

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In the name of ALLAH, the Most Gracious and Most Merciful,

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TABLE OF CONTENTS

CHAPTER 3 - MATERIALS AND METHODS **50**

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS

α	Alpha
β	Beta
%	Percentage
˚C	Degree Celsius
&	And
=	Equal
+	Plus
-	Minus
/	Divide
<	Less than
x	Multiply
µ	Specific Growth Rate
CH_3COOH	Acetic Acid
CO	Carbon Monoxide
CO_2	Carbon Dioxide
H_2SO_4	Sulphuric Acid
HCl	Hydrochloric Acid
He	Helium
KH_2PO_4	Potassium Dihydrogen Phosphate
$MgO_4S \cdot 7\ H_2O$	Magnesium Sulphate Heptahydrate
Na_2CO_3	Sodium Carbonate
$NaClO_2$	Sodium Chlorite
NaOH	Sodium Hydroxide
NO_x	Nitrogen Oxides
O_2	Oxygen

td	Doubling Time
$Y_{p/s}$	Product Yield Coefficient
$Y_{x/s}$	Biomass Yield Coefficient

LIST OF ABBREVIATION

µm	Micrometer
ACE	American Coalition for Ethanol
AFEX	Ammonia Fibre Explosion
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
B.C.	Before Century
B10	10% biodiesel-blend fuel
B5	5% biodiesel-blend fuel
B7	7% biodiesel-blend fuel
C	Carbon
cm	Centimetre
CMC	Carboxymethyl cellulase
CO	Carbon Monoxide
CO_2	Carbon Dioxide
DBKL	Dewan Bandaraya Kuala Lumpur
DNS	3,5-dinitrosalicylic acid
E-10	10% ethanol-blended gasoline
E-85	85% ethanol-blended gasoline
EFB	Empty Fruit Bunches
EISA	Energy Independence and Security Act
EMP	Embden-Meyerhof-Parnas
ETBE	Ethyl Tertiary Butyl Ether
EU	European Union

FELDA	Federal Land Development Authority
FFB	Fresh Fruit Branch
FFVs	Flexible Fuel Vehicles
g	Gram
g/L	Gram per Litre
g/L/h	Gram per Litre per Hour
GC	Gas Chromatography
GDP	Gross Domestic Product
GISS	Goddard Institute for Space Studies
h	Hour
h^{-1}	per Hour
HPLC	High Performance Liquid Chromatography
JIS	Japan Industrial Standard
Kg	Kilogram
kPa	Kilopascal
L	Litre
M	Molar
MDF	Medium Density Fibreboard
mg	Miligram
MINDEF	Malaysian Ministry of Defence
mL	Millilitre
mm	Millimetre
mmol	Millimole
MPa	Megapascal
MPOB	Malaysian Palm Oil Board

MPOC	Malaysian Palm Oil Council
OD	Optical Density
OH	Hydroxyl
OPEC	Organization of the Petroleum Exporting Companies
OPF	Oil Palm Frond
OPFH	Oil Palm Frond Hydrolysate
OPFJ	Oil Palm Frond Juice
OPT	Oil Palm Trunk
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PKC	Palm Kernel Cake
PKS	Palm Kernel Shell
PMSF	Phenyl Methane-Sulfonyl Fluoride
POME	Palm Oil Mill Effluent
POMW	Palm Oil Mill Waste
PORIM	Palm Oil Research Institute of Malaysia
PORLA	Palm Oil Registration and Licencing Authority
PPF	Palm Pressed Fibre
RFS	Renewable Fuel Standard
rpm	Revolution per Minute
RTFO	Renewable Transport Fuel Obligation Programme
SIRIM	Standard and Industrial Research Institute of Malaysia
SmF	Submerged Fermentation
SSF	Solid State Fermentation
TAPPI	Technical Association of the Pulp and Paper Industry

U/g	amount of enzyme that produce 1µmol substrate per minute
US	United States
USM	Universiti Sains Malaysia
UV	Ultraviolet
v/v	Volume per Volume
w/v	Weight per Volume
WRF	White-rot Fungi

PRARAWATAN PELEPAH KELAPA SAWIT OLEH *Phanerochaete crysosporium* CK01 DAN HIDROLISIS ENZIMATIK UNTUK PENGHASILAN ETANOL OLEH *Saccharomyces cerevisiae* HC10

ABSTRAK

Pelepah kelapa sawit (PKS) merupakan satu daripada bahan buangan yang paling banyak didapati daripada ladang kelapa sawit. PKS boleh digunakan sebagai bahan mentah untuk penghasilan bioetanol dan dalam masa yang sama dapat menangani isu pembuangannya. Dalam projek ini, PKS telah dibahagikan kepada dua jenis bahan mentah; biojisim dan jus dengan menggunakan pemerahan secara mekanikal. Permulaannya, biojisim yang kering telah dirawat dengan menggunakan kulat reput-putih; *Phanerochaete chrysosporium* CK01 untuk biodelignifikasi. Dua parameter telah diuji; kesan saiz inoculum dan tempoh fermentasi. Parameter terbaik yang diperolehi adalah saiz inokulum sebanyak 1.0×10^6 spora/mL dan dalam tempoh fermentasi selama 3 minggu telah memberi delignifikasi sebanyak 27.87%. Pada peringkat kedua, biojisim yang telah dirawat tadi, dihadapkan kepada hidrolisis enzimatik secara individu atau gabungan untuk menghasilkan hidrolisat yang bergula tinggi. Dua parameter diuji adalah kandungan enzim (dalam hidrolisis enzimatik secara individu) atau nisbah enzim (dalam hidrolisis enzimatik secara gabungan; ; Cellulase A "Amano" 3 dan Hemicellulase "Amano" 90) dan tempoh hidrolisis. Keputusannya menunjukkan bahawa hidrolisis enzimatik gabungan dapat menghasilkan jumlah gula yang lebih tinggi (5.15g/L) dengan nisbah enzim sebanyak 1:4 (selulase:hemiselulase) bersama tempoh hidrolisis selama 120 minit berbanding dengan hidrolisis enzimatik individu (3.26g/L). Sebelum permulaan peringkat yang

ketiga, jumlah gula dalam hidrolisat (HPKS) dan jus (JPKS) telah dibandingkan dengan menggunakan analisis Kromatografi Cecair Prestasi Tinggi (HPLC). JPKS telah didapati sebagai medium yang lebih baik dan telah dipilih untuk difermentasi di peringkat yang seterusnya. Dalam peringkat ketiga, fermentasi JPKS telah dijalankan dengan menggunakan dua jenis yis iaitu *Saccharomyces cerevisiae* HC10 dan *Scheffersomyces stipitis* ATCC 5837. Perbandingan antara dua fermentasi ini menunjukkan bahawa *S. cerevisiae* HC10 mampu menghasilkan sebanyak 12.20% (v/v) etanol, ialah agen perfermentasian yang lebih baik berbanding *S. stipitis* ATCC 5837 yang menghasilkan hanya sebanyak 9.75% (v/v) etanol. Penggunaan gula individu oleh setiap perfermentasian menunjukkan bahawa keupayaan yis untuk menggunakan heksosa dalam JPKS adalah penting untuk menghasilkan jumlah etanol yang lebih tinggi dengan cekap. Seterusnya, perfermentasian *S. cerevisiae* HC10 dalam JPKS telah dibandingkan dengan fermentasi dalam media Potato Dextrose Broth yang diubahsuai untuk mengkaji kinetic pertumbuhan. Hasil keseluruhan etanol (kecekapan penapaian) yang diperolehi adalah 35.26% dalam perfermentasian JPKS dan 44.99% dalam media PDB yang diubahsuai, sekaligus menunjukkan bahawa walaupun JPKS boleh difermentasikan tanpa sebarang pengubahsuaian atau nutrient tambahan, perfermentasian tidak begitu berkesan jika dikomersialkan untuk pengeluarkan bioetanol. Kesimpulannya, projek ini telah berjaya untuk merungkai potensi baik pelepah kelapa sawit sebagai bahan mentah untuk perfermentasian.

PRETREATMENT OF OIL PALM FROND BY *Phanerochaete crysosporium* CK01 AND ENZYMATIC HYDROLYSIS FOR ETHANOL PRODUCTION BY *Saccharomyces cerevisiae* HC10

ABSTRACT

Oil palm frond (OPF) is one of the most abundant waste generated from an oil palm plantation. OPF can be utilized as a feedstock for bioethanol and can simultaneously address its disposal issue in the plantation. In this project, OPF was divided into two types of feedstock; biomass and juice by mechanical pressing. Firstly, the dried biomass was biologically treated using white-rot fungi; *Phanerochaete chrysosporium* CK01 for biodelignification. Two parameters were tested; effect of inoculum size and fermentation duration. The best parameters were inoculum size of $1.0x10^6$ spore/mL and 3 weeks of fermentation duration which gave 27.87% of delignification. In the second stage, the treated biomass was subjected to enzymatic hydrolysis by either individual or in combination to produce a sugar hydrolysate. Two parameters tested were enzyme loading (in individual enzymatic hydrolysis) or enzyme ratio (in combined enzymatic hydrolysis; Cellulase A "Amano" 3 and Hemicellulase "Amano" 90) and hydrolysis time. The result shows that the combined (cellulase and hemicellulase) enzymatic hydrolysis produces higher amount of simple sugar (5.15g/L) using a combination of enzyme ratio of 1:4 (cellulase:hemicellulase) with a hydrolysis time of 120 minutes compared to individual enzymatic hydrolysis (3.26g/L). Prior to beginning of the third stage, the amount of simple sugar in the hydrolysate (OPFH) and juice (OPFJ) were compared using high performance liquid chromatography (HPLC) analysis. OPFJ were found to be the better medium and thus be chosen for fermentation in the final stage. In the third stage, OPFJ fermentation was done using two types of yeast which are *Saccharomyces cerevisiae* HC10 and *Scheffersomyces stipitis* ATCC 5837. The comparison between the two fermentations show that *S. cerevisiae* HC10 which managed to produce 12.20% (v/v) ethanol, is a better fermentation agent compared to *S. stipitis* ATCC 5837 which produced 9.75% (v/v) ethanol. The individual sugar utilization by each fermentation shows that the ability of the yeast to ferment hexoses in the OPFJ is crucial to produce a higher amount of ethanol efficiently. Later, *S. cerevisiae* HC10 fermentation on OPFJ was compared with a fermentation on a modified PDB media to study the growth kinetics. An overall ethanol yield (fermentation efficiency) obtained was 35.26% in the OPFJ fermentation and 44.99% in modified PDB media respectively, suggesting that although OPFJ can solely be fermented without any modification or additional nutrient, the fermentation was not highly efficient if it were to be commercialised for production of bioethanol. In conclusion, this project has managed to unveil the good potential of oil palm frond as fermentation feedstock.

CHAPTER 1: INTRODUCTION

1.1 Introduction

Unstable oil prices, gradual depletion of fossil fuel sources and increased greenhouse gas emissions are the major factors that stimulated the global efforts in finding a sustainable alternatives way to replace fossil fuels (Fasahati *et al*., 2015). The conversion of plant (lignocellulosic) material into biofuels and biochemical has gained attentions due to the feasible alternative processes available to convert the complex biomass into these desired products (Chaturvedi and Verma, 2013). There were a variety of materials that have been used for the biofuel production previously whereby each group of materials were categorized from 'first generation' biofuel to the 'fourth generation' biofuel.

The 'first generation' bioethanol are produced using raw materials containing simple sugars or starches which are mainly came from consumable resources (Lee and Lavoie, 2013). On the other hand, 'second generation' bioethanol are those that are mainly produced using non-consumable sources such as lignocellulosic (Lee and Lavoie, 2013). Another category of bioethanol is known as the 'third generation' bioethanol. These bioethanol are produced from algal biomass which has a very distinctive growth yield as compared with the second generation bioethanol (Brennan and Owende, 2010). Finally, the 'fourth generation' biofuel are identified as photobiological solar fuels and electrofuels (Aro, 2016). The principle behind this infant-staged idea is that through synthetic biology, solar energy will be directly converted into fuel (Aro, 2016).

Lignocellulosic biomass was chosen as one of the preferred materials to be used for bioethanol production in Malaysia because of the country's global-scale palm oil plantation industry. With huge oil palm plantation area, comes high generation of wastes or by-products. The palm oil mill waste (POMW) can be divided into 2 categories; liquid-based waste and solid-based (biomass) waste (Lorestani, 2006). palm oil mill effluent (POME) is the liquid-based waste (Lorestani, 2006) while others such as empty fruit bunches (EFB), palm pressed fibres (PPF), oil palm trunk (OPT), oil palm fronds (OPF) and shells are biomass wastes. Around 51 % of these wastes are come from OPFs (Abdullah and Sulaiman, 2013). The disposals of OPF are either done by natural-decaying process or by on-site burning. The distinctive drawback is that the natural-decaying process consumes time and does not effectively decompose the whole OPF. In addition, on-site burning of the OPF is a clear violation of environmental law. Thus, alternative ways to utilize and/or dispose OPF are needed.

Nowadays, OPF waste has attracted researcher's attention for utilizing the source as feedstock for bioethanol production (Jamaludin, 2010). However, only a few studies have been focusing on utilizing the lignocellulosic components of OPF (Lim *et al*., 2012). Almost all plant materials are composed of cellulose, hemicelluloses and lignin. Cellulose and hemicelluloses are the sugar component that can be hydrolysed into simple sugars for bioethanol fermentation. On the other hand, lignin is a complex, aromatic polymer (Crawford and Crawford, 1976) that does not possess any fermentative value. Generally, lignin is responsible for the overall protection of the plant cell in such a way that it provides mechanical strength, resistance toward water permeation across cell, and protection against pathogen attack (Crawford and Crawford, 1976). Due to its protective nature for the plant cell, lignin somehow prevents the hydrolysis of sugars by preventing the access of enzyme into cellulose and hemicellulose. Therefore, pretreatment steps are required for lignin removal.

There are a wide range of pretreatment techniques that can be used, for example, physical, chemical, physicochemical and biological pretreatment. In biological pretreatment, white-rot fungus is preferred since the fungus produces ligninolytic enzymes that are effective for lignin degradation. Specific enzymes such as cellulase and hemicellulase are often used to enhance the hydrolysis of cellulose and hemicellulose to simple sugars for fermentation. Hydrolysate; the liquid derived from the enzymatic treatment of pretreated biomass can be fermented by microbiological agent such as yeast and bacteria. Yeast known as *Saccharomyces cerevisiae*, is notable for its application in bioethanol fermentation due to its robustness and rapid rates of ethanol production (Stanbury *et al*., 1995). *Saccharomyces cerevisiae* are mainly used to convert 6-carbon sugars to ethanol. Another group of yeast such as *Scheffersomyces stipitis* have been widely used to ferment 5-carbon sugars. Therefore, the main objective of this study was to utilise oil palm frond for bioethanol production. Oil palm frond can be divided into two types of feedstock which will contain different compositions of sugar. Hydrolysate resulting from the treatment of oil palm frond biomass will contain a lot of 5-carbon sugars. Meanwhile, oil palm frond juice may contain an abundance of 6-carbon sugars. Thus, two types of yeast as mention previously are chosen to ferment these two types of feedstock to produce bioethanol.

1.2 Research Background and Objectives

In this project OPFs were harvested from a private local plantation in Bukit Minyak, Penang. The fronds were pressed using a conventional sugarcane pressing machine for its juice and biomass fibre. The biomass fibre was subjected to biological pretreatment followed by an enzymatic hydrolysis to produce a liquid medium; OPF hydrolysate. In the biological pretreatment, white rot fungi known as *Phanerochaete chrysosporium* CK01 was used for delignification. Then, commercial enzymes such as cellulase and hemicellulase were used to hydrolyse the treated biomass for a high-sugar hydrolysate production. Next, the hydrolysate (OPF hydrolysate) and juice (OPF juice) collected earlier were analysed for their sugar composition and overall amount of fermentable sugar. In the final stage, a submerged fermentation by two types of yeast: *Saccharomyces cerevisiae* HC10 and *Scheffersomyces stipitis* ATCC5837 was done using a liquid medium derived from either the OPF juice or OPF hydrolysate to produce bioethanol. Fermentation by two types of yeast were compared and the better yeast was identified. Growth kinetics for the better yeast was calculated to gain better understanding of the fermentation efficiency.

This project comprises of four objectives which were:

1. To study the biological pretreatment of OPF biomass for biodelignification using white rot fungi; *Phanerochaete chrysosporium* CK1 by studying two factors which are fermentation duration and inoculums size.
2. To study the effect of enzymatic hydrolysis on the resulting pretreated OPF biomass using commercial enzyme such as Cellulase A "Amano" 3 and Hemicellulase "Amano" 90 (Amano, Japan) by either individually or in combination for the production of reducing sugar.
3. To identify the best yeast (*S. cerevisiae* HC 10 or *S.stipitis* ATCC 5837) for ethanol fermentation of OPF juice and further investigate the growth profile and kinetics of the selected yeast.

CHAPTER 2: LITERATURE REVIEW

2.1 Biofuel

A simple definition of biofuel can be stated as an energy source derived from organic material. It differs from fossil fuel in such a way that biofuel is produced in a shorter period of time (days to months) as compared to fossil fuel which take millions of years to be made. Due to relatively shorter duration of time taken to produce this energy, biofuel is categorized as one of the renewable energy resources; non-depletable forms of energy (Kovács, 2001). Biofuel is a form of biomass energy. Other forms of renewable energy resources include solar energy, geothermal energy, wind energy, hydro energy etc. Biofuels are classified into technology generations namely; first generation, second generation, third generation, and fourth generation. Classification were based on feedstock, technological advancement used to produce the respective biofuel (Aro, 2016; European Academies Science Advisory Council, 2012), and the carbon balance of the overall process (Biopact, 2007).

2.1.1 First to Fourth Generation of Biofuel

First generation biofuel is produced from edible sources of crop plants such as sugars from sap or juices, starches, and oils. Specifically, bioethanol is produced from the fermentation process of sugars or starches while biodiesel is produced from conversion of oils by transesterification process (Biopact, 2007). The first generation biofuel was hugely explored until the food security issue was raised. Increasing demand for food to meet the needs of a growing global population suggest that it should no longer be used in biofuel production. In fact, a recent study stated that the crop calories used for biofuel production in 2013 can be used to fed approximately one fourth of the malnourished population at that time (Rulli *et al*., 2016). Despite the raised issue, production of first-generation biofuel still dominates the global biofuel supply (Rulli *et al.*, 2016). This phenomenon might be due to its lower technological complexity for production compared to other generations of biofuel. Realizing the issue in food security, research around the world moves towards utilizing non-edible resources for biofuel; the second generation biofuel.

For second generation biofuel, the feedstock used includes lignocellulosic biomass such as grasses, forestry waste, straw, husks, plant sap etc. Purpose-grown energy crops such as vegetative grasses and short rotation forests also belongs to the feedstock of second generation biofuel (Aro, 2016). The second generation biofuel uses the same technology in the first generation biofuel production with little to no significant technological stride. Nevertheless, it does known to be carbon-neutral (net carbon dioxide produced is same as consumed) which makes the carbon balance to be overall acceptable. Some of the major drawbacks of second generation biofuel production are high overall cost needed for pre-treatment of lignocellulosic material and its debatable energy-conversion efficiency. Currently, research on secondary biofuel are still ongoing to optimize the production; focusing on maximising the amount of renewable carbon and hydrogen that can be converted to fuels from the feedstock (Aro, 2016) and the fermentation process itself.

Next, the definition of third generation biofuel does not really seems to be exact up until today. Most references define third generation biofuel as algae-based while some literatures (Biopact, 2007; Renewable Energy Index, 2010) define it as those that are produced from genetically modified crops. It can be concluded that the third generation biofuel is composed of these two types of feedstock. The reason behind most sources defined it as algal-based biofuel is due to majority of the feedstock both in research and implementation comes from algal-based. Many potential pathways exist for the conversion from algal biomass to different type of biofuel; biodiesel, biogas, and bioethanol. The pathways can be classified into the following three general categories depending on the type of biofuel produced: (1) algal extracts such as lipids and carbohydrates are processed to produce either biodiesel or bioethanol (Alaswad *et al*., 2015; Gouveia, 2011), (2) the whole algal biomass are subjected to anaerobic fermentation to produce biogas (Alaswad *et al*., 2015; Gouveia, 2011) and (3) direct production of biofuel; bioethanol and biogas from algae without the need for extraction (Gouveia, 2011). As compared to previous biofuel-generation, the third generation biofuel are known to give a higher energy-conversion efficiency than in land plants (Aro, 2016). Research are still ongoing for improving both the metabolic production of fuels and the downstream separation processes to lower the production cost (Aro, 2016; Medipally *et al*., 2015).

Nowadays, new technology arises in the field of biofuel research. The fourth generation biofuel is said to be very energy-efficient that it could be the game changer to the industry itself. Similar to the third generation, the exact definition of this biofuel seems blurry. During the emergence of this new technology (in the 2000's), the fourth generation biofuel was known to possess the same second and third generation processing technology but differs in such a way that it involves genetic modification of the feedstock (Biopact, 2007; Dutta *et al*., 2014). Currently, a published journal by Aro in 2016 defines the fourth generation biofuel as those that uses direct solar and synthetic biological technique to produce biofuel. There are 3 ways of producing this biofuel: (1) by using specially designed photosynthetic microorganism to produce photo biological solar fuel, (2) by combination of photovoltaic and electrobiofuel technology, and (3) by synthetic cell organelle that are specially created or modified to produce biofuel. Apart of all the definitions, the common ground is that this generation of biofuel does offers much higher energy-conversion efficiency and is carbon-negative (net carbon dioxide produced is less than as consumed) (Renewable Energy Index, 2010).

2.1.2 Bioethanol and Other Types of Biofuel

Ethanol is an alcohol that has been a major liquid biofuel used in current days. An ethanol derived from biomass raw materials is known as bioethanol (Detchon, 2006). Ethanol can be produced using bioprocess method which is known as fermentation. In general, fermentation is a process whereby sugars are enzymatically converted into ethanol by some microorganisms under an anaerobic condition. Yeasts for example *S. cerevisiae*, are among the most frequently used in the process due to its safety and high efficiency in fermenting sugars to ethanol (El-Mansi *et al.*, 2009). In addition to that, sugars are needed as the main nutrient for fermentation apart from other nutrient to support the growth of *S. cerevisiae.* The research efforts to find renewable source for fuels are vital to ensure continuity of our highly industrialized world. Apart from fermentation, ethanol can also be produced synthetically by gasification process whereby ethene is reacted with steam with phosphoric acid as catalyst. However, this method is not environmentally friendly, and the production is based on non-renewable resources.

Apart from bioethanol, other biofuels such as biodiesel, biogas and biobutanol are also considered to be a renewable energy source. However, they all differ in how they are produced, their chemical and physical properties, and usage intention. A biodiesel is produced mainly from crops with seeds that contain oil, used cooking oil and animal fats (Detchon, 2006). They are used as substitute to diesel as a pure fuel or sometimes blended with petroleum for diesel engine. Biogas on the other hand, exists in gaseous state. They are produced anaerobically. Commonly they are referred to as methane although there are other gases constituents that exist in small amount such as hydrogen and carbon monoxide (Biogas Renewable Energy, 2009). Biogas is mainly used for energy production due to its combustibility, thus it is used to powered vehicle, lighting as well as cooking. Other than that, recently, biobutanol has been added into the list as one of the renewable fuel. Biobutanol is made from the same feed stocks as bioethanol but with a modification in the fermentation and distillation process (Detchon, 2006). Instead of having two carbon atoms as in bioethanol, biobutanol possess four carbon atoms, thus it can produce more energy than bioethanol. Currently, biobutanol is used as fuel blended with gasoline. One of the advantages of biobutanol over bioethanol is that biobutanol can easily blend with diesel (Hal Bernton, 2007) thus making biobutanol to be a more versatile fuel.

2.2 Important Applications of Bioethanol

Bioethanol has been applied in various fields nowadays ranging from being burnt for power generation up to being consumed everyday around the world. Fuel is one of the main applications of ethanol. "Flexible-fuel vehicles (FFV) are vehicles that can run on both ethanol-blended gasoline (E-10 and E-85) as well as pure gasoline. The term "flexible" refers to the ability of the engine to run on pure gasoline whenever ethanol-blended fuel is unavailable. "Because E85 is not widely available, the FFVs were designed to work efficiently on gasoline as well" (Detchon, 2006). In Brazil, more than 80% of all new cars sold are FFVs that are designed for capability to run on 100% ethanol (He, 2013). Studies claims that ethanol-blended gasoline helps improve the fuel combustion by aiding a more complete burning thus reducing the carbon monoxide emissions that are toxic to human (Guerrieri *et al.*, 1995). Other tailpipe emissions from combustion of fossil fuels such as gasoline are carbon dioxide (CO_2), hydrocarbons, and nitrogen oxides (NO_x). CO_2 has been synonym with global warming as they are one of the major greenhouse gases nowadays thus it is vital for us to start reducing the emission of CO_2 wherever possible. Using the ethanol-blended fuel is one of the best ways to counter this problem.

Unsurprisingly, bioethanol has been globally applied in our daily life. Other than in the fuel industry, bioethanol is also produced for beverages and other applications. Since its discoveries decades ago, its application was mainly for beverages. On the early days, ancient starts to make fermented beverages such as wine from grapes. Archaeologists believe that wines and beers have existed for more than 10,000 years. Around 4000 B.C. the Sumerian was the first known civilization to produce alcoholic beverages. Various types of alcoholic beverages known today are difference in their ethanol content as well as the raw material used for brewing. Alcoholic beverages with lower ethanol content are usually referred to as fermented alcoholic beverages, meanwhile those with higher (more concentrate) amount of ethanol are called as distilled alcoholic beverages. Some of the examples of fermented alcoholic beverages that can be differentiated from their feedstock are wine, beer, soju, and toddy. Wine which includes red and white wine is generally made from fermented grapes. Meanwhile, beer and its derivatives such as ale and wheat beer are fermented alcoholic beverages from the fermentation of grains such as barley and wheat respectively. Apart from that, soju which are common among the Koreans, are fermented from vegetable; sweet potato. On the other hand, alcoholic beverages can also come from other sources such as plant sap. Toddy for example, is fermented from palm sap.

Ethanol application as solvent has been covering a much broader spectrum nowadays in various industries. They key to its ability as a good solvent lies on its chemical properties. Since ethanol has both polar end (OH group) as well as non-polar end (hydrocarbon chain), thus it can be a good solvent for both polar and non-polar substances. In cosmetics industry, most of the personal care products such as fragrance, deodorant, and hairsprays contain ethanol (American Coalition For Ethanol). Meanwhile in food industries, colourings and flavourings are mainly polar in nature, thus ethanol can be used as a substitute to water. Besides, ethanol also has been applied in the pharmaceutical field. Drugs can be polar and non-polar, a whole spectrum of medicines including cough treatments, decongestants, iodine solution, and many others uses ethanol as the solvent (American Coalition For Ethanol). Other industry also uses ethanol as a solvent in manufacturing of many substances such as paints and explosives (American Coalition For Ethanol).

2.3 The Need for Biofuel (Bioethanol)

The phenomenon of climate mitigation, and energy security has led most of the developed countries; United States and European Union to issue a mandate for blending of bioethanol into petroleum fuel which triggers the growing demand of bioethanol (Larson, 2006). By the end of year 2020, the European Union (EU) has aimed to reduce greenhouse gases by 20 to 30% (Lewis, 2008). United Kingdom has launched the Renewable Transport Fuel Obligation Programme (RTFO) policy in 2008; the fuel suppliers must ensure that a certain percentage of the total sales are from biofuel (Department for Transport, 2012; International Energy Agency, 2015b; Lewis, 2008). Biofuel sales to transportation company must be at a minimum of 2.5% in year 2008 to 2009 and has increased to a newly proposed 6% in year 2017 to 2018 (United Kingdom Department for Transport, 2017). Meanwhile, Germany has introduced an act; Biofuels Quota Act (Biokraftstoffquotengesetz), which basically introduces a minimum use of biofuels in road transportation in the country (International Energy Agency, 2015a). The Finance Committee of the German Parliament has increased the domestic biofuels quota from 6.25% in 2009 to 8% from 2015 onwards (Nations, 2008). In the United States, an act was issued in 2007; Energy Independence and Security Act (EISA) and a modified Renewable Fuel Standard (RFS) was introduced (International Energy Agency, 2017; Lewis, 2008; U.S. Congress, 2007). The minimum standard level of biofuel to be used in transportation sector set by RFS has risen from 9 billion gallons in 2008 to 36 billion gallons in 2022 (International Energy Agency, 2017; Lewis, 2008; U.S. Congress, 2007).

Currently, ethanol is used as the vehicle fuel in two ways which are as blend with unleaded petrol (E-10 Unleaded), and as an additive for petrol called ethyl tertiary butyl ether (ETBE). Apart from government policies, increasing number of flexible-fuel vehicle (FFV) is one of the factor that drives the global bioethanol demand. Evolution of technology has offered us with the idea of using an electric car to solve the global issues on climate mitigation and energy security. But although the idea of using electric car is emerging, but it is very unlikely that 100 percent of electric car to be used by year 2020 (United Kingdom Department of Transport, 2016).

Another factor that might increase the global bioethanol demand is the volatile nature of the global oil price. The global oil price basically depends on the tide of supply and demand. When a nation is growing, it consumes more oil than ever. Oil are mainly used for industries and transportation. In 2000’s (particularly in 2008), the oil prices shockingly rise. One of the reasons behind the rise is due to very high consumption by the United State, China, India, Japan and Russia (United State Energy Information Administration, 2015). As a nation grows, it becomes highly industrialized and with such scale, the transportation sector blooms. Realizing this, from 2003 to 2008 the Organization of the Petroleum Exporting Countries (OPEC) increases the oil price to economically benefit their members (Khusanjanova, 2011) thus, the whole world faced high oil price crisis. Another key contributor to the unstable global oil price is the occurrence of war. War on middle east greatly affects the global oil price (Jones, 2012). In 2003, the invasion of United State in Iraq has causes the OPEC (which the members are mainly from Arab League) to raise the oil price as an indirect act of protest (Cleveland, 2009).

On the other hand, the global drop in oil price also gives negative impact. In 2015, the world witnessed the decrease in oil price. Surprisingly, the phenomenon did not benefit most of the oil-importing countries since most of the nations were facing deleveraging and balance-sheet repair (Cerdeiro and Plotnikov, 2017). The 2015 global drop in oil price had shown that developed countries with advance economies survived the price shock but negative effects were faced by developing economies (Cerdeiro and Plotnikov, 2017). These suggests that adopting biofuel such as bioethanol as an alternative energy source for both developing and under developed countries is one of the best way for the nations to survive the unstable waves of global oil price.

There is a huge opportunity awaits other nations to invest on the production of bioethanol. Investing in bioethanol industry is advantageous not only will it benefit the country in exporting the fuel, but also good for domestic usage by reducing the dependency (import) of fuel from other country. For example, in 2004 Guatemala has started to produce bioethanol form molasses to reduce its national expenditure on petroleum import (Lewis, 2008). Today, Guatemala’s petroleum import has been gradually decreasing; from 2868.1 to 2970.8 and 2016.0 million US Dollar from year

2014 to 2016 respectively (United Nation Statistic Division, 2016). In addition to that, since 2010, Canada has been moving towards bioethanol industry and managed to produce about 2 percent of the global bioethanol production (Rachel Gantz, 2016). However according to a report, the nation will not produce its bioethanol in excess for international export market, rather it was merely to reduce their dependency on foreign oil (Dessureault, 2016). Statistics shows that Canada has manage to decreased its bioethanol import around 100,000 million litres from year 2014 to 2015 and 2016 (Dessureault, 2016).

Apart from economic advantages, bioethanol industry also contributes to social sector. The industry opens opportunity for employment and economic growth in rural area. In Brazil, sugarcane bioethanol industry employs approximately 1 million workers (Moreira, 2005). Meanwhile in United States, “In 2015, the production of 14.7 billion gallons of ethanol supported 85,967 direct jobs in renewable fuel production and agriculture, as well as 271,440 indirect and induced jobs across all sectors of the economy”(Rachel Gantz, 2016). The jobs offered mainly to those that are of low-skill workers, helping them to gain better annual salary. In addition, local farmers that already own a farm or plantation that generates lignocellulosic waste suitable for bioethanol production can benefit from this industry by offering them alternative income and a better way of disposing waste.

2.4 Malaysia’s Biofuel Growth in the Transportation Sector

Malaysia is known as one of the developing countries and it is committed to address the global environmental issues such as the climate mitigation. Statistics reveal that the total carbon dioxide emission from fossil fuels in Malaysia has risen annually since 2001 (130.8 million tons) till the year in 2010 (166.5 million tons) (Masjuki *et al.*, 2013). “It is predicted that the emissions of greenhouse gases by fossil fuels will increase by 39% in 2030 if no tremendous effort are thrown in to mitigate it” (Masjuki *et al*., 2013). Currently, the Malaysian government has issued several policies on biofuel. The policies created were largely focused on biodiesel. One of the example is the National Biofuel Policy that was launched on 2006 (Chin, 2011). The policy introduces four strategies; 1) to produce a biodiesel fuel blend comprises of 5% processed palm oil and 95% diesel. 2) to encourage biofuel usage with incentives. 3) to establish an industrial standard for biodiesel quality, monitored by Standard and Industrial Research Institute of Malaysia (SIRIM). 4) to build a palm oil biodiesel plant in Labu, Negeri Sembilan. The policy was supported by a government-linked company; PETRONAS. In 2009, the PETRONAS Dagangan Berhad marketed its first biodiesel (B5) to be used in vehicles of the Ministry of Defence (MINDEF) and Dewan Bandaraya Kuala Lumpur (DBKL) (Chin, 2011; Tye *et al*., 2011). Throughout the years, Malaysia has introduced higher biodiesel-blend fuel; B5 in 2011, B7 in 2014, and B10 expected to be in 2017 (Malaysian Biodiesel Association, 2017). The Malaysian Biofuel Industry Act that are introduced in 2006, governs the mandate to be obeyed by Malaysians.

In terms of bioethanol-blend fuel, Malaysia is currently neither producing nor consuming it (Wahab, 2013). Cellulosic ethanol (bioethanol) production in Malaysia is currently facing obstacles such as lack of governmental initiative and high capital investment. The push in Malaysia seems to veer towards biodiesel without much news about bioethanol. This is due to the fact that biodiesel production are heavily relies on crude palm oil industry. The country is well known for its palm oil industry since it is the second world’s largest palm oil producer in the world. The rationale behind the government decision to invest more in biodiesel is to help lower down the nation's palm oil stocks and support prices in the international market (Ching, 2016). Next, high capital investment needed for the industry has made bioethanol production to be neglected. Malaysia struggles with financial mechanism can be seen in the failure to achieve its objective in the Five Fuel Diversification Strategy (Lau *et al*., 2015; Tye *et al*., 2011). The objective originally was issued in the Eight Malaysia Plan (2001-2005) and were later carried on in the Ninth Malaysia Plan (2006-2010) and into the Tenth Malaysia Plan (2011-2015) and has yet to achieve the target (Economic Planning Unit, 2016). Therefore, utilisation of biofuels is highly depending on political decisions.

2.4.1 The Potential for Malaysian Bioethanol Industry

Over the last few decades, although the technologies that gave birth to third and fourth generation biofuel emerges, second generation biofuel (specifically bioethanol) is still considered to be feasible. Approximately 7 to 18 billion tons per year of lignocellulosic biomass are available globally to be browbeaten (Lin and Tanaka, 2006). The third and fourth generation fancy over better overall production efficiency, but these current technologies require humongous cost and intelligence transfer for its operation. While it might be possible for developed countries such as the United States, United Kingdom and Germany, it would be a liability for developing and underdeveloped countries.

Malaysia as one of the developing countries is more suited to engage the second generation bioethanol production. Two important factors identified to be the driving force for bioethanol production in Malaysia are the alarming state of energy security of the country and the current economical downfall in the country. It is known that Malaysia has abundance of energy supply about a decade ago. It has its own oil fields in various states such as in Terengganu, Sarawak and Sabah. However, the country's oil and gas reserves are depleting. Nowadays, Malaysia starts importing oil to caters its ever-growing domestic demand. This phenomenon shows that energy security is a critical issue that needs immediate attention. Majority of the energy consumed in Malaysia is through transportation sector which accounts for 40.6% (2000), 40.5% (2005) and 41.1% (2010) (United Nations Development Programme, 2006). The Malaysian government has introduced biodiesel for the transportation sector as a positive effort to address this problem. However, studies show that from year 2005 to 2020 the anticipated petrol oil consumption is more than of diesel oil (Malaysia-Danish Environment Cooperation Programme, 2006; Tye *et al*., 2011). Petrol oil is the major liquid fuel in Malaysia rather than diesel, thus development of bioethanol need to be prioritized.

In addition, the remaining oil fields in Malaysia are of lower quality due to high its high carbon dioxide content (Economic Planning Unit, 2010; Tye *et al*., 2011). These oil fields are relatively small, scattered and far away from the existing facilities, therefore to develop a new oil extracting facilities in these area is a huge economical and geological challenge (Economic Planning Unit, 2010). The United States for example has invented the fracking (hydraulic fracturing) technique to recover oil from shale rock. Since the millennial era, fracking has been used in the country until today and currently, United Kingdom was planning to adopt this technology (BBC News, 2015). Although this technique offers an opportunity to solve the current problem in Malaysia, fracking will be least likely to be introduced with its given disadvantages such as high capital cost and potentially harmful environmental impact. Given the current state of economic downturn in Malaysia, delaying an alternative for petrol fuel (bioethanol) while continuing to rely on import fuel can made the impact more severe for years to come. Producing bioethanol in industrial scale can be an economical challenge. It is known that production cost for lignocellulosic ethanol is higher than of fossil fuel but the overall cost is highly dependent on the cost of feedstock. Almost 46 percent of production cost comes from the cost of feedstock (Tye *et al*., 2011). Thus, using a cheap and highly abundant raw material may reduce the overall cost of producing lignocellulosic bioethanol. This strategy might be the key in making the process to be economically feasible. In conclusion, developing the bioethanol industry in Malaysia may support the growth of its economy in future. The new industry not only fix the energy security issue, but it will indirectly strengthen the oil palm industry and offering more job opportunities for Malaysians.

2.5 Oil Palm Industry

Oil palm tree, known as *Elaeis guineensis* is believed to be originated from West African region. In the times of British Industrial Revolution, huge demand for palm oil emerges mainly for machinery lubricant in various industries (Kiple and Ornelas, 2000). Apart from its industrial uses, palm oil was the main ingredient to make candles (Kiple and Ornelas, 2000) that were used to light up houses before the discovery of electricity. The native West African farmers were the pioneer in supplying palm oil in the early nineteenth century. Later, oil palm plantations were spread across Central Africa and in the Southeast Asia by the Europeans during the colonization era. Oil palm tree is known to be a reliable source of oil production because it can be used for producing oil up to a minimum of 25 years (Abdullah, 2010). In contrast, the productions of oil from other crops such as soybean, olive and coconut have much lower reliability due to their short life and production fluctuations caused by uncertain weathers (Abdullah, 2010).

Nowadays oil palm industry is proven to be a strong industry since its usability in many areas. Oil palm are not just used as food or lubricant as they were in the early nineteenth centuries, in fact they have a broader spectrum in global demand when the idea of sustainable and renewable resources has been introduced (Arip *et al.*, 2013). The oil palm industry starts off with a slow progress due to low processing technology. In the early days of the industry, traditional method was practiced where by loose fruits are collected from the ground and only a few bunches were harvested from the tree (Kiple and Ornelas, 2000). Starting from the early 1920s, the United Africa Company and the Nigerian British colonial officials introduces better technology to enhance the production in terms of labour use and oil yield (Kiple and Ornelas, 2000). The industry starts to rises due to the development of more sophisticated factories, which able to deal with the mass production of fruit on modern plantations and to produce a better and standardized quality of oil (Kiple and Ornelas, 2000).

Agriculture is an important sector to a country. Traditionally, this sector provides the nation its main food source. In the era of highly industrialized world, having a strong and stable agricultural sector is beneficial since it is one the important driving force for the nation's economic and social development. Malaysian oil palm industry has been the country's biggest agricultural contributor for its economy. In 2015, the agricultural sector contributes 8.9% to the overall Gross Domestic Product (GDP) (Department of Statistics Malaysia, 2016). Oil palm industry was a main contributor to the GDP of agriculture sector at 46.9 per cent followed by other agriculture (17.7%), livestock (10.7%), fishing (10.7%), rubber (7.2%) as well as forestry and logging (6.9%) (Department of Statistics Malaysia, 2016).

2.5.1 World and Malaysia Oil Palm Industry

Four decades ago, palm oil industry has been expended and has become as one of the main global oils and fats resources (Kiple and Ornelas, 2000). The reason behind this phenomenon is due to the growing of world population, rapid economic growth, increasing of biofuel production (Chuangchid *et al*., 2012), high demand for agricultural food as well as the oleochemical industry. Statistics shows the prediction for consumption of world edible oil will exceeds the production from 2015 onwards (MPOC, 2014). Nowadays, the global plantation of oil palm is dominated by Indonesia and Malaysia. Both countries produce about 85% of the world's palm oil while the remaining percentage are produced by other countries which include Thailand, Columbia, Nigeria, Papua New Guinea and Ecuador (Sime Darby Plantation, 2014). On the other hand, the major global palm oil consumers are China, India, Indonesia, and the European Union (Sime Darby Plantation, 2014).

Malaysia starts exploiting the oil palm industry in 1917 (Basiron, 2007). In 1961, the industry bloomed when the Malaysian government appointed Federal Land Development Authority (FELDA) to allocate 375 hectares of land for oil palm plantation (Otieno *et al*., 2016). The government also encouraged a policy which seen rubber plantation fields being replaced by oil palm plantation (Basiron and Weng, 2004). The aim was to diversify and commercialize the country's agricultural sector. In 1956, Malaysian Palm Oil Council (MPOC) was established and charged with spearheading the promotional and marketing activities of Malaysian palm oil. The governmental effort in palm oil sector continued to grow; in 1979, the Palm Oil Research Institute of Malaysia (PORIM) was established. In year 2000, PORIM and Palm Oil Registration and Licensing Authority (PORLA) was merged together under a new name known as the Malaysian Palm Oil Board (MPOB) (MPOC, 2014).

MPOB's objectives are to conduct and promote research and development in oil palm tree breeding, palm oil nutrition and potential oleochemical use. Consequently, Malaysia became the world largest oil palm producer until the year 2006, when Indonesia took the leading position (Abdullah, 2010).

Being the world's second top palm oil producer, Malaysia managed to produce around 19.7 million tonnes of oil palm while Indonesia produces an approximate of 29.3 million tonnes in year 2014 (Department of Statistics Malaysia, 2016). Since the oil palm industry in Malaysia has become the largest agricultural force of the country, plantation area for oil palm trees are increasing steadily since early 90's (Basiron and Weng, 2004). Total plantation area was gradually increased up to 4.17 million hectares in 2006, where by Sabah and Sarawak were the 2 states that undergo massive expansion compared to other states (Abdullah and Sulaiman, 2013). About half a decade afterwards (2012), the oil palm planted area has reached 5.08 million hectares and the expansion continues in 2013, recording a total of 5.23 million hectares (MPOB, 2014). Nowadays, total oil palm plantation area in Malaysia has reached 5.74 million hectare in 2016 (Din, 2017). Table 2.1 (Din, 2017) shows the distribution of oil palm plantation area in 2016.

Table 2.1: Malaysian oil palm plantation area in 2016 (Din, 2017).

State	Plantation Area (hectare)
Perlis	652
Kedah	87,786
Pulau Pinang	14,135
Perak	397,908
Selangor	138,831
Negeri Sembilan	178,958
Melaka	56,149
Johor	745,630
Pahang	732,052
Terengganu	171,943
Kelantan	155,458
Sarawak	1,506,769
Sabah	1,551,714
Total	**5,737,985**

Several factors that driven the success of Malaysian palm oil industry are the ideal tropical climate, improved technologies and facilities, efficient management skills, ongoing research and development, and commitment from the government itself (Abdullah and Sulaiman, 2013).

2.5.2 The Oil Palm Tree and Its Applications

Oil palm tree is well known for its ability to yield high amount of oil. Scientifically, the tree is called as *Elaeis guineensis* and it belongs to the Arecaceae family of the Plantae kingdom. Oil palm tree has a monoecious nature in which the male and female flowers are on the same tree. The tree produces fruits in bunches containing around one to three thousand fruitlets. Unripe fruitlets are purplish-black in colour and as the fruit ripens, it changes into orangish-red colour (Figure 2.1). In a closer look, the individual fruitlets are composed of a hard kernel (seed), a shell (endocarp) and a fleshy mesocarp (Figure 2.2). The kernel is the innermost proportion of the fruitlets whereby the shell is the middle portion and the flesh of the fruitlet is the outermost layer.

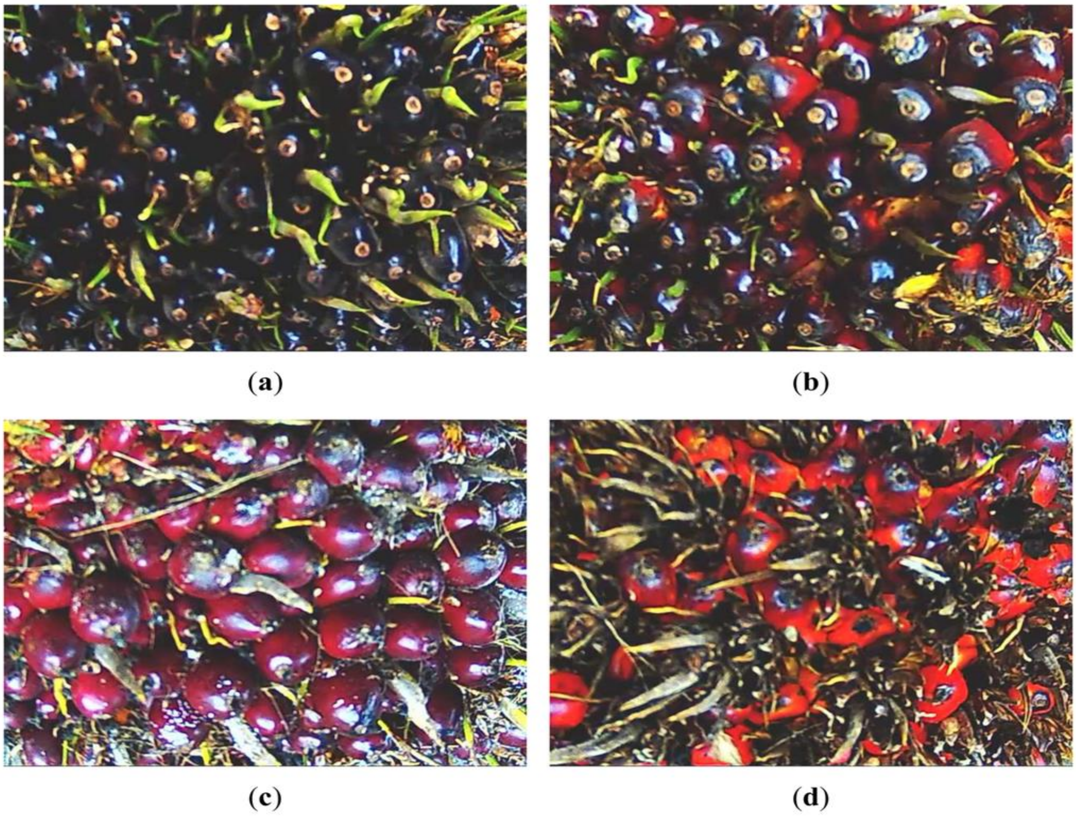

Figure 2.1: Oil palm fruitlets images for four ripeness categories: (**a**) Unripe; (**b**) Under-ripe; (**c**) Ripe; (**d**) Overripe (Fadilah *et al.*, 2012)

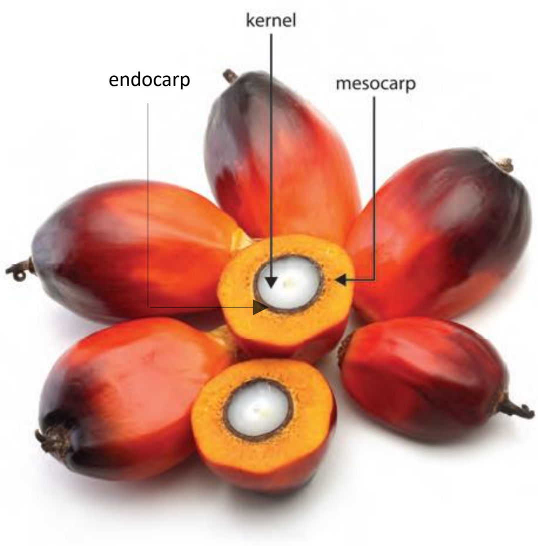

Figure 2.2: Oil palm fruitlets; endocarp, kernel, and mesocarp (MPOC, 2012a)

The oil palm trunk is hard and strong. It may grow up to 60 feet in height. The top part of the trunk is surrounded by fronds while the lower part of the trunk is wrapped by withered fronds which gives the trunk its rough surface. The fronds consist of the petiole (stem) and the lance-shaped leaves as shown in Figure 2.3. The typical life span of an oil palm tree is approximately 25 to 30 years. The oil palm tree can bear fruits around 30 months of planting (MPOC, 2012b).

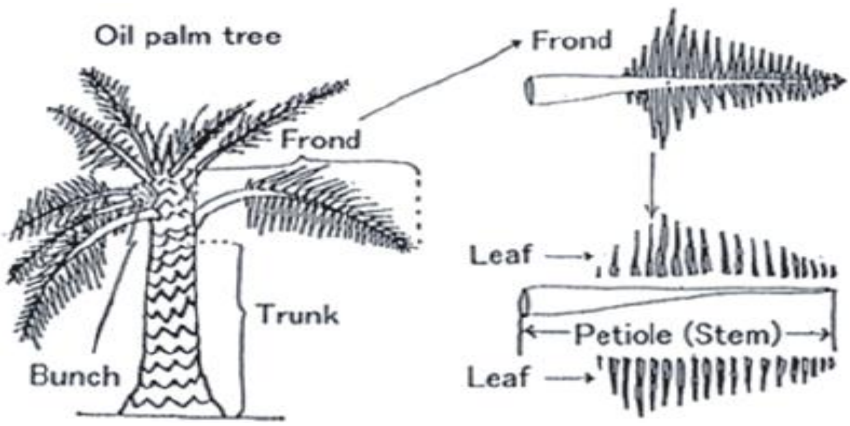

Figure 2.3: Anatomy of an oil palm tree and oil palm frond (OPF) (Ishida and Hassan, 1997; Mohideen *et al.*, 2011)

The oil palm tree possessed huge potential that can benefit mankind. The oil resulting from its fruitlets are the most used for application. It is known that about 80% of palm oil is for edible use and 20% for non-edible use such as oleochemicals manufacture (Basiron and Weng, 2004). Edible applications of the palm oil are mainly being used as cooking oil, shortenings etc. The palm oil has an ability to resist oxidative deterioration and has vitamin E as natural antioxidant thus making it as one of the best edible oil available. On the other side, the non-edible application of palm oil includes usage in soap making, cosmetics, lubricants, oleochemicals etc. Another interesting use of palm oil is as diesel additives and substitute which is known as biodiesel. Nowadays, sustainability and environmental-friendly concern has been quite a norm. Almost every industry is expected to keep up with the "go-green" concept. This includes the oil palm industry. Throughout the year, efforts have been proposed to meet this ideal such as by employing better waste management.

2.5.3 Wastes from Oil Palm Industry

Malaysia's oil palm industry is well known globally with its large plantation area which gives an average annual yield of 19.10 million tonnes of fresh fruit bunches per year since year 2000 (MPOB, 2016). With huge oil palm plantation area, comes high generation of wastes or by-products. The palm oil mill waste (POMW) can be divided into 2 categories; liquid-based waste and solid-based (biomass) waste (Lorestani, 2006). Palm oil mill effluent (POME) is the liquid-based waste (Lorestani, 2006) while others such as empty fruit bunches (EFB), palm pressed fibres (PPF), oil palm trunk (OPT), oil palm fronds (OPF) and shells are biomass wastes. Table 2.2 below shows the breakdown of solid wastes from POMW in 2010 (Agensi Inovasi Malaysia, 2013).