CHAPTER II

THEORY AND LITERATURE REVIEW

2.1 Used frying oil

2.1.1 Generation of used frying oil

Used frying oils and/or fats are generated in large quantities. An estimate of the potential amount of waste frying oil collected in the EU is ~ 700,000-1,000,000 tonnes/year (Supple et al., 2002), in Canada is ~120,000 tonnes/year and in Japan ~ 70,000 tonnes /year (Tsai et al., 2007). However, used frying oil was mainly generated from commercial services, households and food processing plants. In generally used frying oil are generated from restaurants, fast food chains and households as a result of the vegetable oil usage. In recently, resource of used frying oil is considered as one of the commercial/industrial waste reuse types under the authorization of Waste Disposal Act (Tsai et al., 2007).

2.1.2 Disposal and waste management of used frying oil

Due to the large quantity of used frying oil generated per year by any country, these quantities seem to be the disposal problems. Most of this oil generated by society is currently being discharged into the waste water systems,

causing severe problems in waste water treatment plants and blocking sewage systems. Examples of waste management of waste frying oil are:

Landfilling

Landfilling, also known as a dump, is a site for the disposal of waste materials by burial. Historically, landfills have been the most common methods of organized waste disposal and remain in many places around the world. Although the disposal of used frying oil *via* landfill are loss of the by product, this method is the easy way to operated the used frying oil. Generally landfills are classified according to the types of waste disposal material into them. Thus, the disposal of used frying oil *via* landfills without any treatment cauuses several environmental problems such as odor and self-ignition. The others problems can occur from landfill are fatal accidents (i.e. scavengers buried under waste piles), infrastructure damage (i.e. damage to access roads by heavy vehicles), pollution of the local environment (such as contamination of groundwater and/or aquifers by leakage and residual soil contamination after landfill closure), injuries to wildlife and simple nuisance problems (i.e. dust, odor and noise pollution).

Land application

Land application is a form of bioremediation that cleanses waste water by allowing it to flow over a sloped surface. As the water flows over the surface, contaminants are absorbed and the water is collected at the bottom of the slope for

reuse. The land application of used oil has been well researched and put into practice over the past several years. Used oil fraction coats soil particles and percolates only slightly into the soil, while any solids lay on the soil surface. Initially, the oil makes the soil surface water repellent and may cause soil pore blockage. This is an undesirable situation as rain or additional liquid waste will pool on the surface, inhibiting oxygen transfer and percolated to the deeper soil. Thus, proper land application of used oil resembles the composting process. It produces a similar soil amendment and recycling of used oil.

Incineration

Incineration is a waste treatment technology that involves the combustion of waste at high temperatures. The incineration of fat involves some special concerns, including recirculation flue gas back to the boiler since fat carries its own oxygen, the preheating of the oil about 160 degrees F to burn well and the need to maintain a temperature of 85 to 90 degrees F to allow the fat to flow well through the system. As with any incineration process, air emissions would have to be monitored and controlled. Although simple disposal could be the goal of incineration, utilizing spent waste oil as an energy source could be of beneficial use.

Utilization of used frying oil

Used frying oil from restaurants, is a feedstock for rendering plants and biodiesel manufacturers. The rendering plants used this oil to make components of chicken feed and cattle feed. Until recently, the worries of spreading of bird flu disease and mad-cow disease, the lowered demand for rendered products has changed. Thus, used frying oil is mainly uses as biodiesel's feedstock.

2.1.3 Physical and chemical property changes in vegetable oil during frying.

Frying is one of the most popular methods of food preparation in modern times, with the reason being the excellent taste of the fried food. The physical and chemical properties of oil changed during frying. For economical reasons, the same oil/fat is repeatedly or continuously used.

Physical property changes

During deep-frying fat/oil, fat/oil is exposed to light, elevated temperature and atmospheric oxygen; hence, a number of changes take place in the oil, involving a complex pattern of thermolytic and oxidative reaction. Some common physical changes in vegetable oil after frying are:

The increasing in the viscosity of the oils

Viscosity is a measure of internal friction between molecules. Viscosity increases during frying due to oxidation and formation of larger molecules through polymerization. Medeni *et al.* (2005) reported that oil after 50th frying of potato had the increasing of viscosity from 8.341 Pa·sx10^2 to 8.945 Pa ·sx10^2. Moreover, the increasing of viscosity had depended on the duration of frying and the type of oil.

The change in color of the oils

The change in color can be caused by: 1) unsaturated compounds formed in the oil, which are believed to absorb electromagnetic energy over the visible spectrum, 2) darkening of the oil as a result of leaching of pigments from the food into the oil, 3) a Maillard reaction, leading to the formation of the brown pigment melanodin, 4) increased absorption of blue light by conjugated double bonds, giving orange and brown colours to the oil, 5) an increase in polymer content.(Goburdhun *et al.*, 2000)

Chemical properties change

The chemical changes in the frying oil also result in changes of physical characteristic: the color of the oil was darkens with increasing content of polymers; viscosity increases and greater foaming of the oil occurs. Some of the more volatile components such as free fatty acid (FFA), accumulated to the smoke.

The aroma and the flavour of the oil also change with increased frying time. Hydrolysis results in the splitting of the fatty acids from the oil, yielding FFAs, glycerol and mono-and/or diglycerides. The reaction takes place at the junction of the fatty acids and the glycerol apportion of the molecule. In overheated fats, glycerol produced by hydrolysis is decomposed to produce acrolein, a smelling product. irritating to the eyes and mucous membrane (Goburdhun *et al.*, 2000). The major chemical reactions which occurred during frying include:

- Thermolytic reactions: A thermolytic reaction occurs in the absence of oxygen at very high temperatures. If the triglycerides that contain saturated fatty acids are heated at very high temperature in the absence of oxygen, then they produce series of normal alkanes, alkenes, lower fatty acids, symmetric ketones, oxopropyl esters, CO, and CO_2. Unsaturated fatty acids basically form dimeric compounds, including dehydrodimers, saturated dimers, and polycyclic compounds. Unsaturated fatty acids also react with other unsaturated fatty acids *via* the Diels-Alder reaction, forming dimers and trimers. In the case of glycerides, this reaction happens between acyl groups within the same molecule.

- Oxidative reactions: Boiling action in the oil increases aeration in the oil, resulting in increased oxidation of the oil with the formation of hydroperoxides, the primary oxidation product. These peroxides decompose *via* fission, dehydration and formation of free radicals. Which to the chemical products, both volatile decomposition products and non-volatile decomposition, were formed. High levels of volatile decomposition products lead to smoke formation and the strong smell of an abused frying oil. The non volatile decomposition products consist mainly of polymers, dimers and trimers (Kulkarni,

M. G. *et al.*, 2006) In addition, highly oxidized oils may produce polyaromatic hydrocarbons in frying oil. Example of the harmful compounds in oil show in Table.2.1

Table 2.1 The harmful compounds in oil

Year	Type of oil/food	Harmful compound	Amount	Ref.
2006	Frying oil	Total PAHs		Purcaro *et al.*
	1.palm oil/sunflower	- before frying	5.8 ppb	
	oil (60/40)	- after frying	5.4 ppb	
	2. sunflower oil			
		- before frying	22.8 ppb	
		- after frying	16.2 ppb	
	3.soybean oil			
		-before frying	9.5 ppb	
		-after frying	7.1 ppb	
	4.palm oil/soybean oil			
	(50/50)	-before frying	6.3 ppb	
		-after frying	8.2 ppb	

Table 2.1 (cont.)

Year	Type of oil/food	Harmful compound	Amount	Ref.
2006	Frying oil	Naphthalene		Purcaro *et al.*
	1.palm oil/sunflower	- before frying	6.1 ppb	
	oil (60/40)	- after frying	3.5 ppb	
	2. sunflower oil			
		- before frying	7.2 ppb	
		- after frying	4.3 ppb	
	3.soybean oil			
		-before frying	5.9 ppb	
		-after frying	4.7 ppb	
2006	Frying oil	Benzo[a]pyrene		Purcaro *et al.*
	1.palm oil/sunflower	-before frying	0.2 ppb	
	oil (60/40)	- after frying	0.2 ppb	
	2. sunflower oil			
		- before frying	0.3 ppb	
		- after frying	0.3 ppb	
	3.soybean oil			
		-before frying	0.1 ppb	
		-after frying	<0.05 ppb	
	4.palm oil/soybean oil			
	(50/50)	-before frying	0.1 ppb	
		-after frying	0.1 ppb	

Table 2.1 (cont.)

Year	Type of oil/food	Harmful compound	Amount	Ref.
1997	cooking olive oil virgin olive oils (51 sample)	Total PAHs	> 25 ppb	Purcaro *et al.*
1996	Olive oil from	Benzo[a]pyrene	9.7ppb	Pupin *et al.*
1999	Olive oil	Copper	0.28ppb	Castillo *et al.*,
1988	Crude palm oil Refined palm oil	Iron	1.20ppm 4.93ppm	Saleh *et al.*,
1988	Crude palm oil Refined palm oil	Copper	0.70 ppb 1.19 ppb	Saleh *et al.*,

2.2 Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are a class of potentially mutagenic substances emitted from cooking oil heated at high temperatures and well known carcinogenic compounds. Due to the wide distribution of PAHs in the environment and their lipophilic nature, oils can be contaminated with these xenobiotic substances.

2.2.1 Sources of environmental PAHs contamination

Foods can be contaminated by PAH that are present in air (by deposition), soil (by transfer) or water (by deposition and transfer). The sources, natural and mostly anthropogenic, of PAH in the environment are numerous and include:

- Stubble burning and spreading of contaminated sewage sludge on agricultural fields.

- Exhausts from mobile sources (motor vehicles and aircrafts). Close to an emission source such as a motorway, PAHs are very high concentrations in the soil surface layer, but soil at a depth of 4-8 cm was two times less contaminated. Close to highways, concentrations of PAH in the soil in the range of 2-5 mg/kg can be found where as in unpolluted areas, the levels are in the range of 5-100 µg/kg. The distribution and concentration of PAHs in soil, leaf litter, and soil depend on the distance from the roadside.

- Industrial plants (e.g. aluminum foundries, incinerators).

- Wood preservation, use of tar coated wood. Oysters and mussels grown in beds with tar or creosote coated wood posts may be contaminated with PAHs.

- PAHs present in the foods during preparation and cooking.

- Forest fires and volcanic eruptions

2.2.2 Occurrence of PAHs in foods

Raw foods should usually not contain high levels of PAH. In areas remote from urban or industrial activities, the levels of PAHs found in unprocessed foods reflect the background contamination, which originates from long distance airborne transportation of contaminated particles and natural emissions from volcanoes and forest fires.

Processing of food (such as drying and smoking) and cooking of foods at high temperatures (grilling, roasting, frying) are major sources for PAHs generation. Contamination of vegetable oils with PAHs usually occurs during technological processes like direct fire drying, where combustion products may come into contact with the oil. Example of the PAHs contaminated in food and the most contributed PAHs in food shows in Table 2.2 and Table 2.3

Table 2.2 Average values and ranges of PAHs in different food groups (Dennis *et al.*, 1983).

PAHs	Concentration of PAHs (µg/kg)			
	Cereals	Meat	Fruit and sugar	Oils and fats
Fluoranthene	1.38	0.48	0.93	1.76
Pyrene	1.85	0.55	0.83	2.75
Benz[a]anthracene	0.37	0.05	0.11	0.95
Chrysene	0.77	0.15	0.23	1.18
Benzo[e]pyrene	0.22	0.03	ND-1.29	1.10
Benzo[b]fluranthene	0.23	0.04	0.06	0.92
Benzo[a]pyrene	0.32	0.05	0.07	1.57

Table 2.3 Food groups most contributing to PAHs intake

(Dennis *et al.*, 1983).

Ranking	United Kingdom	The Netherlands	Sweden
1	Oils & fats (34%)	Cereals (27%)	Cereals (34%)
2	Cereals (31%)	Sugar & sweets (18%)	Vegetable (18%)
3	Vegetable (12%)	Fruit &sugar (13%)	Oil & fats (16%)

2.2.3 Legal Limits

European countries have set the legislation that shows in Table 2.4.

Table 2.4 The maximum limits for PAHs set in certain EU Member States Commission Regulation (EC) 208/2005 (Wenzl *et.al.*, 2006)

Country	Type of food	PAHs	Max
Czech Republic	Meat products	BaA, BbF, BkF, DhA,DhP, DiP, IcP, CHR	3.0 µg/kg
	Fats and oils	BaA, BbF, BkF, DhA,DhP, DiP, IcP, CHR	2.0 µg/kg
Slovak Republic	Smoked meat products, oils and fats	BaP	1.0 µg/kg

Table 2.4 (cont.)

Country	Type of food	PAHs	Max
Italy	Refined olive pomace oil	BaP, BeP, BaA, BbF, DgP, IcP	2.0 µg/kg
Sweden	Olive pomace oil and refined olive pomace oil	BaA,BbF,BkF, DhA,IcP, BaP, BeP,BgP	2.0 µg/kg
Greece	Olive pomace oil	BaA,BbF,BkF, DhA,IcP, BaP, BeP,BgP	2.0 µg/kg
Spain	Olive pomace oil	BaA,BbF,BkF, DhA,IcP, BaP, BeP,BgP	2.0µg/kg each
United Kingdom France Ireland Cyprus Luxembourg Denmark Slovenia	No legal limits, but some countries have recommended maximum levels		

BaA: Benzo[a]anthracene; BbF: Benzo[b]fluoranthene; BkF:

Benzo[k]fluoranthene; BgP: Benzo[ghi]perylene; BaP: Benzo[a]pyrene; BeP:

Benzo[e]pyrene; DhA: Dibenz[a,h]anthracene; IcP: Indeno[1,2,3-cd]pyrene

2.2.4 The extraction of PAHs from oil.

The most common method for extraction of PAHs from oil has three methods: liquid-liquid partition, caffeine complexation and saponification.

Liquid-liquid partition method

With partition method, the oil sample is dissolved in an organic solvent such as cyclohexane (CH) and PAHs are extracted with a solution of dimethylformamide (DMF)-water (9:1) or dimethyl sulfoxide (DMSO), while most of the lipidic matter (mainly constituted of triglycerides) remains in the organic phase. Isolation is performed by dilution with water, in order to change the coefficients of partition of the PAHs between the two phases, and back-partition into CH, which may be more easily evaporated (at lower temperature). This procedure allows reducing the mass of the residue to a 10% of the initial value.

Caffeine complex formation

Caffeine complex formation is a relatively rapid method for determining PAHs in fats and oils, formation. The sample is dissolved in cyclohexane and the shaking in a caffeine-fomic acid solution. After decomposing the complex, with an aqueous sodium chloride solution (2%), the PAHs are extracted with cyclohexane.

Saponification method

The saponification method allows to reach a considerable concentration of the PAHs fraction. The oil is saponified under reflux during at least 40 min with alcoholic potassium hydroxide and unsaponifiable matter is extracted with cyclohexane.

Moret *et al.* (2000) compared the three extraction procedures above described on an olive oil sample. Sample purification was performed by solid – phase extraction (SPE) clean-up and analytical determination by HPLC and spectrofluorometric detection. The three methods (liquid-liquid partition with cyclohexane and dimethyl formamide-water, caffeine complexation and saponification) resulted to be not equivalent for the degree of purification obtained and the capacity to get a squalene free fraction. Squalene is contained in olive oils at about 50 % of unsaponifiable matter and represents a problem because it co-elutes with PAHs from the cartridge, and is able to trap them, especially the heavier ones. The saponifiction method gave appreciable squalene residue, meaning that an additional purification step is needed, while the liquid-liquid partition method showed a higher purification power, as no appreciable amount of squalene remained in the samples. Liquid-liquid partition followed by SPE clean- up resulted to be the best method. Recoveries ranged between 42.5 and 92.2%, depending on molecular masses except for naphthalene (31.9%) probably due to its volatility.

2.2.5 Properties of PAHs

In generally, PAHs present in many forms of vegetation and fossil fuel. The formations of PAHs also results from the incomplete combustion of hydrocarbons. Thus, many species of PAHs are produced as a result of coal and oil combustion.

Generally, PAHs are lipophilic compounds that show high affinity for organic matter. However, individual PAHs differ substantially in their physicochemical properties. PAHs comprise fused aromatic rings and do not contain hetero atoms or carry substituents. PAHs are containing up to four fused benzene rings are known as light PAHs and those are containing more than four benzene rings are called heavy PAHs. Heavy PAHs are more stable and more toxic than light ones (Wenzl *et al.*, 2006). Low molecular weight (LMW) of PAHs are much more water soluble and volatile than their high molecular weight (HMW) relatives, while the high molecular weight of PAHs show higher hydrophobicity than the low molecular weight compounds. The difference in hydrophobicity is also reflected by the octanol-water partitioning coefficient (K_{OW}). These physico-chemical properties largely determine the environmental behavior of PAHs, and indicate that low molecular weight PAHs transfer and turnover will be more rapid than for the heavier PAHs. The semivolatile nature of low molecular weight of PAHs means that they exist in the atmosphere partly as vapors and are therefore highly susceptible to atmospheric degradation processes. Similarly, in aqueous environments, the low molecular weight PAHs are partly dissolved, making them highly available for various degradation processes. The

high molecular weight of PAHs are primarily associated with particles in the atmosphere and water, and less available for degradation. Furthermore, PAHs adsorbed to particles may be transported over long distances in the atmosphere and are therefore ubiquitous in the environment
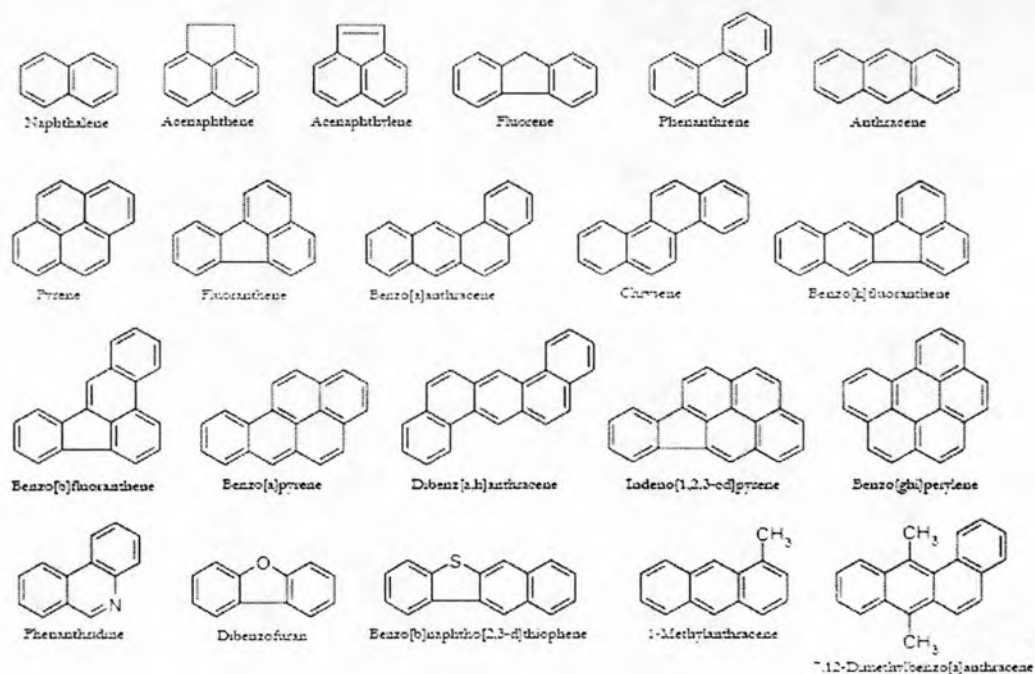

Fig. 2.1 Structures of the 16 US-EPA PAHs and selected alkyl-PAHs and heterocyclic compounds

2.2.6 Toxicity of PAHs

The toxic of PAHs are very well known as ecotoxicants which are harmful to human health. They are causing errors in DNA replication and mutations that initiate the carcinogenic process. Due to the toxicity of PAHs in

used frying oil and the environmental concerns about pollution coming from the oil, the management of this oil in a proper way is very important for the environment. On the others method such as utilization of used frying oil for biodiesel production is the alternative way for management of this waste.

2.3 Utilization of used frying oil for biodiesel production

Biodiesel (fatty acid methyl ester or FAMEs) is defined as "a substitute for or an additive to diesel fuel that is derived from the oils and fats of plants and animals".

2.3.1 The Production processes for biodiesel production

Four main processes to utilized vegetable oils and waste oil as a viable alternative fuel: direct use and blending, micro- emulsification, pyrolysis and transesterification.

Direct use and blending

Direct use and blending process are the use of vegetable oils as an alternative renewable fuel or blending the vegetable oils with diesel fuel. In 1980s, there was beginning to used of vegetable oil as a fuel. At that point, it was not practical to substitute 100% vegetable oil for diesel fuel, but a blend of 20% vegetable oil and 80% diesel fuel was successful.

The advantages of vegetable oils as diesel fuel are: liquid nature-portability, ready availability, renewabitity, higher heat content (about 88 % of diesel fuel), biodegradability. However, the disadvantages of using vegetable oils as diesel fuel are higher viscosity, lower volatility, the reactivity of unsaturated hydrocarbon chains. For used of used frying oil as fuels, two severe problems are waste oil deterioration and incomplete combustion (Adams *et al.*, 1983).

Microemulsion

Microemulsion is defined as thermodynamically stable colloidal dispersion in which the diameter of the dispersed-phase particles is less than one-fourth the wavelength of visible light. Microemulsion fuel is blending of diesel fuel with short chain alcohols aided by ionic or non-ionic amphiphiles. This method can solve the problem of the high viscosity of vegetable oils, but the heavy carbon deposits, incomplete combustion and irregular injector needle sticking were reported.(Ma and Hanna, 1999)

Pyrolysis

Pyrolysis is the conversion of one substance into another by mean of heat or by heat with the aid of a catalyst (Swern *et al.*, 1979). It involves heating in the absence of air or oxygen and cleavage of chemical bonds to yield small molecules. The pyrolysis of triglycerides aims to obtaining products suitable for diesel engines. Thermal decomposition of triglycerides produces compounds of

several classes, including alkanes, alkenes, alkadienes, aromatics, and carboxylic acids.

However, while pyrolyzed vegetable oils possess acceptable amounts of sulphur, water, and sediment, as well as giving acceptable copper corrosion values, they are unacceptable in terms of ash, carbon residues, and pour point. In addition, though the products are chemically similar to petroleum–derived gasoline and diesel fuel, the removal of oxygen during thermal processing also eliminates any environmental benefits of using oxygenated fuel (Ma *et al.*, 1999).

Transesterification

Transesterification is a widely use process for biodiesel production in the world because transesterification reduces the viscosity of triglycerides, thereby enhancing the physical properties of renewable fuels to improve engine performance (Clark *et al.*, 1984). Transesterification is described below.

2.4 Transesterification

2.4.1 Definition of Transesterification

Chemically, transesterification is the process of exchanging the alkyl group (from an alcohol) of an ester with another alkyl, from a different alcohol. In the case of biodiesel, a vegetable oil ester is combined with a simple alcohol and a catalyst, resulting in the breakup of the triglyceride ester (three fatty acids

connected to a single glycerol), and the joining of the fatty acids with the added

simple alcohols. The glycerin alkyls are replaced with the alkyl of the added

alcohol (for example; methyl for methanol, ethyl for ethanol, etc.). The separated

glycerol is the waste product. Transesterification reaction shows in Fig. 2.2:
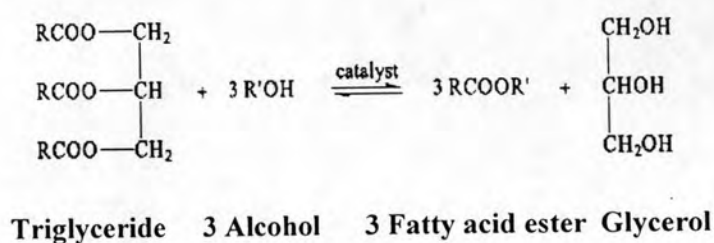
RCOO—CH₂   CH₂OH

RCOO—CH   + 3 R'OH ⇌ catalyst 3 RCOOR' + CHOH

RCOO—CH₂   CH₂OH

Triglyceride   3 Alcohol   3 Fatty acid ester Glycerol

Fig. 2.2. Transesterification reaction of triglyceride.

If methanol is used in this process, it is called methanolysis.

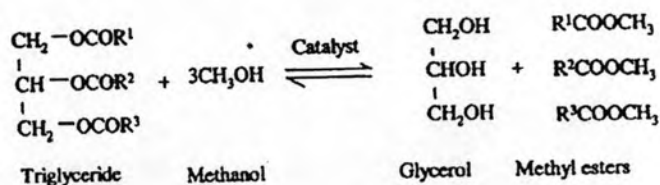Methanolysis of triglyceride is represented in Fig. 2.3.

$$CH_2-OCOR^1$$
$$CH-OCOR^2 + 3CH_3OH \rightleftharpoons Catalyst$$
$$CH_2-OCOR^3$$

CH₂OH   R¹COOCH₃
CHOH + R²COOCH₃
CH₂OH   R³COOCH₃

Triglyceride   Methanol   Glycerol   Methyl esters

Fig. 2.3. Methanolysis of triglyceride

2.4.2 Catalyst for biodiesel production

Catalysts for homogeneous process

Acid catalysts

Acid catalysts used for transesterification include sulfuric, phosphoric,

hydrochloric, and organic sulfonic acids. While the excess amount of alcohol is

required to complete the transesterification and need to be done in absences of

water in order to avoid formation of carboxylic acid, which reduces the yield alkyl ester.

The advantage of the acid catalysts is a high yield of alkyl esters, but the acid catalysts are slower than the alkali catalysts. Although transesterification by acid catalysis is much slower than that by alkali catalysis, acid catalysts is more suitable for glycerides that have relatively high free fatty acid contents and more water (Freedman *et al.*, 1984)

Alkali catalysts

Alkali catalyst transesterification process is more efficient and is less corrosive than acid catalysed process which makes it more applicable to industrial use. Alkali catalysts for transesterification process are including metal hydroxides like sodium or potassium hydroxide, carbonates and metal alkoxides. The commonly catalyst used is sodium hydroxide due to its economical availability.

Metal hydroxides are readily available but are less reactive. Increasing the concentration of the catalyst in the mixture accelerates the reaction. The condition of activity of this catalyst is under anhydrous conditions. However, this can not be avoided as water is formed during the reaction between hydroxides and alcohol. The yield of alkyl esters is reduced as water hydrolyses the ester, forming soap. Soap forms an emulsion with glycerol making recovery of glycerol difficult (Fukuda *et al.*, 2001).

Catalysts for heterogeneous processes

Heterogeneous catalysts are the new trends of biodiesel's production for a few years ago. Although transesterification using homogeneous catalyst such as alkali catalyzed process, gives high conversion levels of triglycerides to their corresponding methyl esters in short times. Thus the reaction has several drawbacks: it is energy intensive; recovery of glycerine is difficult; the catalyst has to be removed from the product; alkaline waste-water requires treatment and free fatty acids and water interfere with the rection.

In order to minimize homogeneous process problem, attempts to use heterogeneous catalyst systems in alcoholysis of triglycerides have been made. These catalysts greatly simplify the post-treatment of the products (separation and purification). They can be easily separated from the system at the end of the reaction and could also be reused. Besides, the use of heterogeneous catalysts does not produce soaps through free fatty acid neutralization or triglyceride saponification (Pinto *et al.*, 2005).

The heterogeneous catalysts are including metal oxides (Mg, Ca, Al, Fe), carbonates ($CaCO_3$), enzymes, etc. (Pinto *et al.*, 2005). In recently, enzymatic catalyst is the most studied heterogeneous catalyst in biodiesel's production, encouraged by pollution and by product separation problems of the chemical catalyzed process (Royon *et al.*, 2007).

Enzymatic Catalyst

Lipases or glycerol ester hydrolase are used as an enzymatic catalyst into transesterification reactions. Thus triglycerides were catalyzed hydrolysis to the corresponding fatty acids and glycerol by lipases in transesterification reaction. In nature, lipases can be found in many different sources such as animal, vegetable cell and microorganisms.

Although lipases are used in the reactions because of their ready availability, high catalytic activity, easy separation, environmetal friendly and saving of chemicals etc., but the problems are the high cost of lipase, slow reaction rates and the deactivation with glycerol. So the attachment of lipase to a solid support called "immobilized lipase" is the other way for enzymatic catalyst (Grandhi et al., 2001).

Immobilization of Lipases

Immobilization of lipases is the attachment of lipases to a solid support over, which a substrate is passed and converted to product. So, immobilized lipase is used more effectively than the free lipase because of they can be repeatedly used without any decreased activity and glycerol can be recovered using mild reaction conditions (Fukuda et al., 2001). Recently, there has been an increasing development of enzymatic catalysts for biodiesel production.

Non-Catalyst for biodiesel process

In a catalytic reaction to produce biodiesel through transesterification, several processe such as the recovery of unreacted reactants and catalysts, purification of the esters and separation are involved. These processes make the production of biodiesel through catalytic transesterification system complicated, thus giving a reason to investigate production of alkyl esters from vegetable oils *via* noncatalytic reactions. Supercritical alcohols have been explored as a reactant for the transesterification process and have proven a conversion rate of triglyceride to be 80-100% (Fukuda *et.al.*, 2001).

2.4.3 Enzyme Kinetics

The mechanism of an enzyme-catalyzed reaction is to determine the rate of the reaction and how it changes in response to changes in experimental parameters, a discipline known as enzyme kinetics. In an equilibrium, an enzyme (E) binds a substrate (S) to form an enzyme-substrate complex (E-S). The E-S complex can dissociate or irreversibly convert the substrate to a product (P) (Fig. 2.4). The Michaelis-Menten equation describes the relationship between the rate of substrate conversion by an enzyme to the concentration of the substrate (Equation 2.1).

Equation 2.1

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

In this equation, V is the rate of conversion, V_{max} is the maximum rate of conversion, [S] is the substrate concentration, and K_m is the Michaelis constant. The Michaelis constant is equivalent to the substrate concentration at which the rate of conversion is half of V_{max}. The meaning of K_m is the strength of binding between the enzyme and substrate. Its value includes the affinity of substrate for enzyme, but also the rate at which the substrate bound to the enzyme is converted to product. A small K_m indicates high affinity, and a substrate with a smaller K_m will approach V_{max} more quickly. Very high [S] values are required to approach V_{max}, which is reached only when [S] is high enough to saturate the enzyme. V_{max} is equivalent to the product of the catalyst rate constant (k_{cat}) and the concentration of the enzyme. The graph of the Michaelis-Menten equation shows in Fig. 2.5.

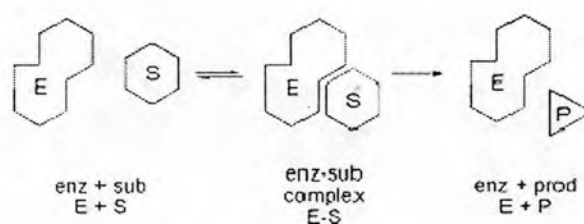enz + sub
E + S

enz·sub
complex
E·S

enz + prod
E + P

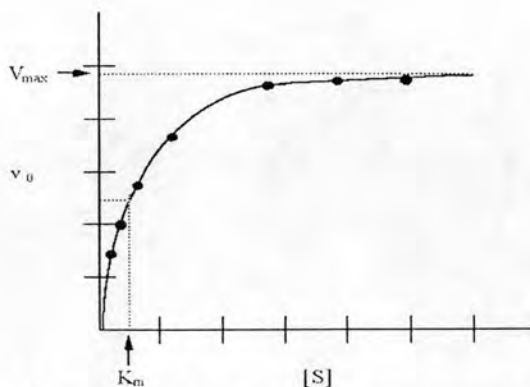Fig. 2.4 A schematic representation of the elements of an enzyme catalyzed reaction.

Fig. 2.5 Michaelis-Menten plot for an enzyme-catalyze reaction

A Michaelis-Menten plot can give an easy way to measure the rate constant for a given enzyme. An immediate difficulty is apparent, however, when Michaelis-Menten plots are used. V_{max} is an asymplote. Its value can only be certain if the reaction is run at an infinite concentration of substrate. Obviously, this is an impossible prospect in lab. In 1934, two individuals, Lineweaver and Burke made a simple mathematical alteration in the process by plotting a double inverse of substrate concentration and reaction rate (velocity). The Lineweaver/Burke equation shows in equation 2.2.

Equation 2.2

$$\frac{1}{V_o} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

This equation fits the general form of a straight line, $y = mx+b$, where m is the slope of the line and b is the intercept (Fig. 2.6) Thus, the Lineweaver/Burke Plot for an enzyme is more useful than Michaelis-Menten, since as velocity reaches infinity, $1/V_{max}$ approaches 0. Moreover, since the plot results in a straight line, the slope is equal to K_m/V_{max}, the y intercept equals $1/V_{max}$ ($1/S=0$). Projection of the line back through the x axis yields the value $-1/K_m$ (when $1/V=0$). These values can easily be determined by using a linear regression plot and calculating the corresponding values for $x=0$ and $y=0$. The inverse of the intercept values will then yield V_{max} and K_m.
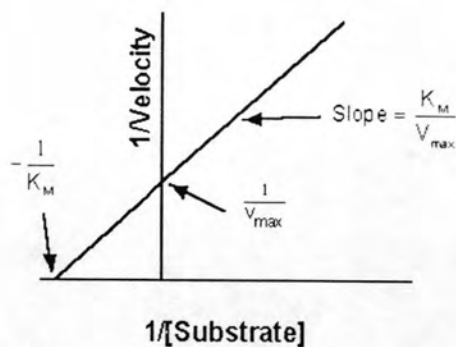
Fig. 2.6 Lineweaver-Burk plot for an enzyme-catalyzed reaction.

2.4.4 Reaction parameter for biodiesel

Some parameter involve in preparation of biodiesel are: type of methanolysis, amount of water in transesterification reaction, dosage of immobilized lipase and stability of immobilized lipase.

Type of methanolysis

Type of methanolysis involved the amount of methanol in the enzymatic production of biodiesel. Low methanolysis is due to the inactivation of immobilized lipase by contact with insoluble methanol which exists as drops in the oil (Shimada *et al.*, 2002). The type of methanolysis was reviewed in Table 2.5

Table 2.5 Review of the type of methanolysis

Year	Type of methanolysis	Immobilized lipase	Oil	Yield	Ref.
2007	Discontinuous batch reactor Continuous batch reactor	Novozyme 435 *C. antarctica*[b]	Cotton seed	95 %[a] 97 %[a]	Royon *et al.*
2005	Three- step	Lipozyme TL (10 wt%)	Soybean	90 %[a]	Du *et al.*
2004	Three-step One- step	Lipozyme TL (4 wt%)	Soybean	~70 %[a] ~ 35 %[a]	Xu *et al.*
2003	One-step	*R. oryzae*[c] lipase	Palm Soybean Rapeseed	55 wt % 25 wt % 30 wt %	Pizarro *et al.*
2002	Three – step	Novozyme 435 *C.* antarctica[b]	Soybean	95.9 %[a]	Watanabe *et al.*
2000	Two- step Three- step	Novozyme 435 *C.* antarctica[b]	Vegetable oil	> 95 %[a] > 95 %[a]	Watanabe *et al.*

a: percentage of FAME conversion.

b: *Candida antractica*

c: *Rhizopus oryzae*

Amount of water in transesterification reaction

Water content is one of the most important parameters affecting the enzyme activity. Enzymes need a small amount of water to maintain their active conformations. The initial velocity increased with respect to the increasing amount of water (Morten *et al.*, 2001). On the other hand, water in the methanolysis decreased the reaction rate, which could result from the hydrolysis side reaction occurring (Du *et al.*, 2005).

Dosage of immobilized lipase

Immobilized lipase activity was affected by the dosage of immobilized lipase. The high amount of immobilized lipase limited the intraparticle diffusion or enzyme aggregation. Then, the optimum dosage of immobilized lipase reviewed in Table 2.6

Table 2.6 Review of dosage of immobilized lipase.

Year	Immobilized lipase	Oil	Optimum dosage of immobilized lipase	Ref.
2007	Novozym 435	Soybean (ethyl acetate as the acyl donor)	10%(w/w)	Modi *et al.*
2003	Novozym 435	Soybean	30 % (w/w)	Xu *et al.*
2003	Lipozyme TL IM	Soybean	4%(w/w) for stepwise methanolysis 10%(w/w) for three step methanolysis	Xu *et al.*
2002	Novozym 435	Cotton seed	30 %(w/w)	Köse *et al.*

Stability of immobilized lipase

During repeated use, lipase lost its activity because of the glycerol in the reaction. Some efforts have been made to remove glycerol from lipase during enzymatic transesterification by washing with the solvent. The operation stability

of immobilized lipase was studied, due to the high cost of the enzyme. Then the

stability of immobilized lipase reviewed in Table 2.7.

Table 2.7 Review of the stability of immobilized lipase.

Year	Immobilized lipase	Oil	Washing by	Cycle No.	Ref
2006	*Candida* sp.	Soybean	Only sucksion	15	Yang *et.al*
2004	*Candida antractica* or Novozym 435	Soybean (methanol as acyl donor)	Sucksion	4-5	Du *et.al*
2004	*Candida antractica* or Novozym 435	Soybean (methyl acetate as acyl donor)	Sucksion	>100	Du *et.al*
2003	*Thermomyces lanuginosus* or LipozymeTL IM	Soybean	Isopropanol	8	Du *et.al*

Table 2.7 (cont.)

Year	Immobilized lipase	Oil	Washing by	Cycle No.	Ref
2002	*Candida antractica*	Tuna oil (ethanol as acyl donor)	Sucksion	> 50	Shimada *et.al.*

Due to the interested of enzymatic catalyst, immobilized lipase (Lipozyme RM IM and Novozym 435) were chosen as the catalyst in this studies. According to the method from Watanabe *et al.* (2000), we studied methanolysis type. Then the method of stability of immobilized lipase was adopted from the method in Table 2.7. Moreover, we studied the important parameter in the transesterification such as amount of water in the reaction, dosage of immobilized lipase, temperature and concentration of methanol. In generally, used frying palm oil was reacted with three moles of methanol using 20 % immobilized lipase at 40°C in this studied.

2.5 Biodiesel quality

Standards are of high significance for producers, suppliers and users of biodiesel for the assessment of safety, risks and environmental pollution. Standarardization of biodiesel is important so that engine manufacturers have reference fuels for engine systems development and also standards play a vital

role in warranty issues and regulatory compliance. The most widely used standard

is ASTM D 6751-03 (Table 2.8) (Fernando *et al.*, 2007).

Table 2.8 Requirements for Biodiesel (B100) blend stock as listed in ASTM D

6751-03.

Property	ASTM Method	Limits	Units
Flash point	D 93	130 (min)	^{o}C
Water and sediment	D 2709	0.050 (max)	% volume
Kinematic viscosity, 40 ^{o} C	D 445	1.9-6.0	mm^2/s
Sulfated ash	D 874	0.020 (max)	wt. %
Total Sulfur	D 5453	0.05 (max)	wt %
Copper strip corrosion	D 130	No.3 (max)	
Cetane number	D 613	47 (min)	
Could point	D 2500	Report to customer	^{o} C
Carbon residue	D 4530	0.05 (max)	wt.%
Acid number	D 664	0.8 (max)	mg KOH/g
Free glycerin	D 6584	0.2	wt.%
Total glycerin	D 6584	0.24	wt.%
Phosphorus content	D 4951	0.001 (max)	% mass