CHAPTER II

THEORY AND LITERATURE REVIEWS

2.1 Biodiesel

Biodiesel, an alternative diesel fuel, is made from renewable biological sources such as vegetable oils and animal fats. It is biodegradable and nontoxic which has low emission profiles and so is environmentally beneficial. Biodiesel has been defined as the monoalkyl esters of long-chain fatty acids derived from renewable feedstocks, such as vegetable oils or animal fats, for use in compression-ignition (diesel) engines. The biodiesel that is considered as a possible substitute or extender of conventional diesel fuel is commonly composed of fatty acid methyl esters that are prepared from the triglycerides in vegetable oils by transesterification with methanol. The resulting biodiesel is quite similar to conventional diesel fuel in its main characteristics. Biodiesel is compatible with conventional diesel and both can be blended in any proportion. A number of plants are manufacturing biodiesel worldwide. These units are using sunflower oil, used-frying oil, jatropha oil, etc. as a source of triglycerides. [2]

2.1.1 Raw materials for biodiesel

Biodiesel is derived from biological sources, such as vegetable oils or fats, and alcohol. Commonly used feedstocks are shown in Table 2.1

Table 2.1 Feedstocks used for biodiesel manufacture vegetable oils animal fats other sources [6]

Vegetable Oils	Animal Fats	Other Source
Soybeans Rapeseed Canola Oil (a modified version of rapeseed) Safflower Oil Sunflower Seeds	Lard Tallow Poultry Fat	Recycled Restaurant Cooking Oil

Vegetable oils are primarily water-insoluble hydrophobic substances that are made of one mole glycerol and three moles of fatty acids and are commonly called triglycerides. Fatty acids vary in carbon chain length and in the number of unsaturated bonds (double bonds). The fatty acids found in vegetable oils and typical fatty acid compositions of common oil sources are summarized in Table 2.2.

Table 2.2 Percentage of fatty acid type for different oils. [7]

Vegetable oil	Fatty acid composition, % by weight								
	16:0	18:0	20:0	22:0	24:0	18:1	18:2	18:3	22:1
Corn	12	2	1	0	0	25	61	1	0
Cottonseed	28	1	0	0	0	13	58	0	0
Crambe	2	1	2	1	1	19	9	7	59
Rapeseed	4	1	0	0	0	64	22	8	0
Soybean	12	3	0	0	0	23	56	7	0
Sunflower seed	6	3	0	0	0	17	74	0	0
Canola oil	6	2	1	1	0	55	24	9	1
Palm	44	4	1	0	0	40	10	0	0
Butter	30	30	2	1	0	30	3	0	2
Peanut	6	6	10	10	0	66	38	0	0
Linseed	9	1	0	0	0	9	8	45	0
Tung	0	0	0	0	0	13	15	72	0

The fatty acids were also found in microorganism, as algae, fungus etc. Table 2.3 showed the fatty acids compositions of microorganism oil.

Table 2.3 Fatty acids compositions of microorganism oil

microorganism oil	Fatty acid composition								
	14:0	16:0	18:0	18:1	18:2	18:3	20:4	20:5	22:1
Algae [8]	-	/	/	/	/	/	-	-	-
Yeast [9]	/	/	/	/	/	/	/	-	-
Fungus [4]	/	/	/	/	/	/	/	/	-

Remark ; / = the fatty acids were also found in microorganism.

Table 2.4 Names and structures of the most common fatty acids [10]

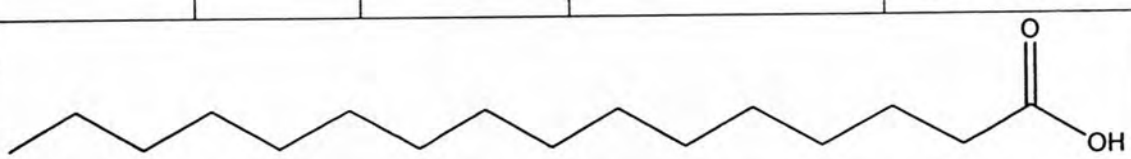
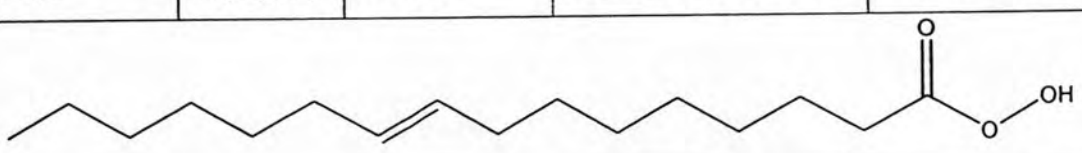
No. of C:No. of double bound	Molecular formular	Molecular mass	Systematic name	Other names
C12:0	$C_{12}H_{24}O_2$	200.32	Dodecanoic acid	Lauric acid, n-Dodecanoic acid
				
C14:0	$C_{14}H_{28}O_2$	228.38	Tetradecanoic acid	Myristic acid
				
C16:0	$C_{16}H_{32}O_2$	256.43	Hexadecanoic acid	Palmitic acid, Hexadecylic acid, Cetylic acid
				
C16:1	$C_{16}H_{30}O_2$	254.41	9-hexadecanoic acid	Palmitoleic acid
				

Table 2.4 Names and structures of the most common fatty acids (*Continued*)

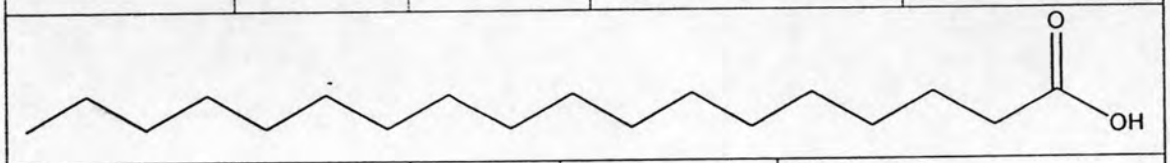
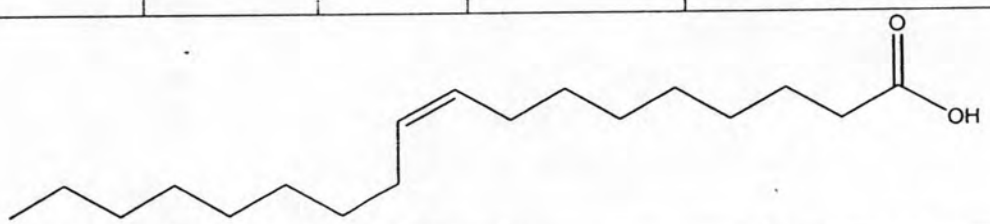
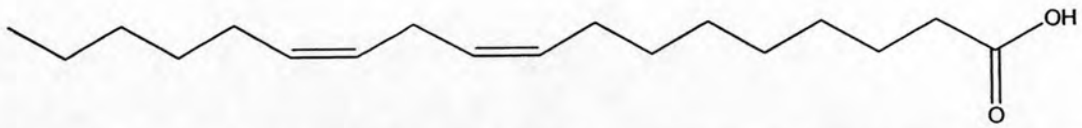
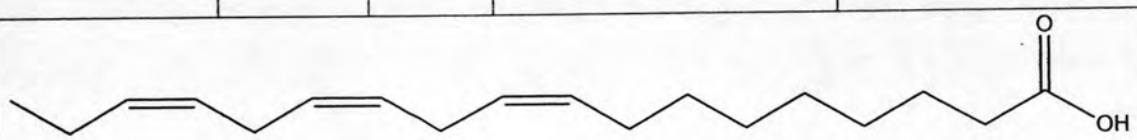
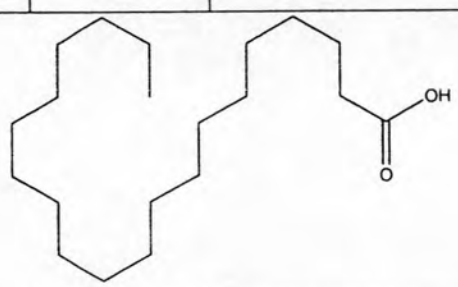
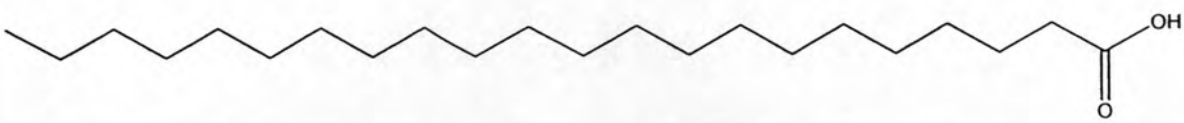
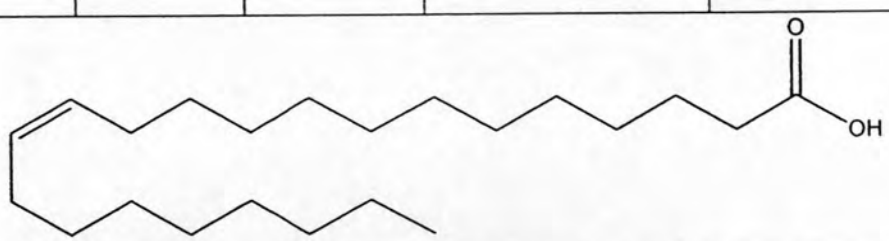
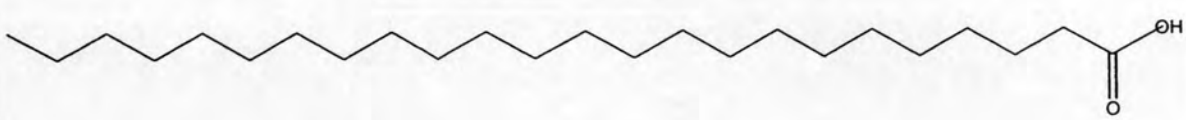
No. of C:No. of double bound	Molecular formular	Molecular mass	Systematic name	Other names
C18:0	$C_{18}H_{36}O_2$	284.48	Octadecanoic acid	Stearic acid
				
C18:1$^{\Delta 9}$	$C_{18}H_{34}0_2$	282.46	9-octadenoic acid	Oleic acid, (9Z)-Octadecenoic acid, (Z)-Octadec-9-enoic acid, *Cis*-9-octadecenoic acid, Cis-Δ9-octadecenoic acid
				
C18:2$^{\Delta 9,12}$	$C_{18}H_{32}O_2$	280.46	(9,12)-Octadecadienoic acid	Linoleic acid, Cis-cis-9,12-Octadecadienoic acid
				
C18:3$^{\Delta 9,12,15}$	$C_{18}H_{30}0_2$	278.44	(9,12,15)-Octadecatrienoic acid	α-Linolenic acid, Cis,cis,cis-9,12,15-Octadecatrienoic acid
				

Table 2.4 Names and structures of the most common fatty acids (*Continued*)

No. of C:No. of double bound	Molecular formular	Molecular mass	Systematic name	Other names
C20:0	$C_{20}H_{40}O_2$	312.54	Eicosanoic acid	Arachidic acid, Arachic acid Eicosanoic acid, *n*-eicosanoic acid
				
C22:0	$C_{22}H_{44}0_2$	340.60	Docosanoic acid	Behenic acid
				
C22:1	$C_{22}H_{42}0_2$	338.58	13-Docosenoic	Erucic acid *Cis*-13-Docosenoic
				
C24:0	$C_{22}H_{48}O_3$	368.63	Tetracosanoic acid	Lignoceric acid
				

2.1.2 Biodiesel production [11]

The major steps required to synthesize biodiesel are as follows:

1. Purification

If waste vegetable oil is used, it is filtered to remove dirt, charred food, and other non-oil materials often found. Water is removed because its presence causes the triglycerides to hydrolyze to give salts of the fatty acids instead of undergoing transesterification to give biodiesel. At home, this is often accomplished by heating the filtered oil to approximately 120°C. At this point, dissolved or suspended water will boil off. When the water boils, it spatters (chemists refer to it as "bumping"). To prevent injury, this operation should be done in a sufficiently large container (at most two thirds full) which is closed but not sealed.

In the laboratory, the crude oil may be stirred with a drying agent such as magnesium sulfate to remove the water in the form of water of crystallization. The drying agent can be separated by decanting or by filtration. However, the viscosity of the oil may not allow the drying agent to mix thoroughly.

2. Neutralization of free fatty acids

A sample of the cleaned oil is titrated against a standard solution of base in order to determine the concentration of free fatty acids (RCOOH) present in the waste vegetable oil sample. The quantity (in moles) of base required to neutralize the acid is then calculated.

3. Transesterification

While adding the base, a slight excess is factored in to provide the catalyst for the transesterification. The calculated quantity of base (usually sodium hydroxide) is added slowly to the alcohol and it is stirred until it dissolves. Sufficient alcohol is added to make up three full equivalents of the triglyceride, and an excess is added to drive the reaction to completion. The solution of sodium hydroxide in the alcohol is then added to a warm solution of the waste oil, and the mixture is heated (typically 50°C) for several hours (4 to 8 typically) to allow the transesterification to proceed. A condenser may be used to prevent the evaporative losses of the alcohol. Care must be taken not to create a closed system which can explode.

4. Workup

Once the reaction is complete, the glycerol should sink. When ethanol is used, it is reported that an emulsion often forms. This emulsion can be broken by standing, centrifugation, or the addition of a low boiling (easily removed) nonpolar solvent, decanting, and distilling. The top layer, a mixture of biodiesel and alcohol, is decanted. The excess alcohol can be distilled off, or it can be extracted with water. If the latter, the biodiesel should be dried by distillation or with a drying agent.

2.1.3 Biodiesel properties [12]

Biodiesel is made up of fourteen different types of fatty acids, which are transformed into fatty acid methyl esters (FAME) by transesterification. Different fractions of each type of FAME present in various feedstocks influence some properties of fuels. Table 2.5 shows some of the properties defined in the ASTM standards for diesel and biodiesel. These properties are described in the remainder of this section, and will be referred to later in this report.

Table 2.5: Comparison of fuel properties between diesel and biodiesel

Fuel Property	Diesel	Biodiesel
Fuel Standard	ASTM D975	ASTM PS 121
Fuel composition	C10-C21 HC	C12-C22 FAME
Lower Heating Value, Btu/gal	131,295	117,093
Viscosity, @ 40° C	1.3-4.1	1.9-6.0
Specific Gravity kg/l @ 60° F	0.85	0.88
Density, lb/gal @ 15° C	7.079	7.328
Water, ppm by wt	161	0.05% max
Carbon, wt %	87	77
Hydrogen, wt %	13	12
Oxygen, by dif. wt %	0	11
Sulfur, wt %	0.05 max	0.0 - 0.0024
Boiling Point (°C)	188-343	182-338
Flash Point (°C)	60-80	100-170
Cloud Point (°C)	-15 to 5	-3 to 12
Pour Point (°C)	-35 to -15	-15 to 10
Cetane Number	40-55	48-65
Stoichiometric Air/Fuel Ratio wt./wt.	15	13.8
BOCLE Scuff, grams	3,600	>7,000

2.1.4 Biodiesel advantages [3]

1. Conventional diesel engines can be operated without much, if any, modification on biodiesel.

2. Biodiesel can be used pure or in a mixture with hydrocarbon-based diesel fuels.

3. Biodiesel is nontoxic, safe to handle and biodegradable.

4. No evaporation of low-boiling components takes place.

5. Exhaust gas is free of SO_2 and halogens.

6. There is substantial reduction of soot, unburnt hydrocarbons, and also of carbon monoxide (when an oxidation catalyst is used) in the exhaust gases.

7. NO_X emissions increase slightly if there are no changes in the engine setting.

8. Good performance in auto-ignition of fatty esters results in a smooth running diesel engine.

9. Biodiesel consumption is similar to hydrocarbon-based diesel fuels.

2.2 Transesterification

In organic chemistry, transesterification is the process of exchanging the alkoxy group of an ester compound by another alcohol. These reactions are often catalyzed by the addition of an acid or base.

Figure 2.1 Transesterification reaction.

Acids can catalyst the reaction by donating a proton to the carbonyl group, thus making it more reactive, while bases can catalyst the reaction by removing a proton from the alcohol, thus making it more reactive.

Transesterification is used in the synthesis of polyester, in which diesters undergo transesterification with diols to form macromolecules. For example, dimethyl terephthalate and ethylene glycol react to form polyethylene terephthalate and methanol, which is evaporated to drive the reaction forward. The reverse reaction (methanolysis) is also an example of transesterification, and has been used to recycle polyesters into individual monomers.

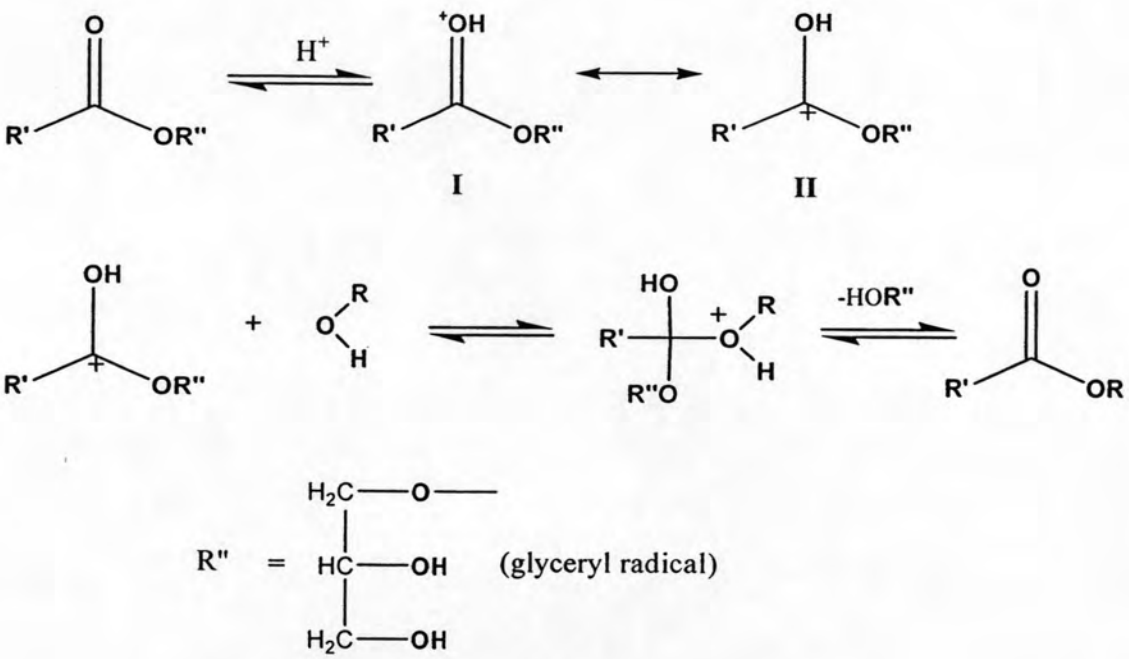
One of the first uses of transesterified vegetable oil (biodiesel) was to power heavy-duty vehicles in South Africa before World War II. The name "biodiesel" has been given to transesterified vegetable oil to describe its use as a diesel fuel.

Biodiesel is produced from vegetable oils by converting the triglyceride oils to methyl (or ethyl) esters with a process known as transesterification. In the transesterification process alcohol reacts with the oil to release three "ester chains" from the glycerin backbone of each triglyceride. The reaction requires heat and a strong base catalyst (e.g., hydroxide or lye), to achieve complete conversion of the vegetable oil into the separated esters and glycerin. The glycerin can be further purified for sale to the pharmaceutical and cosmetic industries. The mono-alkyl esters become the biodiesel, with one-eighth the viscosity of the original vegetable oil. Each ester chain, usually 18 carbons in length for soy esters, retains two oxygen atoms forming the "ester" and giving the product its unique combustion qualities as an oxygenated vegetable based fuel. Biodiesel is nearly 10% oxygen by weight.[13]

2.2.1 Type of transesterification [14]

1. Acid-catalyzed processes

The processes are catalyzed by Bronsted acids, preferably by sulfonic acids, sulfuric acid and hydrochloric acid. These catalysts give very high yields in alkyl esters but the reactions are slow and requiring temperature above 100°C and more than 3 h to reach the complete conversion. Transesterification process under acid-catalyzed condition needs to be done in absences of water because the water reduces the yield alkyl ester. The alcohol/oil molar ratio is one of the main factors that influence the transesterification. An excess alcohol favors the formation of products. On the other hand, an excessive amount of alcohol makes the recovery of glycerol difficult.



R' = carbon chain of the fatty acid

R = alkyl group of the alcohol

Figure 2.2 Mechanism of the acid catalyst transesterification. [14]

2. Base-catalysed processe

This reaction is base catalysed. Any strong base will do, e.g. NaOH, KOH, sodium methoxide etc. Commonly the base (KOH and NaOH) is dissolved in the alcohol to make a convenient method of dispersing the otherwise solid catalyst into the oil. The ROH needs to be very dry. Any water in the process promotes the saponification reaction and inhibits the transesterification reaction.

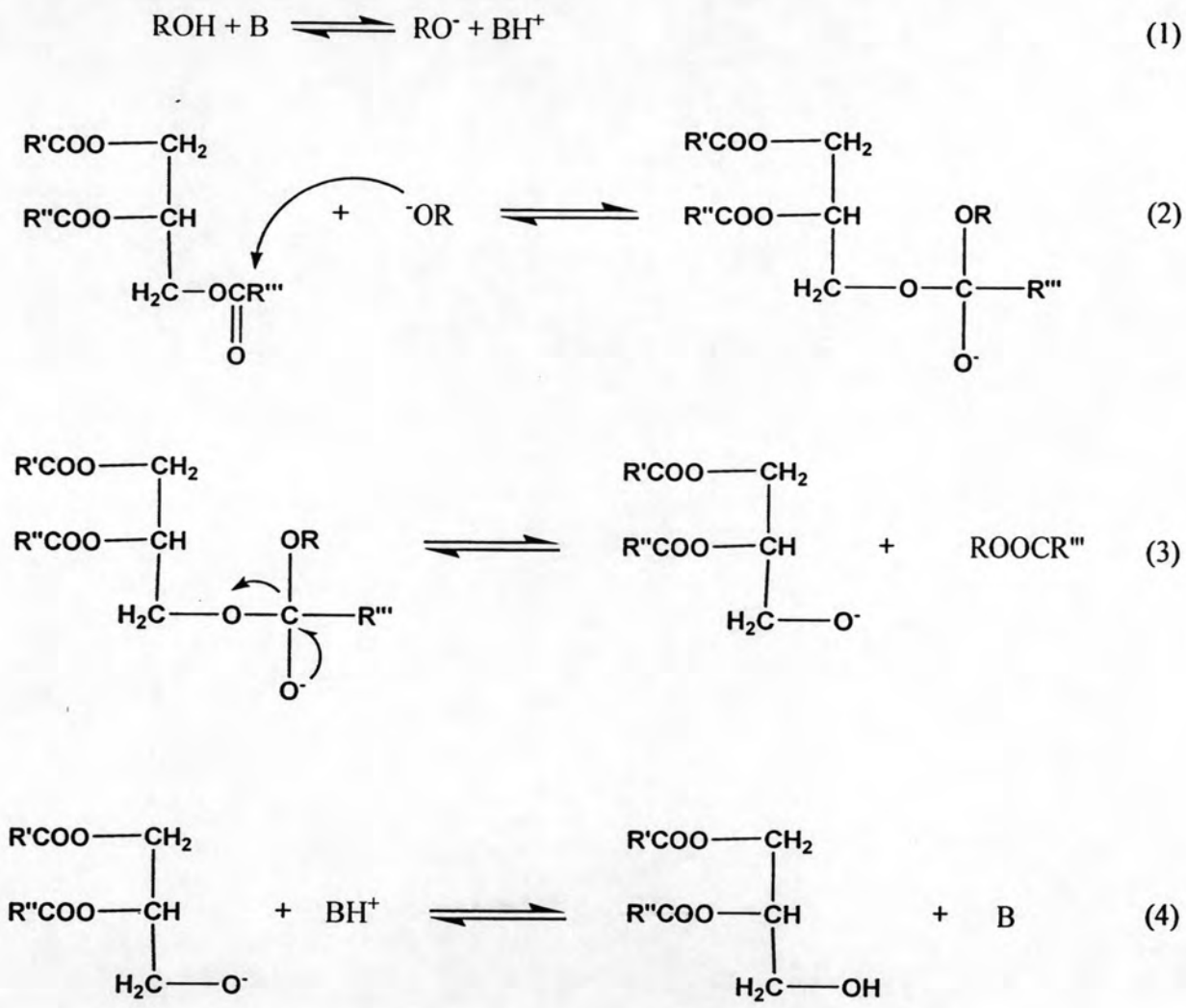


Figure 2.3 Mechanism of the base catalyst transesterification. [14]

3. Lipase-catalyzed process

Although chemical transesterification using an alkali-catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters in short reaction times, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the acidic or alkaline catalyst has to be removed from the product, alkaline wastewater requires treatment, and free fatty acids and water interfere with the reaction. The example of lipase catalysts includes PS 30, Novozyme 435-catalyzed. Recent studies have indicated the use of biocatalyst on the production of biodiesel. The use of whole cell biocatalyst immobilized within biomass support particle, like lipase is of advantage to the biodiesel industry. Immobilized *Pseudomonas fluorescence* lipases is very popular as a biocatalyst relative to mobilized biocatalyst as its activity is more effective and it can be repeatedly used without any decrease activity. Further studies to genetically engineer of this product are being are being done.

4. Non-ionic base-catalyzed process

A great number of organic bases has been developed and used as catalyst or reactant for organic synthesis. These provide for a mild reaction condition to simplify manipulation of the factors involved in increasing the yield of the alkali ester. Bases were used in this process including amines such as triethylamine, piperidine, amidines such as 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 1,5-diazabicyclo[4.3.0]non-5-ene (DBN), guanidines such as 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) and 1,1,3,3-tetramethylguanidine (TMG) and amino- and nitroguanidines such as tris(dimethylamino)methyliminophosphorane (Me_7P).

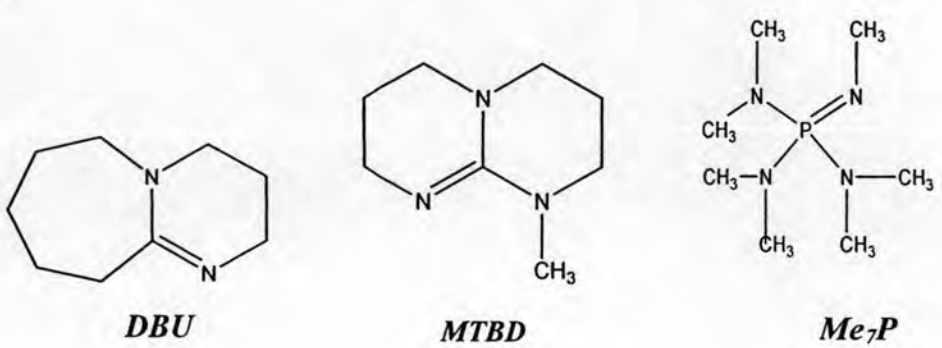


Figure 2.4 Samples of non-ionic base-catalysts. [14]

5. Heterogeneously catalyzed processes

Although transesterification using a conventional base-catalyzed process gives high conversion levels of triglycerides to their corresponding alkyl ester in short times, 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 FFAs and water interfere with reaction. In order to minimize problems, attempts to use heterogeneous catalyst system in alcoholysis of triglycerides have been made. They can be easily separated from the system at the end of the reaction and could also be reused even they could not be for a long duration of time as they leach out and the reaction is incomplete and the phases are difficult to separate.

6. Noncatalytic transesterification process [15]

With the aim of developing a novel methanolysis process for oil without using any catalyst, made a fundamental study of biodiesel production in supercritical methanol. They demonstrated that preheating to a temperature of 350°C and treatment for 240 second in supercritical methanol were sufficient to convert oil to methyl ester. Moreover, while the methyl esters produced were basically the same as those obtained in the conventional method with a basic catalyst, the methyl ester yield of the supercritical methanol method was higher. Kinetic analyses of the reactions in subcritical and supercritical methanol revealed that the rate of oil conversion to methyl esters increased dramatically in the supercritical state. A reaction temperature of 350°C and a molar ratio of methanol to oil of 42 to 1 were considered to be the best conditions.

We can summarize the preparation of biodiesel according to used process, as shown in Table 2.6.

Table 2.6 Classification of processes use different catalysts for the preparation of biodiesel

Homogeneous processes	Heterogeneous processes
Acid-catalyzed process Base-catalyzed process Non-ionic base-catalyzed process Noncatalytic process	Immobilized lipase-catalyzed process Heterogeneous process

Normally, transesterification of vegetable oils is used base-catalyzed processes in commercial due to give a very high yields and very active. After transesterification of vegetable oils, the product is a mixture of esters, glycerol, alcohol, catalyst and tri-, di- and monoglyceride. It is not easy to gain pure ester without impurities such as di- and monoglycerides. [16]

2.3 Oil in microorganism

Oils are compounds that have a significant part of their structure as aliphatic hydrocarbon. They may be substituted with other reactive groups such as hydroxyls and carboxyls; they may be saturated or unsaturated, having double bonded carbons; they may have rings and multiple rings; and they may be combined with carbohydrate, amino acids, and other moieties. This heterogeneous assemblage of diverse structure and function is united primarily by their solubility in nonpolar solvents. They may be divided into a variety of classes, of which I discuss the aliphatic hydrocarbons, the fatty acids, the glycerooils, the sphingooils, and the isoprenoids. Fatty acids are monocarboxylic aliphatic acids whose straight or branched chains may be saturated or unsaturated to various degrees. The chain lengths are usually of an even number of carbons from 10 to 22, in contrast to the predominance of odd-numbered carbon chains in aliphatic hydrocarbons. The fatty acids of fungi do not differ greatly from those of other organisms. The principal ones are palmitic (C16:0), oleic (C18:1) and linoleic (C18:2) acid. Fatty acids are the most abundant oils in nature, but they are commonly found combined with glycerol as fats, oils and phosphooils with long-chain amino alcohols as sphingooils or with sugars or sterols. Free fatty acids are not abundant, although fungal membranes contain

unusually large quantities of them. Their functions depend on their combined states. The oil content of cell wall preparations varied considerably, up to 19% of the dry weight of the wall fractions. [17]

Oils play significant roles as structural as well as storage devices and are also important metabolic intermediates in oil-bearing organisms. Oils are accumulated as oil bodies in almost all eukaryotic organisms at some point during their life cycle. In yeast and bacteria, for example, it has been observed that oil accumulation occurs when the organism is under conditions of stress (e.g., when there is a high carbon to nitrogen ratio in the growth medium).

Triacylglycerol (TG) is the major oil component in oleaginous cells such as those of oleaginous microorganisms, plant oil seeds, and mammalian oil storage tissues. TG is synthesized and assembled in organelles called oil bodies, oil bodies, and oil droplets. The TG structure is characterized by a combination of fatty acyl residues at the *sn*-1, *sn*-2, and *sn*-3 positions, which creates numerous molecular species. The distribution of TG molecular species has been determined in a variety of cells by high performance liquid chromatography (HPLC), or more recently HPLC mass spectrometry, although very little is known about their biosynthetic pathways and regulation. The combination of enzymes for newly formed fatty acids and those for acylation/deacylation reactions yielding TG determines the formation of each TG molecular species. Fatty acids are generated by fatty acid synthase, elongases, desaturases, hydrolases, *etc.* and acylation reactions yielding TG are catalyzed by diacylglycerol acyltransferase, transacylases, *etc.* Although some of these enzymes have been characterized in detail, the combination of these enzymes for the synthesis of each TG molecular species is unknown. In plant seeds, phosphatidylcholine (PC) provides unsaturated fatty acids for TG molecular species, and phosphooil:diacylglycerol acyltransferase was recently found to play an important role in such a mechanism. In addition, some unusual fatty acids, mainly included in TG, may be distributed through channeling mechanisms. In spite of the growing evidence that TG molecular species may be differentially synthesized through specific enzyme pathways, TG molecular species have not been directly quantified. [18]

Triglycerides (TG) are synthesized from dietary fatty acids by the following synthetic pathway in Figure 2.5.

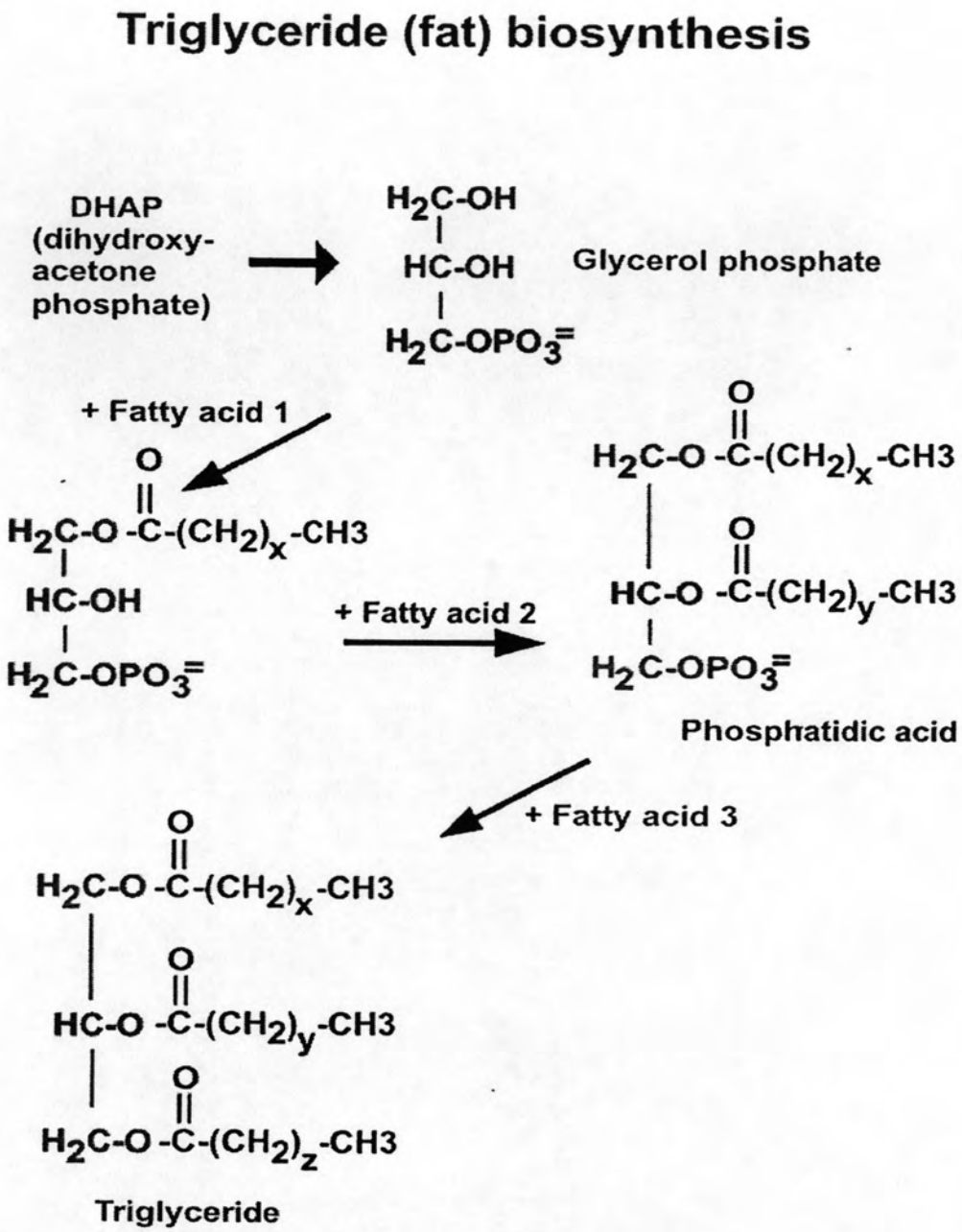


Figure 2.5 Triglyceride biosynthetic pathways. [19]

2.4 Endophytic fungus

2.4.1 Definition of endophytic fungus [20]

The biological and ecological diversity of endophytes is reflected in varying emphasis and heterogeneity of concepts among researchers concerned with studying them. Often the terms "endophyte" and "endophytic" are used with particular meaning by different workers and for particular groups of hosts and microbes. Workers investigating asymptomatic fungal infections of grasses caused by species of Clavicipitaceae and those investigating other microbes, such as endophytic bacteria, have adopted the term "endophyte" and "endophytic" and application of the same terms to different system has contributed to some confusion and controversy. Contemporary application of the terms is not always consistent or accepted among all workers, although as commonly used the terms generally apply to microbes capable of symptomless of occupation of apparently healthy plant tissue.

Important papers published during the past 20 years or so by various authors have stressed particular concepts of endophytism. These have varied in degree of inclusiveness depending on whether primarily organismal interactions or descriptive ecology of endophyte assemblages was emphasized, and on whether the research methodology was primarily histological or based on cultural isolation. The use of "endophyte" and "endophytic" in these varied contexts has contributed to a sense of ambiguity in the application of the terms.

2.4.2 Advantages of endophytic fungus

Endophytic fungus is recognized as one of the most chemically promising groups of fungi in terms of diversity, pharmaceutical and agricultural potential etc. Some examples of bioactive products from endophytic fungus are described below.

1. Metabolite of endophytic fungus as antibiotics [21]

Recently, two endophytic fungi, isolated from monsoonal and tropical rainforests, were reported to produce volatile antibiotics. *Muscodor roseus* was isolated from two monsoonal rainforest tree species in Northern Australia, while *Muscodor albus* was obtained from *Cinnamomum zeylanicum* in Honduras. These endophytes produce a mixture of volatile antimicrobials that effectively inhibit and

kill a wide spectrum of plant associated fungi, bacteria and viruses that effect and protozoans that affect humans and animals.

2. Metabolite of endophytic fungus with anticancer activities [22]

The diterpenoid taxol is an important anticancer agent used widely in the clinic. Taxol and some of its derivatives represent the first major group of anticancer agents that are produced by endophytes. Taxol was isolated from *Taxus brevifolia.*

3. Metabolite of endophytic fungus with antioxidants activities [23]

Pestalotiopsis microspora, an endophytic fungus native to the rainforest of Papua New Guinea, produces a 1,3-dihydroisobenzofuran. This product, pestacin, is 1,5,7-trisubstituted and exhibits moderate antifungal properties and antioxidant activity 11 times greater than the vitamin E derivative trolox. Antioxidant activity is proposed to arise primarily via cleavage of an unusually reactive C–H bond and, to a lesser extent, through O–H abstraction.

4. Metabolite of endophytic fungus with insecticidal activities [23]

Several endophytes are known to have anti-insect properties. Nodulisporic acids, novel indole diterpenes that exhibit potent insecticidal properties against the larvae of the blowfly, work by activating insect glutamate-gated chloride channels.

5. Metabolite of endophytic fungus with antidiabetic activities [23]

A nonpeptidal fungal metabolite was isolated from *Pseudomassaria* sp. This compound acts as insulin mimetic destroyed in the digestive tract and may be given orally.

6. Metabolite of endophytic fungus with immunosuppressive activities [24]

Immunosuppressive drugs are used today to prevent allograft rejection in transplant patients and in the future they could be used to treat autoimmune diseases such as rheumatoid arthritis and insulin-dependent diabetes. The endophytic fungus *Fusarium subglutinans* isolated from *Tripterygium wilfordii* produces the immunosuppressive.

2.5 Literature reviews

2.5.1 Triglyceride in microorganism

In 1988 Sajbidor and coworkers [25] studied the production of oil and fatty acid composition on maltose, lactose, glucose, soluble starch and sodium acetate as a sole carbon source. *Mucor mucedo, Mucor Plumbeus, Mortierella Ramanniana* and *Rhizopus Arrhizus* were tested for growth, oil production and fatty acid composition. In *Mucor mucedo*, sodium acetate gave the best result that gave amount of oil up to 27.1%dry wt. *Mucor Plumbeus*, maltose gave the best result that gave amount of oil up to 23.5%dry wt. *Mortierella* sodium acetate gave the best result that gave amount of oil up to 17.4%dry wt. and maltose gave the best result (amount of oil up to 21.2%dry wt).

In 1996 Rasheva and coworkers [26] studied the lipid biosynthesis of *Monascus purpureus* with various C:N ratios in medium. The 72% (w/w) of lipid production indicated more efficient lipid synthesis at C:N ratio 80:1. The investigation of the fatty acid content showed that the major fatty acids were C18:1 (45.5%) and C16 (22.1%) and a percentage of saturated fatty acids as 51%.

In 1998 Dempster and Sommerfed [27] studied the optimization of growth rate and oil yield in Nile Red mucroalgae as *Nitzschia communis*. Epifluorescent microscopy, utilized with the fluorochrome Nile Red mucroalgae, revealed that neutral oils were a major form of carbon storage in *Nitzschia communis*. Prior to nitrogen deprivation (prestress), rapidly growing cells exhibited multiple (10–15) small neutral oil bodies comprising a small portion (10%–20%) of total cell volume. Storage oil bodies increased in size and coalesced after 2 days of nitrogen deprivation (poststress), creating two to three large neutral oil deposits that made up 60%–75% of total cell volume.

In 2002 Somashekar and coworkers [28] studied the effect of various culture conditions on growth, oil production and fatty acid composition in *Mucor rouxii* and *Mucor* sp. were studied. Total oil production was higher in media containing potassium nitrate for both the cultures (30%) and cultures grown on plant seed oil produced more than 44% oil. Among the carbon sources tested, γ-linolenic acid (GLA) production was maximal in cultures grown on glucose. The major fatty acids produced by these two cultures were palmitic, stearic and oleic acids. Levels of GLA in *M. rouxii* and *M.* sp. were in the range of 3-17% under different culture conditions. Lactose was a poor promoter for biomass and oil production in both cultures. No GLA was found in fungal cultures grown on sesame oil. The optimal conditions for the production of GLA was standardised in these cultures.

In 2005 Dyal and coworkers [29] studied maximizing the production of γ-linolenic acid in *Mortierella ramanniana* var. *M. ramanniana*. was evaluated as a potential industrial producer of γ-linolenic acid (GLA). Six growth variables (pH, temperature, carbon source, nitrogen source, and metal ions and oil supplementation) were systematically manipulated. The results indicated that the GLA production for this particular strain, could be maximized by using a basal growth medium consisting of 5% dextrose and 1% yeast extract, supplemented with 5 mg/L Mn^{2+} with incubation at 20°C. The oil yield under optimum conditions was 54.2% of the total dry biomass and consisted of 84.3% unsaturated fatty acids.

In 2006 Mat-arhin studied metabolite of endoohytic fungus. The mycelium was extracted by hexane that gave amount of oil up to 30% dry weight in malt extract broth (MEB).

2.5.2 Biodiesel production

In 2004 Ramadhas and coworkers [30] studied production of fuel-quality biodiesel from high FFA. It was found that the feedstocks with high FFAs could not be transesterified with the commercially available alkaline catalyst transesterification process. A two-step transesterification process is developed to convert the high FFA oils to its esters. The first step (acid catalyzed esterification via H_2SO_4 1%w/w)

reduces the FFA content of the oil to less than 2%. The alkaline catalyst transesterification process converts the products of the first step to its mono-esters and glycerol. Excess addition of sulphuric acid darkens the product. It has been also found that the conversion efficiency is strongly affected by molar ratio of alcohol to oil. The molar ratio of 6:1 favors the completion of alkaline catalyzed esterification process with in half an hour. The maximum ester conversion is achieved at the reaction temperature of 45-50 °C. The flash point of biodiesel (about 130 °C) is greater than that of diesel and the calorific value is slightly lower than that of diesel. This two-step esterification method reduces the overall production cost of the biodiesel, as it uses low cost unrefined non-edible oils.

In 2005 Miao and Wu [31] studied method for the production of biodiesel from heterotrophic microalgal oil. *Chlorella protothecoides* is a microalgae that can be photoautotrophically or heterotrophically grown under different culture conditions. Heterotrophic growth of *C. protothecoides* resulted in the accumulation of a large amount of oil in cells. Oil content in heterotrophic cells reached as high as 55.20%. Large amount of microalgal oil was efficiently extracted from these heterotrophic cells by using n-hexane. Biodiesel comparable to conventional diesel was obtained from heterotrophic microalgal oil by acidic transesterification. Good quality of biodiesel could be obtained in the presence of 100% acid catalyst (on oil basis) at high temperature. The best combination of factors was 100% catalyst quantity (based on oil weight) with 56:1 molar ratio of methanol to oil at temperature of 30°C, which reduced product specific gravity from an initial value of 0.912 to a final value of 0.8637 in about 4 h of reaction time.

In 2006 Veljkovic and coworker [32] studied the production of fatty acid methyl esters (FAME) from crude tobacco seed oil (TSO) having high free fatty acids (FFA) was investigated. Due to its high FFA, the TSO was processed in two steps: the acid-catalyzed esterification (ACE) followed by the base-catalyzed methanolysis (BCM). The first step reduced the FFA level to less than 2% in 25 min for the molar ratio of 18:1. The second step converted the product of the first step into FAME and glycerol. The maximum yield of FAME was about 91% in about 30 min. The tobacco biodiesel obtained had the fuel properties within the limits prescribed by the latest American (ASTM D 6751-02) and European (DIN EN 14214) standards, except a

somewhat higher acid value than that prescribed by the latter standard (<0.5). Thus, tobacco seeds (TS), as agricultural wastes, might be a valuable renewable raw material for the biodiesel production.

In 2007 Berchmans and Hirata [33] studied biodiesel production from crude *Jatropha curcas* oil (CJCO) with a high content of FFA. In alkali base catalyzed transesterification process, the presence of high concentration of FFA reduced the yield of methyl esters of fatty acids significantly. A two-stage transesterification process was selected to improve the methyl ester yield. The first stage was acid pretreatment process (H_2SO_4 1%w/w as catalyst), which could reduce the FFA level of CJCO to less than 1%. The second stage, alkali base catalyzed transesterification process gave 90% methyl ester.

In 2007 Wang and coworkers [34] studied a two step catalyzed process for biodiesel from waste cooking oil. Acid value in oil was 75.92±0.036 mgKOH/g. The free fatty acids of WCO were esterified with methanol catalyzed by ferric sulfate in the first step, and the triglycerides (TGs) in WCO were transesterified with methanol catalyzed by potassium hydroxide in the second step. The results showed that ferric sulfate had high activity to catalyze the esterification of free fatty acids (FFA) with methanol, The conversion rate of FFA reached 97.22% when 2 wt% of ferric sulfate was added to the reaction system containing methanol to TG in10:1 (mole ratio) composition and reacted at 95°C for 4 h. The methanol was vacuum evaporated, and transesterification of the remained triglycerides was performed at 65°C for 1 h in a reaction system containing 1 wt% of potassium hydroxide and 6:1 mole ratio of methanol to TG. The final product with 97.02% of biodiesel, obtained after the two step catalyzed process, was analyzed by gas chromatography. This new process has many advantages compared with the old processes, such as no acidic waste water, high efficiency, low equipment cost and easy recovery of the catalyst.