

Production of Biodiesel by Lipase-Catalyzed
Transesterification using Immobilized
Pseudomonas fluorescens

And
Comparison of the Biodiesels and Quality Analysis

Project Report

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Project Report

*In partial fulfillment of the
Curricular Requirement for the Degree of
Bachelor of Technology*

By

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DECLARATION

I hereby declare that my dissertation entitled “*Production of Biodiesel by Lipase-Catalyzed Transesterification using Immobilized **Pseudomonas fluorescens** And Comparison of the Biodiesels and Quality Analysis*” is not substantially the same as any that I have submitted for a degree or diploma or other qualification at any other colleges or university.

I further state that no part of my dissertation has already been or is being concurrently submitted for any such degree or diploma or other qualification.

Except where explicit reference is made to the work of others, this dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration.

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Abstract

Biodiesel is an eco-friendly alternative fuel for diesel engines consisting of the alkyl monoesters of fatty acids from vegetable oils or animal fats. Most of the biodiesel that is currently made uses soybean oil, methanol, and an alkaline catalyst. The high value of soybean oil as a food product makes production of a cost-effective fuel very challenging. However, there are large amounts of low-cost oils and fats such as waste vegetable oils and animal fats that could be converted to biodiesel. The problem with processing these low cost oils and fats is that they often contain large amounts of free fatty acids (FFA) that cannot be converted to biodiesel using an alkaline catalyst. In this study, a newer approach is investigated to reduce the free fatty acids content of these feedstocks using a microbial lipase catalyzed enzymatic method of transesterification process and produce biodiesel fuel of standard quality.

Though efficient in terms of reaction yield and time, the chemical approach to synthesizing alkyl esters from triglycerides has drawbacks, such as difficulties in the recovery of glycerol, the need for using reactive catalysts, and the energy-intensive nature of the process. On the other hand, biocatalysts such as microbial lipase allow for synthesis of specific alkyl esters, easy recovery of glycerol, and transesterification of glycerides with high free fatty acid (FFA) content. This newer approach could be extended to transesterification of greases, which are even less expensive than edible vegetable oils, like soybean oil. This process can further be used to synthesize other value-added products, including biodegradable lubricants and additives. Lipase can also be used to introduce other functionalities into alkyl esters that may further improve the cold temperature properties of the resulting biodiesel, which remain a matter of further investigation.

Keywords: Alternative fuel, Biodiesel, Diesel, High free fatty acid, Animal fat, Waste vegetable oil, Transesterification.

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Chapter 1

INTRODUCTION

1.1 General Background

1.1.1 Introduction to Biodiesel

General Definition of Biodiesel

Biodiesel is a domestic, renewable fuel for diesel engines derived from natural oils and which meets the specifications of ASTM (American Society of Testing and Materials) D 6751.

Clarifying language to general definition

Biodiesel can be used in any concentration with petroleum based diesel fuel in existing diesel engines with little or no modification. Biodiesel is not the same thing as raw vegetable oil. It is produced by a chemical process which removes the glycerin from the oil.

Technical Definition for Biodiesel (ASTM D 6751) and Biodiesel Blend

Biodiesel — a fuel comprised of **fatty acyl mono-alkyl esters (FAME)** of long chain fatty acids derived from vegetable oils or animal fats, designated as B100, and meeting the requirements of ASTM D 6751.

Biodiesel Blend — a blend of biodiesel fuel meeting ASTM D 6751 with petroleum-based diesel fuel, designated BXX, where XX represents the volume percentage of biodiesel fuel in the blend.

Clarifying language to technical definition

Biodiesel, as defined in D 6751, is registered with the US EPA as a fuel and a fuel additive under Section 211(b) of the Clean Air Act.

Biodiesel is typically produced by a reaction of a vegetable oil or animal fat with an alcohol such as methanol or ethanol in the presence of a catalyst to yield mono-alkyl esters and glycerin, which is removed.

Blends ^[1.1]

Much of the world uses a system known as the "B" factor to state the amount of biodiesel in any fuel mix:

- 100% biodiesel is referred to as **B100**, while
- 20% biodiesel is labeled **B20**
- 5% biodiesel is labeled **B5**
- 2% biodiesel is labeled **B2**

Obviously, the higher the percentage of biodiesel, the more ecology-friendly the fuel is.

[Note: August 10th has been declared "*International Biodiesel Day*".]

1.1.2 Comparison between Biodiesel, Vegetable Oils & Petro-diesel ^[1,2]

Property	Biodiesel	Vegetable oils (SVO, WVO)	Petro-diesel
Meant to be used in	Standard diesel engines	Converted diesel engines	Standard diesel engines
Source	Plant oil or animal fat	Plant oil	Petroleum
Chemical nature	Mono-alkyl ester	Saturated fat Monounsaturated fat Polyunsaturated fat Trans fat	75% saturated hydrocarbons (primarily paraffins including n, iso, and cycloparaffins), and 25% aromatic hydrocarbons (including naphthalenes and alkyl benzenes).
Glycerin Removal	Yes	No	No
Transesterification	Yes	No	No
Freezing point	-5 to 0°C	-10°C to -12°C	0°C
Free fatty acid content	Very low	High	Low
Lubricating properties	Very good	Moderate	Low
Cetane ratings	High (45-65)	Low (~40)	Moderate (~45)
Iodine Value	Very Low (10-15)	Very High (125-150)	Low (<25)
Calorific value	33-40 MJ/L	37 MJ/L	38.3 MJ/L
Viscosity @ 40°C	Low (4-6 mm ² s ⁻¹)	Very High (37 mm ² s ⁻¹)	Low (4-5 mm ² s ⁻¹)
Affects Engines	No	Yes	Yes
Preheating Necessary	No	Yes	In Winter

1.1.3 Energy Content

The energy content (also referred to as heating value) of biodiesel fuel is its heat of combustion; the heat released when a known quantity of fuel is burned under specific conditions.

The heating value is usually expressed as British thermal units (Btu) per pound or per gallon at 60°F (International metric [SI] units are kilojoules per kilogram or per cubic meter at 15°C). For gross (high) heating value, the water produced by the combustion is assumed to be re-condensed to a liquid. For the net (lower) heating value, the water remains as a gas. Since engines exhaust water as a gas, the net heating value is the appropriate value for comparing fuels.

The three main factors that affect vehicle fuel economy, torque, and horsepower are the type of engine (i.e. gasoline or diesel), the efficiency of the engine turning energy in the fuel into usable work, and the fuel's volumetric energy content or heating value. The energy content of conventional diesel can vary up to 15% from supplier to

supplier or from summer to winter. The efficiency of diesel engines is the same whether using biodiesel, diesel, or biodiesel blends so differences in horsepower, torque or fuel economy are due to entirely volumetric energy content. The energy content of biodiesel is much less variable than that of petrodiesel, and with biodiesel meeting ASTM D 6751 standards the energy content is more dependent upon the feedstock used than the particular process.

Average Density and Heating Value of Biodiesel and Diesel Fuel ^[1,3]

Fuel	Density (in g/cm³)	Net Heating Value (Btu/gal)	% Difference vs. Petro-diesel Avg.
Petro-diesel	0.850	129,500	-
Biodiesel	0.880	118,296	8.56%
B20 Blend	0.856	127,259	1.73%
B2 Blend	0.851	129,276	0.17%

1.1.4 Performance

Successful alternative fuels fulfill environmental and energy security needs without sacrificing operating performance. Operationally, biodiesel blends perform very similar to low sulfur diesel in terms of power, torque, and fuel without major modification of engines or infrastructure.

Biodiesel offers similar power to diesel fuel

One of the major advantages of biodiesel is the fact that it can be used in existing engines and fuel injection equipment with little impact to operating performance. Biodiesel has a higher Cetane number than most fuel. In more than 50 million on-road miles and countless marine and off-road applications, biodiesel shows similar fuel consumption, horsepower and torque as conventional diesel fuel.

Biodiesel provides significant lubricity improvement over petroleum diesel fuel

Lubricity results of biodiesel and petroleum diesel using industry test methods indicate that there is a marked improvement in lubricity when biodiesel is added to conventional diesel fuel. Even biodiesel levels as low as one percent can provide up to a 65 percent increase in lubricity in distillate fuels.

Compatibility of biodiesel with engine components

The switch to low sulfur diesel fuel has caused most OEMs (*Original Equipment Manufacturers*) to switch to components suitable for use with biodiesel. In general, pure biodiesel will soften and degrade certain types of elastomers and natural rubber compounds overtime. Using high percent blends can impact fuel system components (primarily fuel hoses and fuel pump seals), that contain elastomer compounds incompatible with biodiesel. Manufacturers recommend that natural or butyl rubbers not be allowed to come in contact

Blends of B20 or lower have not exhibited elastomer degradation and need no changes. If a fuel system does contain these materials and users wish to fuel with blends over B20, replacement with compatible elastomers is recommended.

Biodiesel in cold weather

Cold weather can cloud and even gel any diesel fuel, including biodiesel. Users of a 20 percent biodiesel blend with #2 diesel will usually experience an increase of the cold flow properties (cold filter plugging point, cloudpoint, pour point) approximately 2 to 10°F. Precautions employed for petroleum diesel are needed for fueling with 20 percent blends. Neat (100 percent) biodiesel will gel faster than petrodiesel in cold weather operations. Solutions for winter operability with neat biodiesel are much the same as that for low-sulfur diesel (i.e., blending with diesel, utilization of fuel heaters, and storage of the vehicle in or near a building). These same solutions work well with biodiesel blends, as do the use of cold flow improvement additives.

1.1.5 Advantages of Biodiesel over Petrodiesel^[1,3]

Reduction of Pollution as Compared with Petroleum

The use of biodiesel in a conventional diesel engine results in a substantial reduction of unburned hydrocarbons, carbon monoxide, and particulate matter compared to emissions from diesel fuel. Bio-diesel is considered clean fuel since it has almost no sulphur, no aromatics and has about 10% built-in oxygen, which helps it to burn fully. Its higher Cetane number improves the ignition quality even when blended in the petroleum diesel.

The exhaust emissions of sulfur oxides and sulfates (major components of acid rain) from biodiesel are essentially eliminated compared to diesel. Of the major exhaust pollutants, both unburned hydrocarbons and nitrogen oxides have reduced substantially. Emissions of nitrogen oxides are either slightly reduced or slightly increased depending on the duty cycle of the engine and testing methods used.

Mitigation of “Global Warming”

Biodiesel is the best greenhouse gas mitigation strategy for today’s medium and heavy duty vehicles. A 1998 biodiesel lifecycle study, jointly sponsored by the U.S. Department of Energy and the U.S. Department of Agriculture, concluded biodiesel reduces net carbon dioxide emissions by 78 percent compared to petroleum diesel. This is due to biodiesel’s closed carbon cycle. The CO₂ released into the atmosphere when biodiesel is burned is recycled by growing plants, which are later processed into fuel.

Health perspective

Scientific research confirms that biodiesel exhaust has a less harmful impact on human health than petroleum diesel fuel. Pure biodiesel emissions have decreased levels of polycyclic aromatic hydrocarbons (PAH) and nitrated PAH compounds that have been identified as potential cancer causing compounds. Also, particulate matter, an emission linked to asthma and other diseases, is reduced by about 47 percent, and CO, a poisonous gas, is reduced by about 48 percent.

1.1.6 Customer Usage

Storage

In general, the standard storage and handling procedures used for petroleum diesel can be used for biodiesel. The fuel should be stored in a clean, dry, dark environment. Acceptable storage tank materials include aluminum, steel, fluorinated polyethylene, fluorinated polypropylene and Teflon. Copper, brass, lead, tin, and zinc should be avoided.

Use of Biodiesel in Petroleum Based Engines

Biodiesel addition reduces fuel system wear, and in low levels in high pressure systems increases the life of the fuel injection equipment that relies on the fuel for its lubrication.

Biodiesel blends of up to 20 percent work in any diesel engine with no modifications to the engine or the fuel system. Biodiesel has a cleansing effect that may release deposits accumulated on tank walls and pipes from previous diesel fuel usage. The release of deposits may end up in fuel filters initially, so fuel filters should be checked more frequently at first.

1.1.7 Biodiesel Emission

Biodiesel is the first and only alternative fuel to have a complete evaluation of emission results and potential health effects submitted to the U.S. Environmental Protection Agency (EPA) under the Clean Air Act Section 211(b). These programs include the most stringent emissions testing protocols ever required by EPA for certification of fuels or fuel additives. The data gathered

Complete the most thorough inventory of the environmental and human health effects attributes that current technology will allow.

EPA has surveyed the large body of biodiesel emissions studies and averaged the Health Effects testing results with other major studies. The results are seen in the table below.

Average biodiesel emissions compared to conventional diesel according to EPA ^[1,4]

Emission Type	Biodiesel	Petrodiesel
<i>Regulated</i>		
Total Unburned Hydrocarbons	-67%	-20%
Carbon Monoxide	-48%	-12%
Particulate Matter	-47%	-12%
NO _x	+10%	+2% to -2%
<i>Non-Regulated</i>		
Sulfates	-100%	-20%
PAH (Polycyclic Aromatic Hydrocarbons)	-80%	-13%
nPAH (nitrated PAH's)	-90%	-50%
Ozone potential of HC	-50%	-10%

1.1.8 Benefits of Biodiesel

Environmental Benefits

In 2000, biodiesel became the only alternative fuel in the country to have successfully completed the EPA-required Tier I and Tier II health effects testing under the Clean Air Act. These independent tests conclusively demonstrated biodiesel's significant reduction of virtually all regulated emissions, and showed biodiesel does not pose a threat to human health.

Biodiesel contains virtually no sulfur or aromatics, and use of biodiesel in a conventional diesel engine results in substantial reduction of unburned hydrocarbons, carbon monoxide and particulate matter. A U.S. Department of Energy study showed that the production and use of biodiesel, compared to petroleum diesel, resulted in a 78.5% reduction in carbon dioxide emissions. Moreover, biodiesel has a positive energy balance. For every unit of energy needed to produce a gallon of biodiesel, at least 4.5 units of energy are gained.

Advantages of biodiesel on environment as compared with petrodiesel in terms of their emissions

- *The ozone (smog) forming potential of biodiesel hydrocarbons is less than diesel fuel*

The ozone forming potential of the hydrocarbon emissions is 50 percent less than that measured for diesel fuel.

- *Sulfur emissions are essentially eliminated with pure biodiesel*

The exhaust emissions of sulfur oxides and sulfates (major components of acid rain) from biodiesel are essentially eliminated compared to diesel.

- *Criteria pollutants are reduced with biodiesel use*

Tests show the use of biodiesel in diesel engines results in substantial reductions of unburned hydrocarbons, carbon monoxide, and particulate matter. Emissions of nitrogen oxides stay the same or are slightly increased.

Carbon Monoxide – The exhaust emissions of carbon monoxide (a poisonous gas) from biodiesel are on average 48 percent lower than CO emissions from diesel.

Particulate Matter – Breathing particulate has been shown to be a human health hazard. The exhaust emissions of particulate matter from biodiesel are about 47 percent lower than overall particulate matter emissions from diesel.

Hydrocarbons – The exhaust emissions of total hydrocarbons (a contributing factor in the localized formation of smog and ozone) are on average 67 percent lower for biodiesel than diesel fuel.

Nitrogen Oxides – NO_x emissions from biodiesel increase or decrease depending on the engine family and testing procedures. NO_x emissions (a contributing factor in the localized formation of smog and ozone) from pure (100%) biodiesel increase on average by 10 percent. However, biodiesel's lack of sulfur allows the use of NO_x

control technologies that cannot be used with conventional diesel. Additionally, some companies have successfully developed additives to reduce NOx emissions in biodiesel blends.

Biodiesel reduces the health risks associated with petroleum diesel

Biodiesel emissions show decreased levels of polycyclic aromatic hydrocarbons (PAH) and nitrated polycyclic aromatic hydrocarbons (nPAH), which have been identified as potential cancer causing compounds. In Health Effects testing, PAH compounds were reduced by 75 to 85 percent, with the exception of benzo (a) anthracene, which was reduced by roughly 50 percent. Targeted nPAH compounds were also reduced dramatically with biodiesel, with 2-nitrofluorene & 1-nitropyrene reduced by 90 percent, and the rest of the nPAH compounds reduced to only trace levels.

Energy Security Benefits

With agricultural commodity prices approaching record lows, and petroleum prices approaching record highs, it is clear that more can be done to utilize domestic surpluses of vegetable oils while enhancing our energy security. Because biodiesel can be manufactured using existing industrial production capacity, and used with conventional equipment, it provides substantial opportunity for *immediately* addressing our energy security issues.

If the true cost of using foreign oil were imposed on the price of imported fuel, renewable fuels, such as biodiesel, probably would be the most viable option. For instance, in 1996, it was estimated that the military costs of securing foreign oil was \$57 billion annually. Foreign tax credits accounted for another estimated \$4 billion annually and environmental costs were estimated at \$45 per barrel. For every billion dollars spent on foreign oil, America lost 10,000 – 25,000 jobs.

Economic Benefits

The biodiesel industry has contributed significantly to the domestic economy. The 51,893 jobs that are currently supported by the US biodiesel industry reflect the beginning of the industry's potential to create jobs and economic growth in the US economy. Biodiesel has added \$4.287 billion to the Gross Domestic Product (GDP). Biodiesel has the potential to support more than 78,000 jobs by 2012. A stable, thriving biodiesel industry is necessary if the U.S. is to eventually benefit from the commercial scale production of algal-based biofuels. The NBB estimates that for every 100 million gallons of biodiesel that is produced from algae, 16,455 jobs will be created and \$1.461 billion will be added to the GDP.

Quality Benefits

Biodiesel is registered as a fuel and fuel additive with the EPA and meets clean diesel standards established by the California Air Resources Board (CARB). B100 (100 percent biodiesel) has been designated as an alternative fuel by the U.S. Department of Energy and the U.S. Department of Transportation. Moreover, in December 2001, the American Society of Testing and Materials (ASTM) approved a specification (D675) for biodiesel fuel. This development was crucial in standardizing fuel quality for biodiesel in the U.S. market. As of

2008, there is a specification for B6-B20, and up to B5 is included in the diesel fuel specification (D 975).

The biodiesel industry also utilizes a voluntary quality management certification program for biodiesel producers, marketers, and laboratories called the BQ-9000 Program. The BQ-9000 Program combines internationally accepted quality management principles with the ASTM biodiesel fuel specification to help ensure that customers and end users get the highest quality fuel possible. The National Biodiesel

1.1.9 Biodiesel as Feedstocks for Industry

Biodiesel or Fatty acid methyl esters can be transformed into a lot of useful chemicals, and raw materials for further synthesis, as shown in Fig. 1.1.^[1,5] The alkanolamides, whose production consumes the major part of the methyl esters produced in the world, have a direct application as non-ionic surfactants, emulsifying, thickening and plastifying agents. The fatty alcohols are applied as pharmaceutical and cosmetics additives (C16-C18), as well as lubricants and plastifying agents (C6-C12), depending on the length of their carbon chain. The isopropyl esters are also applied as plastifying agents and emollients. However, they can not be produced in a convenient way by esterification of fatty acids, as an azeotrope formed by water and isopropanol avoids the recycling of the alcohol. The fatty acid methyl esters are further used in the manufacture of carbohydrate fatty acid esters (sucrose polyesters), which can be applied as non-ionic surfactants or edible non-calorific oils, and can be used as an alternative fuel substitute for diesel engines (biodiesel). The glycerol also has important applications, in cosmetics, toothpastes, pharmaceuticals, food, lacquers, plastics, alkyl resins, tobacco, explosives, cellulose processing.

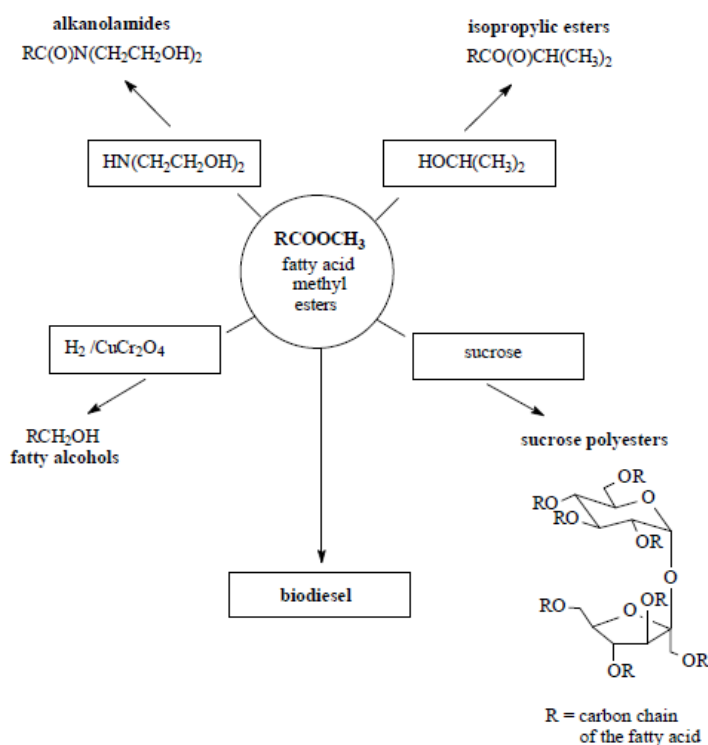


Fig. 1.1: Some fatty acid methyl esters applications.

1.2 Objectives

Primary Objectives of the Project

- i. Production of biodiesel from Soybean oil (or Straight Vegetable Oil, SVO) by Immobilized Microbial Lipase-catalyzed enzymatic transesterification.
- ii. Production of biodiesel from Soybean oil (or Straight Vegetable Oil, SVO) and Waste Vegetable Oil (WVO) by Base-catalyzed chemical transesterification.
- iii. Comparative studies on the cost-effectiveness of using different transesterification processes.
- iv. Comparative studies on the quality parameters of the biodiesels produced from two sources using different transesterification processes.

Secondary Objectives of the Project

- i. Recovery and Purification of glycerin for its suitable use.
- ii. Design, Modeling & Process Simulation of the manufacturing plant for the industrial production of biodiesel.
- iii. Proposal and analysis of the aspects of using genetically modified microorganisms for higher expression of lipase.

Chapter 2

LITERATURE REVIEW

2.1 Sources of Biodiesel

Plant -- as a source of Biodiesel

The main commodity sources for Bio-diesel in India can be non-edible oils obtained from plant species such as *Jatropha curcas* (Ratanjyot), *Pongamia pinnata* (Karanj), *Calophyllum inophyllum* (Nagchampa), *Hevca brasiliensis* (Rubber) etc.

Algae -- as a source of Biodiesel

Algae have emerged as one of the most promising sources for biodiesel production. It can be inferred that algae grown in CO₂-enriched air can be converted to oily substances. Biodiesel can be produce using common species like *Oedogonium* and *Spirogyra*.

Straight Vegetable Oil -- as a source of Biodiesel

Edible oil from sunflower, rapeseed etc. is the raw material used in Europe for industrial scale biodiesel production, whereas soybean is used in USA. Thailand uses palm oil, Ireland uses frying oil and animal fats.

Waste Vegetable Oil-- as a source of Biodiesel

Conversion of waste vegetable oils and fats to biodiesel fuel is one possibility but poses some difficulties such as in the use of toxic or caustic materials and by-product disposal. Conversion to biodiesel may also decrease the economic attractiveness of using waste oils as fuels. An alternative to the conversion of waste vegetable oils into biodiesel by chemical methods can be achieved by using natural sources of enzymes such bacteria and fungi which could mediate the production of biodiesel from WVOs in a more natural and economic way without emanating any toxic hazards.

2.2 Biodiesel Standards ^[2.1, 2.2, 2.3]

Since biodiesel is produced in quite differently scaled plants from vegetable oils of varying origin and quality, it was necessary to install a standardization of fuel quality to guarantee engine performance without any difficulties. Austria was the first country in the world to define and approve the standards for rapeseed oil methyl esters as diesel fuel. As standardization is a prerequisite for successful market introduction and penetration of biodiesel, standards or guidelines for the quality of biodiesel have also been defined in other countries like Germany, Italy, France, the Czech Republic and in the United states.

The parameters, which define the quality of biodiesel, can be divided into two groups. One group contains general parameters, which are also used for mineral oil based fuel, and the other group especially describes the chemical composition and purity of fatty acid alkyl esters. Table 2.1 contains the general and Table 2.2 the vegetable oil specific parameters and the corresponding value of fatty acid methyl esters according to standards of above countries.

Table 2.1

General parameters of the quality of biodiesel						
Parameters	Austria (ON)	Czech republic (CSN)	France (journal official)	Germany (DIN)	Italy (UNI)	USA (ASTM)
Density at 15 °C g/cm ³	0.85–0.89	0.87–0.89	0.87–0.89	0.875–0.89	0.86–0.90	–
Viscosity at 40 mm ² /s	3.5–5.0	3.5–5.0	3.5–5.0	3.5–5.0	3.5–5.0	1.9–6.0
Flash point (°C)	100	110	100	110	100	130
CFPP (°C)	0/–5	–5	–	0–10/–20	–	–
Pour point (°C)	–	–	–10	–	0/–5	–
Cetane number	≥49	≥48	≥49	≥49	–	≥47
Neutralization number (mgKOH/g)	≤0.8	≤0.5	≤0.5	≤0.5	≤0.5	≤0.8
Conradson carbon residue (%)	0.05	0.05	–	0.05	–	0.05

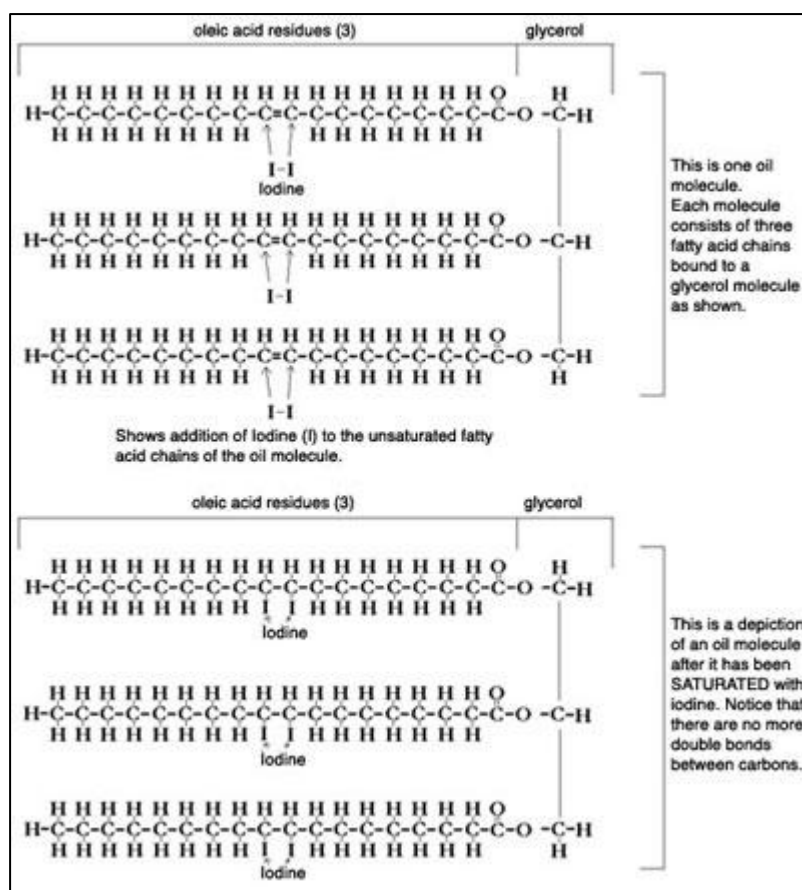
Table 2.2

Vegetable oil specific parameters for the quality of biodiesel						
Parameters	Austria (ON)	Czech republic (CSN)	France (journal official)	Germany (DIN)	Italy (UNI)	USA (ASTM)
Methanol/ ethanol (% mass)	≤ 0.2	–	≤ 0.1	≤ 0.3	≤ 0.2	–
Ester content (% mass)	–	–	≥ 96.5	–	≥ 98	–
Monoglyceride (% mass)	–	–	≤ 0.8	≤ 0.8	≤ 0.8	–
Diglyceride (% mass)	–	–	≤ 0.2	≤ 0.4	≤ 0.2	–
Triglyceride (% mass)	–	–	≤ 0.2	≤ 0.4	≤ 0.1	–
Free glycerol (% mass)	≤ 0.02	≤ 0.02	≤ 0.02	≤ 0.02	≤ 0.05	≤ 0.02
Total glycerol (% mass)	≤ 0.24	≤ 0.24	≤ 0.25	≤ 0.25	–	≤ 0.24
Iodine number	≤ 120	–	≤ 115	≤ 115	–	–

2.2.1 Iodine Value^[2,4]

Chemically, vegetable and animal oils and fats are triglycerides. Animal fat is saturated, meaning that in the fatty acid portion, all the carbon atoms are bound to two hydrogen atoms, and there are no double bonds. This allows the chains of fatty acids to be straighter and more pliable so they harden at higher temperatures. As the number of double bonds in a fatty acid is increased, they reduce that ability for oils to gain a conformation that would make them solid, so they remain liquid.

To test a vegetable oil to see how many double bonds it has (how unsaturated it is) iodine is introduced to the oil. The iodine will attach itself over a double bond to make a single bond where an iodine atom is now attached to each carbon atom in that double bond. Higher iodine numbers do not refer to the amount of iodine in the oil, but rather the amount of iodine needed to "saturate" the oil, or break all the double bonds.



The traditional measure of the degree of bonds available for this process is given by the 'Iodine Value' (IV) and can be determined by adding iodine to the fat or oil. The amount of iodine in grams absorbed per 100 ml of oil is the IV. The higher the IV, the more unsaturated (the greater the number of double bonds) the oil and the higher is the potential for the oil to polymerize.

When it dries the oil irreversibly polymerizes into a tough, insoluble plastic-like solid. At the high temperatures in internal combustion engines the process is accelerated. Steadily accumulating films of tough, insoluble plastic-like solids are not what are intended in the engine and injector pump.

Polymerization happens when the double bonds in unsaturated oil molecules are broken by oxygen from the air or water. The oil oxidizes, forming peroxides (hydroperoxides), and the peroxides polymerize, bonding with carbon to create a long and stable molecule called a polymer (plastic). Another effect of oxidation is that the hydroperoxides attack elastomers, such as rubber seals.

Saturated oils don't polymerize, unsaturated oils do. The level of unsaturation is called the Iodine Value (IV) -- the higher the IV the more unsaturated the oil, the faster it will oxidize and the more it will polymerize.

While some oils have a low IV and are suitable for use as fuel without any further processing other than extraction and filtering, the majority of vegetable and animal oils have an IV which may cause problems if used as a neat fuel.

The IV can be easily reduced by hydrogenation of the oil (reacting the oil with hydrogen), the hydrogen breaking the double bond and converting the fat or oil into a more saturated oil which reduces the tendency of the oil to polymerize. However this process also increases the melting point of the oil.

Oils and their melting points and Iodine Values		
Oil	Approx. melting point deg C	Iodine Value
Coconut oil	25	10
Palm kernel oil	24	37
Mutton tallow	42	40
Beef tallow	-	50
Palm oil	35	54
Olive oil	-6	81
Castor oil	-18	85
Peanut oil	3	93
Rapeseed oil	-10	98
Cotton seed oil	-1	105
Sunflower oil	-17	125
Soybean oil	-16	130

As can be seen from the table below, only coconut oil has an IV low enough to be used without any potential problems in an unmodified diesel engine. However, with a melting point of 25 °C, the use of coconut oil in cooler areas would obviously lead to problems. With IVs of 25-50, the effects on engine life are also generally unaffected if a slightly more active maintenance schedule is maintained such as more frequent lubricating oil changes and exhaust system decoking. Triglycerides in the range of IV 50-100 may result in decreased engine life and in particular to decreased fuel pump and injector life. However these must be balanced against greatly decreased fuel costs and it may be found that even with increased maintenance costs this is economically viable.

2.2.2 Cetane Number

This factor influences ease of starting, duration of white smoking after start-up, drivability before warm-up and intensity of diesel knock at idle. Studies have correlated ignition quality with all regulated emissions. As ignition delay is reduced, the combustion process starts earlier and emissions (primarily carbon monoxide and hydrocarbons) are reduced.

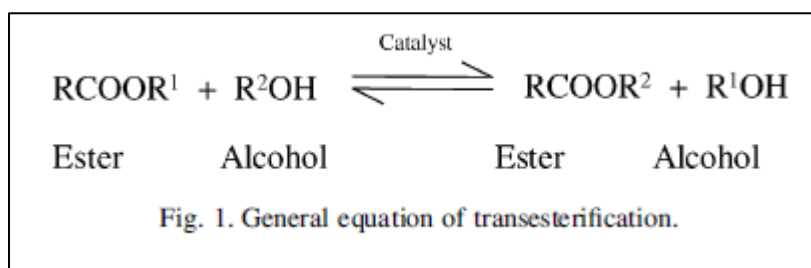
Ignition delay is measured by the Cetane Number (CN) test (ASTM D 613), which uses a single-cylinder, variable compression ratio engine analogous to the Octane Number engine. In this case, the ignition delay of the test fuel is measured at a fixed compression ratio. This result is compared with the results from standard reference fuels consisting of blends of n-Cetane and heptamethylnonane.

2.3 General Methods of Production of Biodiesel

2.3.1 Transesterification

Transesterification or alcoholysis is the displacement of alcohol from an ester by another in a process similar to hydrolysis, except than alcohol is used instead of water ^[2.5].

This process has been widely used to reduce the high viscosity of triglycerides. The transesterification reaction is represented by the general equation as Fig. 1.



If methanol is used in this process it is called methanolysis. Methanolysis of triglyceride is represented in Fig. 2.

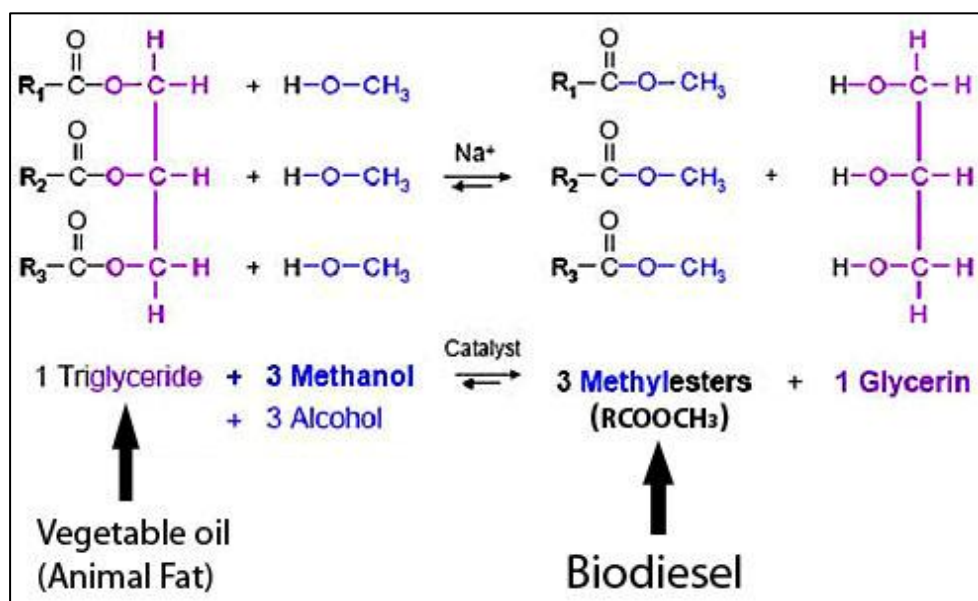
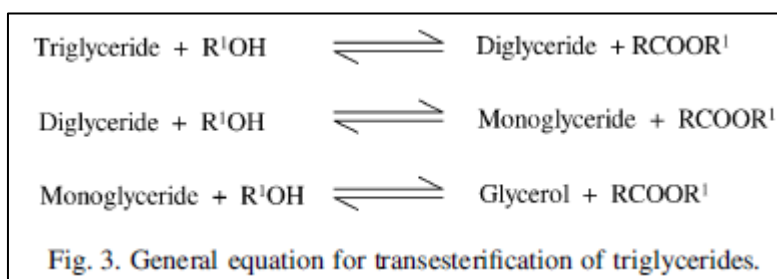


Fig. 2. Methanolysis

Transesterification is one of the reversible reactions and proceeds essentially by mixing the reactants. However, the presence of a catalyst (a strong acid or base) accelerates the conversion.

Transesterification kinetics and mechanism

Transesterification of triglycerides produce fatty acid alkyl esters and glycerol. The glycerol layer settles down at the bottom of the reaction vessel. Diglycerides and monoglycerides are the intermediates in this process. The mechanism of transesterification is described in Fig. 3.



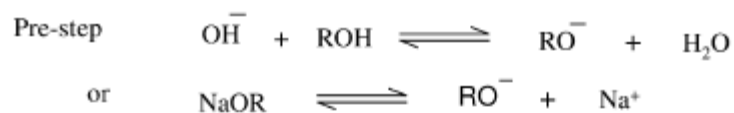
The step wise reactions are reversible and a little excess of alcohol is used to shift the equilibrium towards the formation of esters. In presence of excess alcohol, the forward reaction is pseudo-first order and the reverse reaction is found to be second order. It was also observed that transesterification is faster when catalyzed by alkali ^[2.6].

The mechanism of base-catalyzed transesterification is described in Fig. 4.

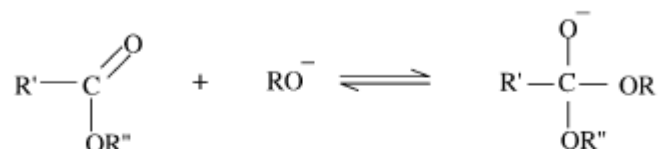
The first step involves the attack of the alkoxide ion to the carbonyl carbon of the triglyceride molecule, which results in the formation of a tetrahedral intermediate. The reaction of this intermediate with an alcohol produces the alkoxide ion in the second step. In the last step the rearrangement of the tetrahedral intermediate gives rise to an ester and diglycerides ^[2.7].

Transesterification can be catalyzed by Brownsted acids, preferably by sulfonic and sulfuric acids. These catalysts give very high yields in alkyl esters but these reactions are slow, requiring typically temperature above 100 °C and more than 3 h to complete the conversion ^[2.8]. The mechanism of acid catalyzed transesterification of vegetable oil (for a monoglyceride) is shown in Fig. 5. However, it can be extended to di- and tri-glycerides.

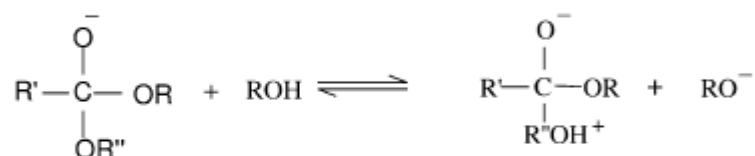
The protonation of carbonyl group of the ester leads to the carbocation, which after a nucleophilic attack of the alcohol produces a tetrahedral intermediate. This intermediate eliminates glycerol to form a new ester and to regenerate the catalyst. The methanolysis of soybean oil in the presence of 1% H₂SO₄ with an alcohol/oil molar ratio 30:1 was studied. At a reaction temperature of 65 °C the conversion was observed to be completed in 20 h, while butanolysis at 117 °C and ethanolysis at 78 °C using the same quantities of catalyst and alcohol, take 3 and 18 h, respectively [2.6].



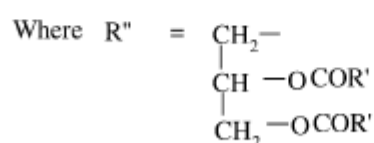
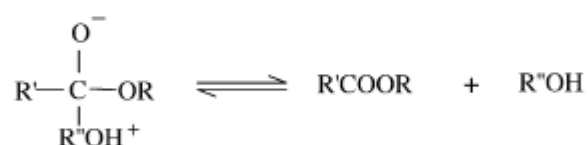
Step. 1.



Step. 2.



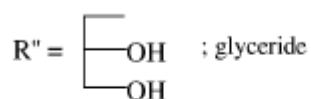
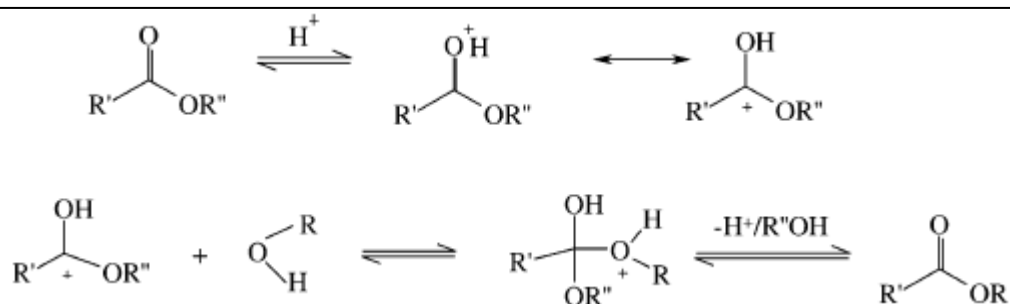
Step. 3.



$\text{R}' =$ Carbon chain of fatty acid

$\text{R} =$ Alkyl group of alcohol

Fig. 4. Mechanism of base catalyzed transesterification.



$\text{R}' =$ carbon chain of fatty acid

$\text{R} =$ alkyl group of the alcohol

Fig. 5. Mechanism of acid catalyzed transesterification.

2.4 New Approaches

With the increasing demand of Biodiesel fuel and growing concerns over hazardous by-products generated from biodiesel production process, new techniques have been proposed which uses lipase enzymes from microbial species like bacteria, fungi and algae.

Lipase enzymes produced from several microbes have been used for the production of biodiesel over the past few years and their efficiencies in biodiesel production have been analyzed.^[2.9]

Lipase from immobilized *Candida antarctica*^[2.10], *Mucor miehei*^[2.11], *Pseudomonas cepacia*^[2.12] and others like *Arthrobacter sp.* have been used to produce biodiesel of good quality.

2.5 Future of Biodiesel

Biodiesel is a substance that preserves air quality. This type of fuel is designed to enhance the richness of a diesel automobile. Various organizations are setup to control air pollution and find new sources of fuels for vehicles. Not only does biodiesel help the environment, it also assists people in using a lower cost means of transportation. It is a more harmless method than the now used petroleum diesel. Biodiesel is created from natural oils and fats. This means that it is a renewable source of power. It is a simple solution to an over growing problem. However, even though this method has begun, it is still not the main source of fuel for diesel powered engines.

Several organizations agree that this will be the only fuel of the future. Nevertheless, it is still in the developmental stages. There are several goals that groups of people are trying to take control of. These people want to preserve the way of life while still using technology to move towards the future. Off-shore drilling will be a thing of the past when the fuels that are present in the kitchen and backyards are used. They believe that the growing concern of high oil prices mixed with the fuel emissions is not worth the trouble any longer.

The future of biodiesel is growing. At this stage, only diesel powered automobiles can use the new fuel. This is expected to change in the upcoming years. The mounting concern of off-shore oil as well as the environmental issues has groups in an uproar. Already there are several types of companies using biodiesel as their main source for transportation. The Yellowstone Nation Park bus system uses a mixture of biodiesel and petroleum to run the whole fleet. Tests by the government have proven that this type of fuel is overall more functional and safe than petroleum based products. As fossil beds run dry, everyday scientists come closer to new alternative. Soon biodiesel will become the new source of power.

Through research and constant testing, biodiesel is more productive than the petroleum based fuel. It has been discovered that this type of product will become the new source of power. Not only for diesel automobiles but for other power sources individuals desperately require living and surviving. Before long, this type of supply will be not only in vehicles but also in homes and factories.

Chapter 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Microorganism

Pseudomonas fluorescens var. **Migula** (NCIM No. 2173) was used for the production of microbial lipase. It was purchased from *National Collection of Industrial Microorganisms (NCIM)*.^[3,1]

It is a Gram-negative, rod-shaped, obligate aerobic bacterium. Optimal temperatures for growth of *Pseudomonas fluorescens* are 25-30 °C. It tests positive for the oxidase test.

Heat-stable lipases and proteases are produced by *Pseudomonas fluorescens*.

3.1.2 Raw Materials

2 liters of Waste Vegetables Oil (WVO) was donated by ‘Tasty Hut’ Food Centre (Behala, Kolkata). 2 liters of Soybean oil (as Straight Vegetables Oils or SVO) was bought from grocery market.

3.1.3 Chemicals and Reagents

Items	Quantity or Composition	Uses or Function
Methanol (>99% pure)	200 ml per liter of WVO or SVO	Used as alcohol reagent for the formation of FAME.
NaOH (97% pure)	5.7 gm for WVO 5.0 gm for SVO [Calculated as 3.5 gm + (titration value)]	Used as catalyst in chemical transesterification.
Hexane	In equal amount of oil	Used as Solvent in lipase catalyzed transesterification.
Isopropanol (>99% pure)	10 ml	Used for dissolving oil during titration.
Distilled water	As per needed	Used for the preparation of nutrition media and Washing and QC tests.
Beef extract	10 gm. (per L of media)	Component of Media
Sodium chloride (NaCl)	5 gm. (per L of media)	Component of Media
Peptone	10 gm. (per L of media)	Component of Media
Agar	20 gm. (per L of media)	Component of Media
Calcium chloride (CaCl ₂)	4 gm	Used for Immobilization
Sodium alginate	2 gm	Used for Immobilization

3.1.4 Equipment and Glassware

- Distillation column
- Laminar Hood Chamber
- Autoclave
- Refrigerator
- Incubator
- Centrifuge
- Electric shaker
- Electronic pH tester
- Micro-pipette
- Water heater
- Thermometer
- Blender
- Sterile Petri-dishes and test-tubes
- Sterile Conical flasks
- Sterile beakers
- HDPE (High-density polyethylene) containers
- PET (Polyethylene terephthalate) containers
- Nylon filter mesh
- Funnels

3.2 Methodology

3.2.1 Cultivation of Microorganism

Preparation of Slant Culture:

Pseudomonas fluorescens containing vials were primarily stored in refrigerator. For the cultivation of this bacteria slant cultures were prepared on Nutrient Agar media.

Composition of Nutrient Agar Media:

Constituents	Quantity (per liter of media)
Beef extract	10 gm.
NaCl	5 gm.
Peptone	10 gm.
Agar	20 gm.
Distilled water	1 liter

pH of the Nutrient agar media was kept in the range of 6.8-7.4.

Growth of the culture in Nutrient Broth media:

1 liter Nutrient broth media was prepared for the mass production of bacterial cells. Media was then inoculated with *Pseudomonas fluorescens* cells from the slant culture in sterile and controlled environment.

Optimum growth condition for *Pseudomonas fluorescens*:

For the optimum growth of bacterial cells, inoculated broth culture was incubated at 30 °C overnight. Dense mass of bacterial colony was subsequently produced.

Subculture preparation:

Primary culture was sub-cultured twice to maintain the qualitative and quantitative requirements of the microbial cells.

3.2.2 Recovery and Immobilization of Microbial Cells

Recovery of Cells:

Cells were recovered from the nutrient broth culture by performing centrifugation at 5000 rpm for 10 minutes. Recovered mass of cells were preserved at sterile condition, until needed.

Immobilization of Cells:

The technique of immobilizing the microbial cells involves trapping them in a matrix of an inert material like Calcium alginate. This makes it easier to remove the active catalysts (or lipase) from the reaction mixture.

Preparation of Sodium-alginate solution:

2 gm (2% w/v) of Sodium-alginate was dissolved in 100 ml of cold, distilled or deionized water to form homogeneous solution of Sodium-alginate. The Sodium-alginate powder must be mixed in water slowly and cautiously with continuous stirring to avoid the powder from coagulating and clamp formation. It is worth carrying out a trial run, and adjusting the concentration of alginate if necessary. If the alginate gel is too dense, the substrate cannot enter the beads.

Preparation of Calcium chloride (CaCl₂) solution:

4 gm (4% w/v) of calcium chloride was dissolved in 100 ml of cold, distilled or deionized water in a 250 ml beaker. Calcium chloride is an irritant – so eye protection is preferable when making up this solution. Once made, this 4% solution is low hazard. Anhydrous calcium chloride (only 3 gm in 200 ml) could be used, but it could make the water boil. The calcium ions need to be in excess for this to work well. They form cross-links of calcium alginate in the gel, trapping the microbial cells within the matrix.

Preparation of Cell Suspension:

As close to the time of use as possible, bacterial cell suspension with sterile distilled water was prepared.

Equal volumes (100 ml each) of Sodium-alginate solution and bacterial cell suspension were mixed to give a final concentration of 2% alginate.

Procedure:

The alginate-cell suspension was then taken into a 25 ml non-medical syringe and allowed to drip through into the calcium chloride solution. The height of the syringe from CaCl_2 surface is maintained properly for the formation of beads of needed size (3 mm diameter). The beaker containing the beads was kept in the refrigerator overnight at 10 °C. Alginate beads were then separated from the calcium chloride solution using a small sieve or tea-strainer and rinsed with a little distilled water. These beads were kept in the refrigerator for future use.

3.2.3 Production of Biodiesel

3.2.3.1 Pretreatment of Oil

3.2.3.1.1 Evaporation:

Waste vegetable oil may contain water, which can slow down the reaction and cause saponification (soap formation). The less the water in WVO/SVO, the better it is.

For assessing the water content of the oil -- half a liter of oil was heated and the temperature rise was monitored with a thermometer.

In the presence of water, it started to "snap, crackle and pop" by 50 °C. If it's still not crackling by 60-65 °C there should be no need to dewater it.

The technique used here requires less energy and doesn't risk forming more Free Fatty Acids (FFA) by overheating. Oil was heated to 60 °C, maintained at that temperature for 15 minutes and then poured into a settling tank. It was allowed to settle for at least 24 hours and then pumped out from the top, leaving the bottom 90% for removal later and re-settling. A stirrer was used to avoid steam pockets forming below the oil and exploding, splashing hot oil out of the container.

Water in vegetable oil can exist as free water, which eventually settle to the bottom of the vessel; as suspended droplets, which may settle if the oil is heated, or the droplets are coalesced; and as water in solution with other impurities in the oil. Free water is the easiest to remove. The droplets are removed most efficiently by coalescing and draining. Suspended droplets that cannot be coalesced and water in solution are more problematic.

Boiling off the water is more difficult than it appears on the surface. Colligative properties of solutions (and some mixtures) can make removal of the last traces of water almost impossible. Water mixed with oil will not boil at the same temperature and pressure as pure water. As water is removed, more heat or lower pressure will be required to remove more water. If the oil contains salts or semi-soluble fatty acids, distillation is even more difficult.

3.2.3.1.2 Filtration:

Oil was then filtered with a 0.2 mm screening mesh to eliminate any suspended particle, if any, of considerable size to ensure the efficiency of centrifugation process.

3.2.3.1.3 Centrifugation:

Excess fat was separated from the overused waste vegetable oils with centrifugation at low speed of 500 rpm for 5 minutes.

Centrifugation must not be carried for too long or even at a higher speed because washing centrifuged fuel may yield very soapy wash-water due to saponification.

3.2.3.2 **Titration**

For processing WVO, it's essential to titrate the oil to determine the Free Fatty Acid (FFA) content and calculate how much extra lye is required to neutralize it.

An electronic pH tester is best, but phenolphthalein solution can also be used as an indicator.

1 gram of pure sodium hydroxide lye (NaOH) was dissolved in 1 liter of distilled water in a beaker. (0.1% w/v NaOH solution).

In a smaller beaker, 1 ml of preprocessed oil was dissolved in 10 ml of pure isopropyl alcohol (isopropanol). This mixture was then warmed gently by keeping it in hot water bath, stirred until all the oil dissolved in the alcohol and the mixture turned clear.

Electronic pH tester electrode was then put in the oil-alcohol mixture and 0.1% NaOH solution was then added drop by drop while stirring until the pH tester read 8.5.

Number of milliliters (2.2 ml for WVO and 1.5 ml for SVO) of 0.1% lye solution used was noted down for future reference.

It is important to add their equivalent amount (in gm) of lye during chemical transesterification process added with the predetermined 3.5 gm of lye (the basic amount of lye needed for fresh oil).The amount of lye required to process per liter of oil was as follows:

$$3.5 + 2.2 = 5.7 \text{ gm for WVO}$$

$$3.5 + 1.5 = 5.0 \text{ gm for SVO}$$

3.2.3.3 Transesterification

3.2.3.3.1 Microbial-Lipase Catalyzed Transesterification:

A typical reaction was run as follows: to a 2 L HDPE Jar, containing preprocessed WVO (500 ml) and hexane (500 ml), as solvent, was added 80 ml of the methanol and the appropriate amount of beads of immobilized bacterial cells. The reaction mixture was shaken at 100 rpm for 24 hrs at 32 °C. Then it was kept at the room temperature with any further agitation. Progress of the reaction was followed everyday. Reaction was allowed to take place until there was a clear layer of biodiesel formed at the upper part of the reaction mixture. For this particular process of transesterification it took almost 3 days (~70 hrs) to obtain a clear almost transparent layer of biodiesel.

Key compositions for lipase catalyzed transesterification process	Quantity
Preprocessed WVO	500 ml
Methanol	80 ml
Beads of immobilized bacterial cells	100 gm
n-hexane	500 ml
Temperature	32 °C
Rotational speed of shaker	100 rpm
Cultivation time	24 hrs.

3.2.3.3.2 Base Catalyzed Transesterification:

At least 97% pure NaOH was measured as per pre-determined quantity. It must be measured quickly as the lye absorbs water rapidly from the atmosphere and water interferes with the biodiesel reaction.

Potassium hydroxide or KOH can also be used. The quantity of KOH required depends on its strength. Following table describes the required quantities of KOH needed depending upon its strength.^[3.2]

Strength or Purity of KOH	Amount needed (per liter of WVO)
99%	4.9 grams
92%	5.3 grams
90%	5.5 grams
85%	5.8 grams

3.2.3.3.2.1 Production of Methoxide

200 ml of methanol was measured and poured into a 1 L HDPE container via the funnel. Methanol also absorbs water from the atmosphere so it must be done quickly and the lid of the methanol container must be closed tightly. Then the lye was carefully added to the methanol and the container was then closed air-tight.

Then the container was swirled round rather than shaking it up and down. The mixture generated heat from the reaction. After swirling it thoroughly for a minute or so, five or six times over a period of time, the lye was completely dissolve in the methanol, forming Sodium Methoxide (Potassium Methoxide in case where KOH is used). Mixing KOH is much faster; it dissolves in the methanol more easily than NaOH and can be ready for use in 10 minutes. These Methoxides are highly reactive agents and must be handled with care with necessary protection taken.

3.2.3.3.2.2 Operating Conditions

1 L of pre-processed WVO was taken up in a 2 L HDPE container. Then the Methoxide was poured slowly and carefully into the oil. A blender was used to mix the oil and methoxide for 20-30 minutes. Temperature was maintained at 55 °C by using a small water heater and a thermometer.

3.2.3.4 Purification of Biodiesel

3.2.3.4.1 Filtration:

As soon as the process was completed, the mixture was poured from the HDPE container into a 2 L PET container after passing through a 0.5 mm nylon filter to get rid of any unwanted particles and beads of immobilized bacterial cells (in case of microbial lipase-catalyzed transesterification) and kept the container for settling after screwing on the lid tightly.

3.2.3.4.2 Settling:

The mixture was allowed to settle for 48 hours (longer is better). Darker-colored glycerin by-product was collected in a distinct layer at the bottom of the container, with a clear line of separation from the paler liquid above, which was the crude biodiesel. The biodiesel varies somewhat in color according to the oil used (and so does the by-product layer at the bottom) but usually it's pale and yellowish (used-oil biodiesel can be darker and more amber). After settling, the top layer of biodiesel was drawn up using a small pump into a clean 1 L PET container, taking care not to get any part of the glycerin layer mixed up with the biodiesel.

Assessment of the Quality of the Crude Biodiesel:

Then wash-test (refer to 3.2.3.5.1) and methanol test (refer to 3.2.3.5.3) were performed on the test sample to assess the quality of the crude biodiesel.

It often takes several attempts to pass the quality tests. For instance, different blenders have different shapes and different rates of agitation, and the processing time required for good process completion can vary accordingly.

3.2.3.4.3 Washing:

After the test sample passed the quality tests, the remaining biodiesels are then washed. For washing, two 2 L PET jars were used in succession, with half a liter of normal/distilled water added for each of the three or four washes required. A small pump was used to draw up and collect the biodiesel part after washing.

- A low speed blender was used to mix the water/fuel mixture for about 5 minutes, to the point of appearing homogenous.
- Then it was allowed to settle for 1 hour or so until transparent layers of water and biodiesel were observed with a fine and thin interface between these two layers.
- Then the washed biodiesel part was drawn up from the top into another container using a small pump and two more cycles were repeated.

3.2.3.4.4 Drying:

When the fuel became clear -- not colorless but translucent, and there's no haze or cloudiness -- then it seemed to be dry. Actually it's never dry: despite what the standards say, it always absorbs some water from the atmosphere, 1200 ppm or more, but this is dissolved water, which is harmless, unlike suspended water, which must be removed.

It got clear by itself in a day or so after it was separated from the final batch of wash-water. It was then heated up to 45-50 °C and allowed to cool in a vented container. Most of the suspended water causing the cloudiness evaporated off and no more water settled out at the bottom – it confirms the dryness of the biodiesel.

3.2.3.4.5 Distillation:

Distillation was performed on the biodiesel obtained after washing to eliminate any dissolved water or methanol, if present at all, to optimize the quality of the final biodiesel. Methanol evaporated at 64.7 °C and water at 100 °C. What remained was pure biodiesel.

3.2.3.4.6 Fate of Glycerol:

What sinks to the bottom of the biodiesel processor during the settling stage is a mixture of glycerin, methanol, soaps and the lye catalyst. Most of the excess methanol and most of the catalyst remains in this layer. Once separated from the biodiesel, adding phosphoric acid to the glycerin layer precipitates the catalyst out and also converts the soaps back to free fatty acids (FFAs), which float on top. A light-colored precipitate was formed at the bottom, glycerin/methanol/water in the middle, and FFA on top. The glycerin obtained was approx. 95% pure.

The 95% pure glycerin was distilled to recover the dissolved methanol and produce 99%-pure glycerin, which is of good marketing value.

Purifying it is no simple matter -- it's difficult to distill it because glycerin has a very high boiling point (290 °C). Solvent purification distillers can purify the glycerin, but they are expensive.

3.2.3.5 **Quality Analysis of Biodiesel**

3.2.3.5.1 Wash Test:

This is the most useful all-round test, and it's very simple and described below:

- 100 ml of unwashed biodiesel (settled for 12 hours or more, with the glycerin layer removed) was poured into a half-liter PET container.
- 100 ml of water (at room temperature) was added into the container, then the lid was tightened and the container was shaken up and down violently for 10 seconds. Then it was allowed to settle.
- The biodiesel separated from the water in half an hour or less, with amber (and cloudy) biodiesel on top and milky water below, and no more than a paper-thin white interface layer between the oil and water.

This is quality fuel, a completed product with minimal contaminants. But if it turns into something that looks like mayonnaise and won't separate, or if it only separates very slowly, with a thick, creamy white layer sandwiched between the water and the biodiesel, it's not quality fuel and the process needs improvement. This problem may arise either if we use too much catalyst or make excess soap, or an incomplete reaction with poor conversion leads to half-processed monoglycerides and diglycerides, fuel contaminants that also act as emulsifiers.

Solutions of this problem are more accurate measurements, better titration, longer processing time, adequate conversion and better temperature control.

3.2.3.5.2 Reprocessing Test:

Reprocessing test is a simple check that tells us if the process went far enough, with good completion of the reaction and good conversion. If not, there will still be unconverted and partly converted material in the fuel, such as diglycerides and monoglycerides, fuel contaminants that can emulsify the fuel during washing so that it won't separate from the wash-water.

The procedure of this test is described below:

- 1 liter of finished settled and separated fuel was taken and processed again as if it were new oil, using 200 ml of methanol and appropriate amount of NaOH or the equivalent of KOH.
- It was then allowed to settle.
- If any more glycerin by-product drops out then it signifies the reaction wasn't as good as it should have been.

3.2.3.5.3 Methanol Test:

Exactly 25 ml of biodiesel was taken and dissolved in exactly 225 ml of methanol in a conical flask glass.

Pure 99%+ methanol with no water contamination must be used for this test.

After dissolving the biodiesel in the methanol, it was allowed to settle for 30 minutes.

The test is most accurate at room temperature, about 20-25 °C.

A clean methanol test result with no deposit at the bottom indicated a well-completed reaction and production of high-quality fuel.

The biodiesel should be fully soluble in the methanol, forming a clear bright phase. If not, there is pollution in the biodiesel. If there is any un-dissolved material at the bottom of the measuring glass the reaction is not complete and this may cause trouble with the water test.

This method does not cover every aspect of quality, but it gives a hint. It is valid only for biodiesel made from vegetable and animal oils. It is not valid for biodiesel made from oils with a very wide fatty acid pattern, such as fish oils.

Biodiesel dissolves easily in methanol, but vegetable or animal oils and fats (triglycerides) won't dissolve in methanol. Any unconverted oil left in the biodiesel will settle out at the bottom of the test flask.

This means the processing didn't go far enough and needs improvement. All the oil should be fully converted to biodiesel.

If some unconverted triglycerides remain in the biodiesel, there will also be partly converted diglycerides and monoglycerides -- this is poor-quality fuel that will not meet the quality specifications and could damage the engines. Diglycerides and monoglycerides also cause emulsions when we try to wash the fuel.

The test can be used with finished biodiesel that's been washed and dried or unwashed biodiesel or samples taken during processing.

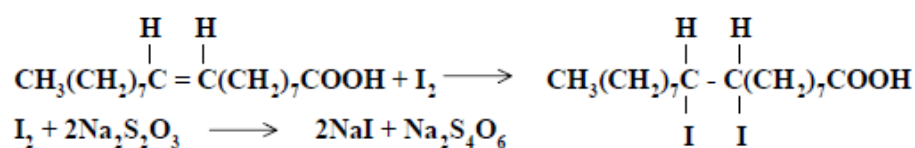
3.2.3.5.4 Iodine Value: ^[3.3, 3.4, 3.5]

The iodine value is expressed in grams of iodine for the amount of halogens linked with 100g test sample, and is used as degree of unsaturated bond of fats and oils or Bio Diesel Fuel.

The test method to determine iodine value of Bio Diesel Fuel (BDF) is standardized as in ASTM D1959-97 Standard Test Method for Iodine Value of Drying Oils and Fatty Acids, ISO 3961:1996 Animal and vegetable fats and oils – Determination of iodine value, and JIS K 0070-1992 Test Method for Acid value, Saponification number, Ester number, Iodine number, Hydroxyl value of Chemical products and Unsaponifiable matter.

The test sample was made out of bio diesel fuel and dissolved in cyclohexane, added in excess with Wijs reagent (0.1mol/L iodine monochloride acetic acid solution), left in a dark room for 30 minutes reaction, then added with potassium iodide, titrated with 0.1mol/L sodium thiosulfate solution for the excessive iodine monochloride to the endpoint, which is the highest inflexion point.

The iodine value of BDF is calculated from titration volume of sodium thiosulfate solution.



Reagent

- i. Titrant: 0.1mol/L sodium thiosulfate (f = 1.003)
- ii. Additive: Cyclohexane
Wijs reagent (iodine trichloride 7.9 gm and iodine 8.9 gm each dissolved in acetic acid, then, mixed together to make it total 1L)
100g/L potassium iodide solution

Cautions in measurement

- i. Wijs reagent (0.1mol/L iodine monochloride acetic acid solution) is added to 50~60% in excess.
- ii. Wijs reagent is liable to change, and blank titration is required accordingly.
- iii. When Wijs reagent and sample react, iodine tends to evaporate. Therefore, a 200mL conical flask with joint stopper was used.

Preparation of iodine monochloride (Dark brown liquid) entails simply combining the halogens in a 1:1 molar ratio, according to the equation $I_2 + Cl_2 \rightarrow 2 ICl$.

Test procedure

- i. 0.3g sample biodiesel was taken in a 200mL conical flask with stopper.
- ii. Approximately 10mL cyclohexane was then added and dissolved.
- iii. Wijs reagent was added and stirred.
- iv. The flask was then plugged with stopper, and left for 30 minutes in a dark room at room temperature.
- v. Then, 20mL of 100g/L potassium iodine solution and 100mL pure water were added.
- vi. Titration was then performed with 0.1mol/L sodium thiosulfate solution to obtain iodine value.

* Blank level was obtained beforehand by performing blank test likewise.

Calculation formula

$$\text{Iodine value (g/100g)} = (\text{BL} - \text{EP}) \times \text{TF} \times \text{C/S}$$

BL: Blank level

EP: Titration volume (mL)

TF: Factor of titrant

S: Sample amount (g)

C: Concentration conversion coefficient (1.269)

Alternative Method for Measuring Iodine Value ^[3.6]

The iodine value method is reproducible only if the exact conditions of the test are carefully followed. Any changes in strength of reagent, sample size or reaction time may produce varying results.

The exact specified weight of the sample is accurately weighed into a glass stoppered iodine flask, and dissolved in chloroform. The measured volume of Hanus reagent is accurately added and after thorough mixing, is placed in the dark for exactly one hour. A corresponding reagent blank is simultaneously prepared.

At the end of the specified time, the reaction is stopped by adding potassium iodide and diluting with water to prevent loss of the free iodine. The amount of iodine present is determined by titrating with sodium thiosulfate using starch indicator. The difference between a reagent blank titration and the titration of the test sample represents the amount of iodine absorbed by the sample. The iodine value is calculated as grams of iodine per 100 grams of sample.

Hanus reagent is a solution of iodobromide in concentrated acetic acid. The iodine combines with double bonds slowly under these conditions. A large excess of the halogens must be present to complete the reaction. At the end of the reaction the unconsumed iodine should be greater than 60% of the total. If the sample titration is less than 60% of the blank titration, take a smaller sample and repeat the analysis.

Apparatus

- i. Iodine flasks, 250 ml. glass stoppered
- ii. 50 ml. Burette with a tolerance of ± 0.07 ml
- iii. 25 ml Class A volumetric pipette with a tolerance of ± 0.07 ml or appropriate dispenser
- iv. Graduated cylinder capable of containing 100 ml
- v. Analytical balance capable of weighing to the nearest 0.1mg
- vi. Dispensing device

Reagents and Solutions

- i. Chloroform
- ii. Hanus Solution
- iii. Potassium Iodide - 10% solution
- iv. Stabilized starch solution
- v. Sodium thiosulfate, 0.1N

Procedure

1. Sample was accurately weighed into a 250 ml conical flask. Sample size was determined from the following table. Weight was recorded accurately to the nearest mg.

<u>Anticipated Iodine Value</u>	<u>Sample Size</u>
0 - 30	0.8 ± .01 g
30 - 50	0.5 ± .01 g
50 - 100	0.25 ± .01 g
100 - 150	0.16 ± .01 g

2. Using the dispensing device, 10 ml chloroform was added and dissolved by swirling (NOTE: 50% aqueous products will not dissolve completely until Hanus solution is added).
3. 25 ml Hanus solution was added and stoppered immediately and then swirled to mix thoroughly and placed in the dark.
4. A corresponding reagent blank was simultaneously prepared containing 10 ml chloroform and 25 ml Hanus solution. Stoppered immediately and placed in the dark with the test sample.
5. After 60 minutes, using the dispensing device, 20 ml 10% potassium iodide was added.
6. By a graduated cylinder, 100 ml water was added rinsing the neck and side walls of the flask.
7. The solution was then titrated using 0.1N sodium thiosulfate until the aqueous layer's color begins to lighten.
8. Using the dispensing device, approximately 3 ml starch solution was added and titration was continued until the blue color of the aqueous layer begins to disappear.
9. Towards the end of the titration, at intervals, the flask was stoppered and shaken to extract any iodine remaining in the chloroform layer.
10. When the end point appeared to have been reached, flask was again stoppered and shaken, allowed the layers to separate and two drops of starch solution were added to verify that no blue color is formed in the aqueous layer.
11. The burette reading was recorded accurately to two decimal places.

Calculations

$$\text{Iodine Value} = \frac{(\text{ml blank} - \text{ml sample}) \times N \text{ sodium thiosulfate} \times 12.69}{\text{g sample}}$$

3.2.3.5.5 Cetane Number:

Ignition delay is measured by the Cetane Number (CN) test (ASTM D 613), which uses a single-cylinder, variable compression ratio engine analogous to the Octane Number engine. In this case, the ignition delay of the test fuel is measured at a fixed compression ratio. This result is compared with the results from standard reference fuels consisting of blends of n-Cetane and hepta-methylnonane.

Diesel engines vary widely in their Cetane requirements, and there is no commonly recognized way to measure this value. In general, the lower an engine's operating speed, the lower the CN of the fuel it can use. Large marine engines can tolerate fuels with CNs as low as 20, while some manufacturers of high-speed passenger car diesel engines specify 55 CN fuel.

Chapter 4

RESULTS AND DISCUSSION

4.1 General

The objective of this study was to investigate the use of low-cost, high FFA feedstocks to produce fuel-quality biodiesel. It was determined that feedstocks with high FFAs could be transesterified with Lipase enzymes produced from immobilized cells of *Pseudomonas fluorescens* as well as with the traditional alkaline catalysts that have been used with good success for vegetable oils.

Base-catalyzed transesterification came up with several problems. Alkaline catalysts formed soap when they reacted with the FFAs. Soap removed the catalyst from the reaction and prevented the separation of the glycerin and the ester with greater perfection. The effects of the methanol molar ratio and reaction time on the reduction of FFA level were studied with a high FFA feedstock consisting of 10% palmitic acid in soybean oil.

4.2 Effect of Microbial Lipase Catalysis

The following specific observations are based on the process development portion of this project:

- i. Enzymatic transesterification mediated by *Pseudomonas fluorescens* lipase produced biodiesel of good quality.
- ii. Enzymatic transesterification comes up with added advantage of eliminating the difficulties in the separation of glycerol and fatty acyl methyl ester or biodiesel.
- iii. Immobilized *Pseudomonas fluorescens* cells are reusable for several cycles of biodiesel production process and thus show higher efficiency and cost effectiveness.
- iv. Enzymatic transesterification process eliminated any necessity of using highly reactive chemical substances like Caustic Soda (NaOH) or Caustic Potash (KOH) and thus much easier to handle.
- v. Enzymatic transesterification process is much energy efficient as there is less requirement of maintenance of higher temperature during the process.
- vi. The reaction yields as well as the reaction times of enzymatic transesterification are still unfavorable compared to the base-catalyzed reaction systems.
- vii. Microbial lipase generally brings about a range of bioconversion reactions such as etherification, transesterification; acidolysis, and aminolysis.

4.3 Effect of Immobilization

Whole cell immobilization of *Pseudomonas fluorescens* by gel entrapment using Calcium Alginate was adopted to produce lipase enzyme with higher efficiency.

One of the most important characteristics of an immobilized enzyme is its stability and reusability over an extended period of time. Experiments were performed to examine the recyclability and the stability of the lipase. After each standard transesterification reaction, lipase containing cells were recovered by filtration and subsequently reused. This procedure was repeated several times to examine the extent of the stability of the immobilized enzyme.

Following advantages of using lipase producing immobilized cells were observed:

- i. Reuse
- ii. Continuous use
- iii. Less labor intensive
- iv. Saving in capital cost
- v. Less contamination chances in products
- vi. More Stability
- vii. Improved process control, and
- viii. High enzyme: substrate ratio.

The effect of diffusional limitation was almost eliminated by optimizing the diameter of the beads of immobilized cells.

4.4 Effect of Catalysts

Catalysts used here were alkali and enzyme. Alkali-catalyzed transesterification was much faster than enzyme-catalyzed transesterification. Alkalis include sodium hydroxide, sodium methoxide, potassium hydroxide, potassium methoxide, sodium amide, sodium hydride, potassium amide and potassium hydride. Sodium methoxide was more effective than sodium hydroxide because of the assumption that a small amount of water was produced upon mixing NaOH and Methanol. Sodium hydroxide was chosen to catalyze the transesterification because it is cheaper. Sodium hydroxide is used widely in large-scale processing. An immobilized lipase was employed to catalyze the methanolysis of soybean oil which resulted in an ester conversion of >98% (Jackson and King, 1996).

4.5 Effect of Reaction Temperature

Transesterification can occur at different temperatures, depending on the oil used. In methanolysis of soybean oil, the reaction proceeded most satisfactorily at 25°C during enzymatic transesterification and at 35°C during base-catalyzed transesterification. Temperature clearly influenced the reaction rate and yield of esters.

4.6 Effect of Oxidation and polymerization

Oxidation and polymerization pose a complicated problem generally faced during production of biodiesel fuel. All oils are not the same. Some are 'drying' oils such as linseed oil. When it dries the oil irreversibly polymerizes into a tough, insoluble plastic-like solid. At the high temperatures in internal combustion engines the process is accelerated. Steadily accumulating films of tough, insoluble plastic-like solids are not desired in engines and injector pumps. ^[4.1]

Polymerization happens when the double bonds in unsaturated oil molecules are broken by oxygen from the air or water. The oil oxidizes, forming peroxides (hydroperoxides), and the peroxides polymerize, bonding with carbon to create a long and stable molecule called a polymer (plastic). Another effect of oxidation is that the hydroperoxides attack elastomers, such as rubber seals.

Without oxygen the oil can't polymerize. Oxidation and polymerization don't only affect the drying oils; there are also semi-drying oils, many of which are commonly used to make biodiesel, including sunflower and soybean oil.

Saturated oils don't polymerize, unsaturated oils do. The level of unsaturation is called the Iodine Value (IV) -- the higher the IV the more unsaturated the oil, the faster it will oxidize and the more it will polymerize. Converting unsaturated oils to biodiesel lessens the polymerizing effect but doesn't prevent it. Mixing biodiesel made from high-IV oils with biodiesel made from lower-IV oils lessens the polymerizing effect to a greater extent.

4.7 Effect of Oil Characteristics

Quality of biodiesel fuel depends largely on the type of oils used for its production. Soybean oil was used in this project both for enzymatic transesterification and base-catalyzed transesterification.

Chemical properties of Soybean oil ^[4.2]

Fatty acid composition, % by weight									Acid ^a	Phos ^b	Peroxide ^c
16:0	18:0	20:0	22:0	24:0	18:1	22:1	18:2	18:3	value	ppm	value
11.75	3.15	0.00	0.00	0.00	23.26	0.00	55.53	6.31	0.20	32.00	44.5

^a Acid values are milligrams of KOH necessary to neutralize the FFA in 1 g of oil sample.

^b Phosphatide (gum) content varies in direct proportion to phosphorus value.

^c Peroxide values are milli-equivalents of peroxide per 1000 g of oil sample, which oxidize potassium iodide under conditions of the test.

Typical fatty acid composition (in % of weight) of Soybean oil ^[4.3]

Lauric	0.1
Myristic	0.1
Palmitic	10.2
Stearic	3.7
Oleic	22.8
Linoleic	53.7
Linolenic	8.6

4.8 Cost estimation and Economic viability

This work put emphasis on production of biodiesel fuel as economically as it could be possible. The major constituents that differ in enzymatic transesterification and base-catalyzed transesterification is the usage of immobilized microbial strain (Calcium alginate immobilized *Pseudomonas fluorescens*) and highly pure (>97%) base (NaOH or KOH).

It might be observed that the cost of biodiesel production using enzymatic transesterification primarily seemed to be high but it was evident that the immobilized microbial cells can be reused over several production cycles, ultimately bringing the overall average costs much lower compared to that of base-catalyzed transesterification.

A question many people are asking is whether bio-diesel fuel is going to be an economically viable option. Some will say “No”, others say “perhaps in time”, and while a third group says that “it already is”. The truth depends on what we consider as economically viable.

In the current scenario of world politics, conventional petro-diesel is culminating lots of concerns over its price instability. The current price of petro-diesel in USA is fluctuating around \$3.58/gallon and that of regular gasoline is \$3.21/gallon whereas price of biodiesel is around \$2.2/gallon. ^[4.4]

One of reasons why it is difficult to say whether or not biodiesel will be economically viable or not is the speed with which this research field is moving forward and all the new and more efficient ways of producing biodiesel fuel, improving production efficiencies by using recombinant bacterial or algal strains etc. that are being discovered . Right now, just a few small breakthroughs may be enough to radically change all calculations.

Though biodiesel is being successfully marketed in USA and EU countries, the current scenario of biodiesel in India presents a confused picture. *Oil and Natural Gas Corporation* (ONGC) of India started a project to add biodiesel to conventional petro-diesel in a certain percentage. This was about a few years ago. The current status is unknown. At the moment, India is barely meeting out food requirements of edible oils and has to revert frequently to imports. If any significant quantity of edible oils is used in the production of biodiesel the situations might be difficult from economic perspective. For this very reason alternative sources for biodiesel production, like non-edible oils (WVO, Jatropha oil) are being investigated to some great extent and different microbial and non-hazardous processes for its production are being researched day by day.

Chapter 5

CONCLUSION

5.1 Conclusion

In conclusion, lipase-mediated transesterification using immobilized *Pseudomonas fluorescens* is a viable method for the production of fatty acyl alkyl esters or biodiesel, of standard marketable quality, from straight vegetable oil (SVO). But for the production of biodiesel from waste vegetable oil (WVO), also, lipase-catalyzed transesterification bears tremendous prospects. However, base-catalyzed transesterification may not always produce biodiesel fuel of usable quality from waste vegetable oils. Work is still ongoing to maximize conversions for lipase enzymes produced from different microbial strains, for specific alcohols, to improve conversions for solvent-free methanolysis, to scale-up reactions, to provide sufficient quantities for determining their cold-temperature properties and to further improve upon these properties, emission, and performance characteristics of the alkyl esters as diesel fuel alternatives. The potential of this technology is also being explored to introduce other functionalities to prepare biodegradable lubricants and additives.

5.2 Scope for Future Work

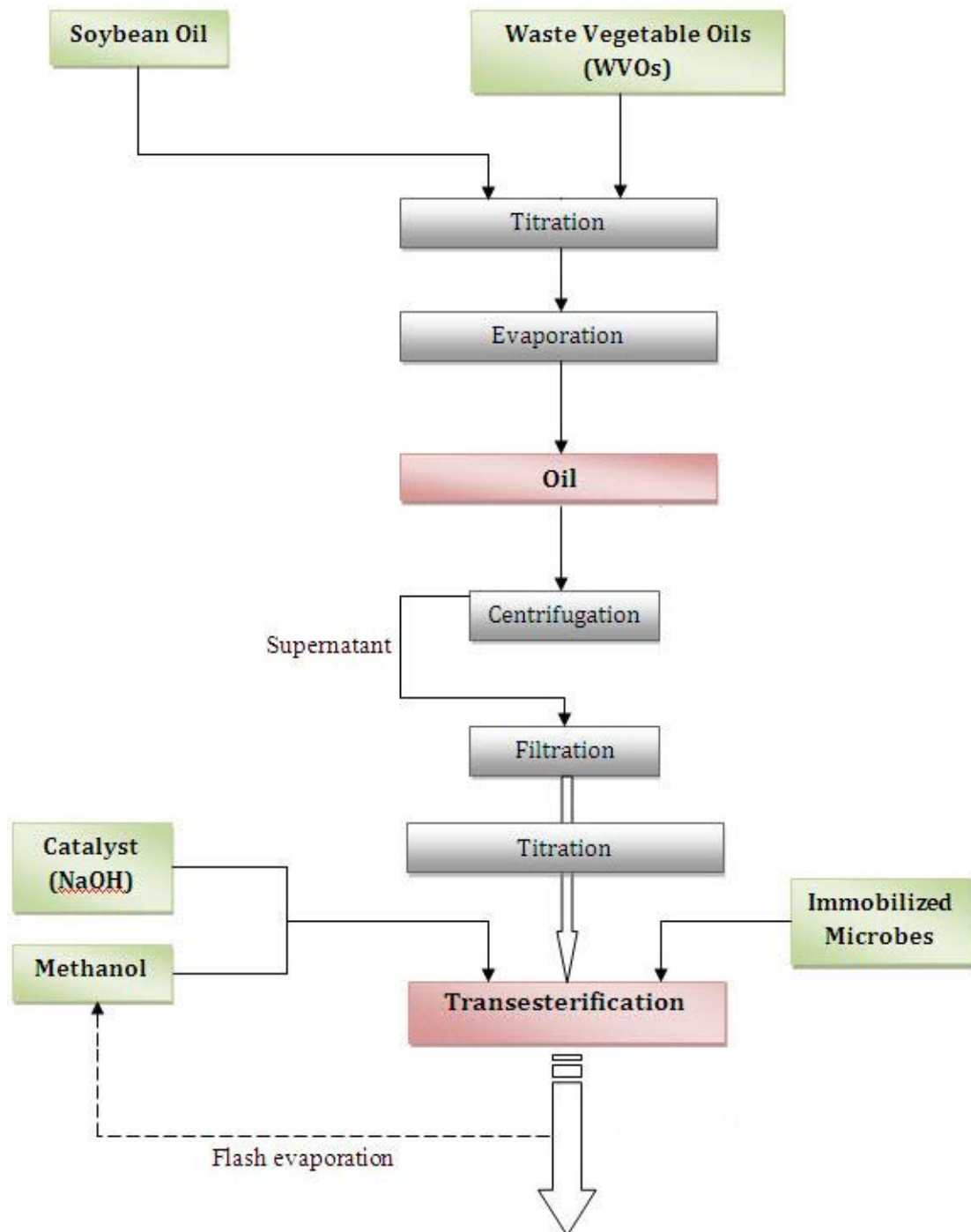
Biodiesel has become more attractive to replace petroleum fuel. As per the reported literature, most of the transesterification studies have been done on edible oils like rapeseed, soybean, sunflower, canola etc. by using methanol and NaOH/KOH as catalyst. There are very few studies reported on non-edible oils or waste vegetable oils (WVOs), for that matter. The use of heterogeneous catalysts is a new area in the development of suitable catalysts in the transesterification of high FFA oil. The quality of biodiesel is most important for engine part of view and various standards have been specified to check the quality.

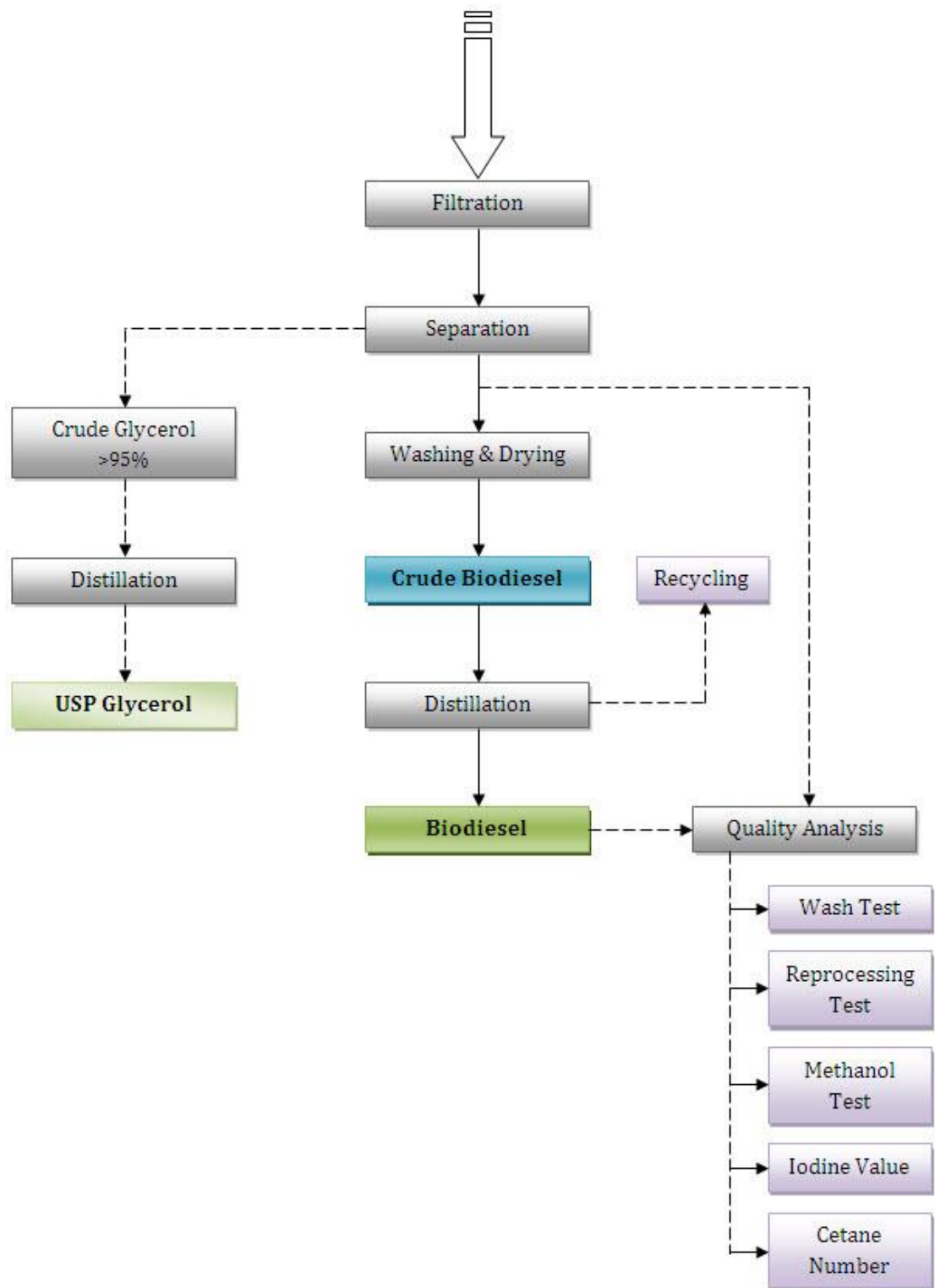
Biodiesel has become more attractive recently because of its environmental benefits and the fact that it is made from renewable resources. The cost of biodiesel, however, is the main hurdle to commercialization of the product. The used cooking oils are used as raw material, adaption of continuous transesterification process and recovery of high quality glycerol from biodiesel by-product are primary options to be considered to lower the cost of biodiesel.

In future, several works on the design, modeling & process simulation of the manufacturing plant for the industrial scale production of biodiesel can be done. Recent progress in genetic engineering opened up larger avenue for the development of genetically modified microorganisms for higher expression of lipase enzymes and much affordable production of biodiesel fuel.

Appendices

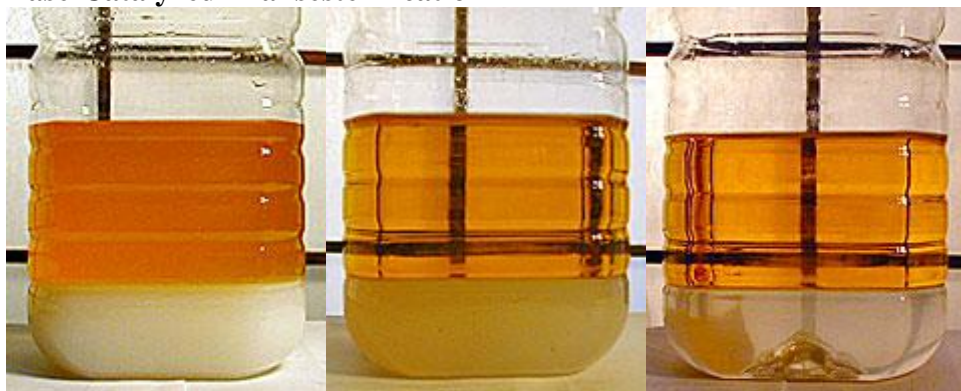
Appendix A: Process flow diagram of production of biodiesel





Appendix B: Figures related to the progress of production of biodiesel

Base-Catalyzed Transesterification



Wash Test



Washing



Lipase-Catalyzed Transesterification



Iodine Test



Glycerin



**Final biodiesel product from SVO
using enzymatic transesterification**



**Final biodiesel product from SVO
using base-catalyzed transesterification**



**Final biodiesel product from WVO
using base-catalyzed transesterification**



Appendix C: List of biodiesel producing companies

Trademet Ltd

Fuel Blends Offered: B100

Contact Details

Joe Shaw (info@trademet.co.uk)

Cherwell Business Village, Southam Road, BANBURY, OXFORDSHIRE, OX16 2SP

Phone: 01295 261901

Website: www.trademet.co.uk

Future Fuel

Fuel Blends Offered: 100% Biodiesel

Contact Details

S. H. (Salimhussain21@hotmail.com)

20 Mulller Road, Bristol, Bristol, BS5 6XP

Phone: 07889042916

Doncaster Bio Fuels

Fuel Blends Offered: B100

Contact Details

Dave Belli (davebelli@hotmail.co.uk)

Unit 3, Jupiter Business Park, Bentley Road, Doncaster, South Yorks, DN5 9TJ

Phone: 07869 282856

Bioflo UK ltd

Fuel Blends Offered: B100, B80

Contact Details

Jamie Daqle (sales@bioflo.net)

Yates Farm, Ashworth Rd, Ashworth, Rochdale, Lancs, OL11 5UN

Phone: 07501132919

Website: www.bioflo.net

Diesel Dan Greenfuel Man

Fuel Blends Offered: Biodiesel B100, SVO

Contact Details

Steve Sharp (steve@dieseldan.co.uk)

5D Westfield Ind Est, Radstock, Radstock, Somerset, BA3 4BH

Phone: 01761 411011

Sujap Group

Fuel Blends Offered: Blends to suit customer specifications.

Products are to EU standards EN 14214 and US standards 6751. The blend of the fuel can be tailored to customer specs.

Contact Details

Sophie Dobbs (sophiedobbs@sujapgroup.com)

Amberly House, Canton, Cardiff, Glamorgan, CF11 9XH

BML Oils Ltd

Fuel Blends Offered: Pure Plant Oil

Produce 1.3 Million Liters a year. Oil is filtered down to 1 Micron.

Contact Details

Llyr Jones (llyr.derwydd@yahoo.co.uk)

Derwydd, Llanfihangel G.M, Corwen, Denbighshire, LL21 9UP

Phone: 014904204569

Website: www.bmloils.co.uk

The Greenbiodiesel Depot

Fuel Blends Offered: B100 Biodiesel to EN14213 and EN14214 standard in 1000 litres IBC delivered.

Contact Details

Steve and Angie (thegreenbiodieseldepot@hotmail.co.uk)

Manchester, Manchester, Lancashire, BL0

Phone: 07760760346

Sundance Renewables

Fuel Blends Offered: 100%

Contact Details

Jan Cliff (info@sundancerenewables.org.uk)

Capel Hendre Industrial Estate, Ammanford, Carmarthenshire, SA18 3SJ

Phone: 01269 842 401

Website: www.sundancerenewables.org.uk

GreenFuel Supply Solutions Ltd

Fuel Blends Offered: 100% Biodiesel

Contact Details

Mark Taylor (mark@greenfuelsupplysolutions.co.uk)

19 Brunel Close, Drayton Fields Industrial Estate, Daventry, Northamptonshire, NN11 8RB

Phone: 0845 838 4671

Website: www.greenfuelsupplysolutions.co.uk

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