Materials and methods

MATERIALS AND METHODS

Plant Material used for the Study

Jatropha Seed oil (*Jatropha curcas*)

Pongamia seed oil (*Pongamia pinnata*)

Extraction of oil – Machineries Required

- Seeds decodecator
- Expeller (Steam boiler, Pre-cooking chamber, Seed collection chamber)
- Controller unit
- Squeezing unit
- Sieves for filtration
- Oil settling tank
- Filter press with pipeline connected to oil storage tank

Procedure for Extraction of *Jatropha* and Karanja oil

The steam boiler should be switched on for one hour before the extraction process. Ten Kilograms of *Jatropha*/Karanja seeds were taken and passed to seed decodecator, to break the seed into two halves.

These broken seeds were sent to the collection chamber after decodecation. Then, the seeds were passed to pre-cooking chamber at the steam of 20-30 per cent, where they were pre-cooked, and sent to the squeezing unit via control unit.

Squeezing unit consists of plates fitted in it, where the unit has high and low - pressure units. The seed materials from the above unit are squeezed from high pressure to low pressure through plates. The waste materials obtained from the seeds are collected and used as the food for the cattle. The oil extracted was filtered through a sieve that is fixed below the squeezing unit. The oil is collected through the oil settling tank.

From the squeezing unit, pipeline was connected to the filter press. The oil from the oil settling tank was sent through pipeline to the filter press for filtration. The filter press consists of 16 filters. The oil was sprayed to each filter through a sprayer. The oil gets filtered and then stored in the storage tank. Then the oil was subjected to transesterification for biodiesel preparation.

General Mycological Techniques

Mycological techniques suggested by Booth, (1971) were generally followed,

Glassware

Scrupulously cleaned Borosil glassware was used throughout the study.

Chemicals

All chemicals used in the present study were of Analar grade.

Water

Deionized distilled water was used.

Sterilization

All the glassware and media used in the experimental work were sterilized in an autoclave at 15 Ibs pressure for 30 minutes. Instrument like forceps, inoculation needles etc., were sterilized over a flame after dipping in alcohol. The inoculation chamber was initially sterilized with alcohol and later with UV light radiation.

I. ISOLATION OF FUNGI FROM SOILS

Microorganisms are found in diverse habitats. In the present study, oil rich soil samples were collected from petrol bunks, edible oil refining factories located in and around Coimbatore district, Tamilnadu, India. Lipolytic fungi were isolated from the collected oil rich soil samples.

Serial Dilution Techniques (Wakesman, 1922)

The isolation process for lipolytic fungi was performed by serial dilution of collected samples and inoculating the diluted soil sample into the plates containing Potato Dextrose Agar medium. The inoculated plates were then incubated at 27^0C for 3 days.

Maintenance of Cultures

The fungal isolates were maintained on Potato Dextrose Agar slants at 4^0C.

Composition of PDA Medium (Riker and Riker, 1986)

Potato	-	250 gms
Dextrose	-	20 gms
Agar	-	15 gms
Distilled water	-	1000 ml
pH	-	6.5

Preparation of the PDA (Potato Dextrose Agar) Medium

250 gms of Potato were sliced and boiled in 500 ml of distilled water and filtered. To this filtrate 15 gms of agar and 20 gms of dextrose were added thus the filtrate was made up to 1000 ml.

II. IDENTIFICATION OF LIPOLYTIC FUNGI

Media used

a) Palmer's Medium

Potassium Hydrogen Ortho Phosphate (K_2HPO_4)	- 0.2 g
Sodium Chloride (NaCl)	- 1 g
Magnesium Sulfate ($MgSO_4$)	- 0.2 g
Ferrous Sulfate ($FeSO_4$)	- trace
Distilled water	- 1000 ml
Ammonium Chloride (NH_4Cl)	- 3 g
Oil (*Jatropha/Pongamia*)	- 1 ml
pH	- 7

The above medium was used to culture fungi in order to identify their ability to produce lipase when oil is used as the only carbon source. Following heat sterilization, the medium was inoculated with the conidia of the isolated fungal strains. The flasks were incubated at 30°C. Culture filtrates were collected at specific intervals and the fungal biomass was filtered, and the culture filtrate was used as the source of enzyme and the activity of lipase was assayed following the method of Vonderwulbech (1992) and the isolates with significant activity were used for further studies.

b) Synthetic Oil Based Medium (SOB) (Mahadik *et al.,* 2002)

Sodium Nitrate ($NaNO_3$)	- 0.05 g
Magnesium Sulfate ($MgSO_4$)	- 0.05 g
Potassium Chloride (KCl)	- 0.05 g
Potassium di hydrogen Phosphate (KH_2PO_4)	- 0.2 g
Yeast extract	- 0.1 g
Bacto peptone	- 0.5 g
Oil (*Jatropha/Pongamia*)	- 1 ml
pH	- 7

c) Malt Yeast Glucose Peptone Medium (MYGP) (Mahadik *et al.,* 2002)

Malt extract	- 0.3 g
Yeast	- 0.3 g
Bacto peptone	- 0.5 g
Oil (*Jatropha/Pongamia*) (Instead of glucose)	- 2 g
pH	- 7

d) Czapek's Dox Medium (Purvis *et al.,* 1964)

Sodium Nitrate ($NaNO_3$)	- 2 g
Potassium Chloride (KCl)	- 0.5 g
Magnesium Sulfate ($MgSO_4$)	- 0.5 g
Potassium di hydrogen Phosphate (KH_2PO_4)	- 1 g
Ferrous Sulfate ($FeSO_4$)	- trace
Distilled water	- 1000 ml
Oil (*Jatropha/Pongamia,* instead of sucrose)	- 1 ml
pH	- 7

The fungal strains with maximum lipolytic activity viz., *Aspergillus oryzae*, *Aspergillus terreus, Mucor racemosus, Penicillium candidum, Rhizopus oryzae* and *Trichoderma viride* were then cultured on the above three media in order to study the variation in lipolytic activity and to find out the suitability of the medium for high lipase production for further studies.

Control

Control was also maintained with inoculated medium with no carbon source.

Liquid State Fermentation

Five days old culture on Potato Dextrose Agar medium in petridishes was used as a source of inoculums. Mycelial spores from growing front were removed using a sterile loop.

Conical flasks (250 ml) containing 20 ml of liquid media (Synthetic Oil Based medium, Malt Yeast Glucose Peptone medium and Czapek's Dox medium with only *Jatropha/Pongamia* seed oil as sole carbon source) were used for liquid state fermentation. There replicates were maintained for each experiment.

One ml of spore suspension containing 10^3/ml of spores of each of the fungal species was transferred aseptically to 250 ml flask containing the above media with *Jatropha/Pongamia* seed oil as carbon source. The inoculated conical flasks were incubated at ordinary laboratory conditions for five days.

III. STANDARDIZATION OF PROCESS PARAMETERS FOR HIGH LIPASE PRODUCTION

Media used with *Jatropha oil*

Synthetic Oil based medium with oil as the carbon source was used for *Aspergillus terreus, Mucor racemosus* and *Rhizopus oryzae* and Malt Yeast Glucose Peptone medium with oil as carbon source was used for *Aspergillus oryzae, Penicillium candidum* and *Trichoderma viride* for this study as these media were found suitable for high lipase production and the effect of the various parameters were tested in these respective media for high lipase production.

Media used with Karanja oil

Synthetic Oil based medium with oil as the carbon source was used for *Aspergillus terreus and Mucor racemosus* and Malt Yeast Glucose Peptone medium with oil as carbon source was used for *Penicillium candidum, Aspergillus oryzae* and

Trichoderma viride and Czapek's Dox medium with oil as the carbon source was used for *Rhizopus oryzae* for this study as these media were found suitable for high lipase production and the effect of the following parameters were tested in these respective media for high lipase production.

pH of the Medium

The effect of acidity and alkalinity of the medium for lipase production was investigated by the control of pH. Dilute hydrochloric acid was added to one set of medium and the initial pH was brought to pH 4, 5, 6, 7, 8 and 9. In other set the initial pH was brought to pH 8 and 9 by adding dilute sodium hydroxide.

Temperature

The inoculated fungal cultures were maintained at different temperature (20, 25, 30, 35, 40 and 45^0C in a BOD incubator and pH of the medium was adjusted to pH 6 and incubated for 5 days.

Effect of Carbon Sources

The lipase activity was standardized by adding carbon sources such as lactose, maltose, sucrose and glucose along with *Jatropha/Pongamia* seed oil in 20 ml of the medium showing high lipase activity by specific fungi.

Effect of nitrogen Sources

20 mg of nitrogen sources viz., $(NH_4)_2HPO_4$ (di-ammonium hydrogen ortho phosphate), $(NH_4)_2SO_4$ (di-ammonium sulfate), NH_4Cl (Ammonium chloride) and

KNO_3 (Potassium nitrate) was amended into 20 ml of specific medium for the different fungi to find out the effect of nitrogenous sources.

IV. LIPASE ASSAY

The production of the enzyme (lipase) by various isolates was determined as follows

Extraction of Enzyme

a) Crude Lipase (Culture filtrate)

Enzyme was extracted following the method of Johri and Pandey (1982). Fungal mat grown in conical flasks containing liquid medium was filtered through double layered muslin cloth and the filtrate was centrifuged at 15,000 rpm for 30 min. The supernatant was used as crude enzyme source.

b) Partial Purification of Enzyme (by Dialysis)

The culture filtrate of the fungal species was filtered through fine sieve and centrifuged at 10,000 rpm for 30 min. The supernatant was then collected and to it 80% saturated ice cold ammonium sulfate was added and left overnight at 4°C to precipitate. The precipitate was then centrifuged at 10,000 rpm for 30 minutes and the pellet was collected and dissolved in phosphate buffer pH 7. This was then dialyzed against phosphate buffer pH 7 for 24 hrs with several changes of buffer, the filtrate thus obtained was measured and its enzyme activity / mg of protein were calculated.

c) Whole cell / Intracellular Lipase (Dry Mycelium) (Mahadik *et al.,* 2002)

The mycelium harvested by filtration was washed with distilled water twice to remove trace of medium and then washed with chilled acetone. The acetone treated mycelium was vacuum dried for 6 hrs to remove acetone and water. This vacuum dried mycelium was used for determining cell bound (intracellular) lipase activity and used as catalysts for biodiesel production using whole cell / intracellular lipase.

Storage of Enzyme

The crude enzyme was stored at 4°C in refrigerator till use (Shewale and Sadana, 1978).

Enzyme Assay

The activity of lipase was found out by following the method of Vonderwulbech *et al.* (1992) using p-nitro phenyl palmitate (pNPP) as substrate.

Spectrophotometric Assay of Lipase Using pNPP as Substrate

The spectrophotometry method (Vonderwulbech *et al.,* 1992) was used for rapid and routine measurements of lipase activity using p-nitrophenyl palmitate (pNPP) as substrate. The substrate solution was prepared by adding solution A (40 mg of pNPP in 12 ml of propane-2-oil) to 9.5 ml of solution B (0.1 g of gum Arabic and 0.4 g of triton x-100 in 90 ml of distilled water) drop wise with intense stirring. The emulsion obtained remained stable for at least 2 hrs. The assay mixture consisted of 0.9 ml of substrate solution, 0.1 ml of suitable buffer (0.5 M) and 0.1 ml of suitably diluted enzyme. The assay mixture was incubated at 50^{0}C for 30 min and the p –

nitrophenol released was measured at 410 nm. The reagent blank was used and it contained distilled water instead of enzyme.

Reagent blank was used to set zero in the spectrophotometer. The difference in OD between substrate blank and the enzyme mixture was noted and the amount of p – nitrophenol released was calculated.

Unit of Enzyme Activity

Lipase activity is expressed in international units. One unit of activity (µ) is expressed as µ mol of p – nitrophenol released per minute under the standard assay condition (Vonderwulbech *et al.,* 1992) and is expressed in IU / mL of enzyme extract. For each experiment three replicates were maintained and the average value of enzyme production was calculated.

Estimation of Protein (Lowry *et al.,* 1951)

Since culture filtrate was used as crude enzyme source the protein component in the culture filtrate was estimated for determining units of lipase available per milligram of protein. Bovine serum albumin was used as standard for this purpose.

Estimation

Reagent A: Alkaline Sodium Carbonate solution (2% Na_2CO_3 in 0.1 NaOH)

Reagent B: Copper sulfate –Potassium sodium tartrate solution

0.5% ($CuSO_4$*$5H_2O$) in 1% Sodium potassium tartrate (freshly prepared).

Reagent C: Alkaline Copper reagent. Mix 50ml of reagent A and 1ml of reagent B (freshly prepared).

Folin–Ciocalteu Reagent

The commercial reagent was diluted with equal volume of water just before use.

Method

One ml of enzyme sample was suspended in test tube and to this 5 ml of reagent C was added. It was allowed to stand for 10 minutes at room temperature. Then 1 ml of dilute Folin Ciocalteu reagent was added to the test tubes and placed it in dark place for 10 minutes. The absorbancy of blue colour was measured at 660 nm in colorimeter. The amount of protein in enzyme sample was calculated from standard graph, prepared using Bovine serum albumin. Respective control was also maintained.

V. METHODS OF TRANSESTERIFICATION

a) Estimation of Free Fatty Acids (Cox and Pearson, 1962)

Objective: Any oil consists of few percent of fatty acid. If its percentage is higher it will lead to soap formation. Therefore, before oil is subjected to transesterification its free fatty acid has to be found out. If it is more than 4% acid – base method is followed. If it is below 4% base method is followed.

Preparation of Neutral Solvent

Mix 25 ml of ether, 25 ml of 95% alcohol and 1 ml of 1% phenolphthalein solution and neutralize with N/10 alkali.

Method

One gram of oil was dissolved in 50 ml of neutral solvent into a 250 ml conical flask and few drops of phenolphthalein were added. The contents were titrated against 0.1N potassium hydroxide and the mixture was stirred well until a pink colour which persisted for 15 seconds was obtained.

Calculation

$$\text{Acid value (mg / KOH / g)} = \frac{\text{Titre value} \times \text{normality of KOH} \times 56.1}{\text{Weight of the sample (g)}}$$

b) Transesterification Using Alkali (Senthilkumar *et al.,* 2003)

Transesterification of *Jatropha/Pongamia* seed oil to obtain biodiesel consists in replacing the glycerol of triglycerides with a short chain alcohol in the presence of a catalyst. Initially, Transesterification was performed using the following procedure and actual methodology was standardized during the course of work.

This involves making the triglycerides of oil to react with methyl alcohol in the presence of a catalyst (sodium hydroxide or potassium hydroxide) to produce glycerol and fatty acid ester. Known quantity of oil (100 ml), methanol (40 ml for *Jatropha* oil and 50 ml for *Pongamia* oil), sodium hydroxide or potassium hydroxide

(1 g for *Jatropha* oil and 450 mg for *Pongamia* oil), (according to FFA present in oil) were taken in a round bottom flask, the contents were stirred till ester formation began. The mixture was heated to 70^0C and held at that temperature without stirring for one hour, and then it was allowed to cool overnight without stirring.

Two layers were formed, the bottom layer consisted of glycerol and top layer was the methyl ester.

c) Transesterification using Acid

100 ml of oil was taken and heated at 35^0C for 15 minutes and 4% by volume of methanol was added to it and mixed for about 5 minutes. 0.1 ml of 95% sulphuric acid was added to oil and stirred well at 35^0C for one hour by heating gently. The above temperature was maintained for an hour and then heating was stopped but stirring was continued for another hour. The mixture was then allowed to settle for at least 8 hours. For making sodium/potassium methoxide solution for *Jatropha* oil 20 ml and 25 ml for *Pongamia* oil by volume of methanol and 750 mg for *Jatropha* oil and 450 *Pongamia* oil of sodium/potassium hydroxide was added and mixed completely. After the oil mixture has settled down for 8 hours, one half of the methoxide solution was added to the unheated oil and mixed for 5 minutes and then the oil mixture was heated to 70°C and the remaining methoxide solution was added to the heated mixture and the temperature was maintained until a straw yellow colour appeared in the mixture. The heating and mixing was then stopped. The mixture was

allowed to settle and the biodiesel component was separated and subjected to several washings with water.

d) Transesterification using Extracellular/Intracellular (Whole cell) Enzyme

Following were the steps involved in converting oil into its methyl ester using crude lipase as catalysts.

100 ml of oil was taken and heated for 15 minutes and cooled. 50 ml of crude enzyme obtained from culture filtrate was taken and to this 40 ml and 50 ml of methanol for *Jatropha* and *Pongamia* oil respectively was added and the content was stirred well. Then, it was poured into the oil and it was stirred for 1 hour. Mixture was heated to 40^0C without stirring for 1 hour and it was poured in separating funnel. Two layers were formed, the top layer was methyl ester and bottom layer consisted of glycerol. Vacuum dried fungal mycelium was mixed well by gently grinding with water/buffer at the rate of 40 mg per 40 ml (*Jatropha* oil) and 50 mg per 50 ml (*Pongamia* oil) and this was used as enzyme source per 100 ml of oil (*Jatropha/Pongamia*) along with 40 ml of methanol (*Jatropha* oil) and 50 ml of methanol (*Pongamia* oil).

e) Transesterification Using Commercial Enzyme and Partially Purified Enzyme (Dialyzed Enzyme) and Purified enzyme

Pretreatment

1 mg of lipase powder (Commercial / Purified) was suspended in suitable buffer (pH 7) and stirred for 30 min at 4^0C, the enzyme solution was then centrifuged at 17 X 1000 g for 20 min and the supernatant was used for biodiesel production using lipase.

100 ml of oil was taken and heated for 15 minutes and cooled, 50 ml of commercial lipase enzyme or 40 ml of dialyzed enzyme was taken and to this 40 ml of methanol was added and the content was stirred well. Then, it was poured into the oil and it was stirred for 1 hour. Mixture was heated to 40^0C without stirring for 1 hour and it was poured in separating funnel. Two layers were formed, the top layer was methyl ester and bottom layer consisted of glycerol.

Washing

The upper layer (Biodiesel) was washed several times with water to get pure biodiesel.

f) Transesterification Using Purified/Lyophilized enzyme

For purification of enzyme, after optimizing the culture conditions the extracellular lipase was purified 1200 fold by ammonium sulfate precipitation, sulfopropyl sepharose chromatography, Sephadox G75 Gel filtration and second sulfopropyl sepharose chromatography and then freeze dried (Hiol *et al.,* 2000).

g) Immobilization

The enzymes was collected by centrifugation (8000 rpm, 10 min). The wet cells (0.1g) were then suspended in 0.85% sterile saline mixed with sodium alginate (2%) in the ratio 1:1. The obtained mixture was then extruded drop wise through a syringe into 0.2M $CaCl_2$ solution from about 5 centimeters height. The calcium alginate beads of size 2mm were formed by adding the drop of enzyme-alginate mixture to calcium chloride solution Na^{2+} ions of sodium alginate were replaced by Ca^{2+} ions of calcium chloride solution. The beads were left in the $CaCl_2$ solution for about 30 min and then thoroughly washed with distilled water three times and used for further investigation on transesterification of *Jatropha*/Karanja oil.

Estimation of Biodiesel Yield

The yield of biodiesel was calculated in terms of weight percentage.

$$\text{Weight of oil + Methanol} = A$$

$$\text{Weight of biodiesel} = B$$

$$\text{Percentage Yield} = \frac{B}{A} \times 100$$

VI. CHARACTERIZATION OF THE BIODIESEL

Biodiesel was characterized based on the following parameters in order to compare with International Standards.

a) Determination of Iodine Value of the Oil (William Horowitz., 1975)

The iodine value is a measure of the degree of unsaturation in oil.

Materials

Preparation of Hanus Iodine Solution

0.6 g of iodine was weighed and dissolved in 825 ml glacial acetic acid by heating and then it was cooled. 25 ml of this solution was titrated against 0.1N sodium thiosulfate. Another portion of 200 ml of glacial acetic acid was measured and 3 ml of bromine solution was added to it. To 5 ml of this solution, 10 ml of 15% potassium iodide solution was added and titrated against 0.1N sodium thiosulfate. The value of bromine solution was calculated, to double halogen content of the remaining 800 ml of the above iodine solution as given below.

X = B / C, Where X = mL of bromine solution required to double the halogen content,

B = 800 X thiosulfate equivalent of 1 ml of iodine solution

C = thiosulfate equivalent of one ml of bromine solution.

- 15% Potassium iodide solution
- 0.1% Sodium thiosulfate
- 1% Starch

Procedure

i. Weighed 0.5 or 0.25 g of oil into an iodine flask and dissolved in 10 ml of chloroform.

ii. Added 25 ml of Hanus iodine solution using a pipette, draining it in a definite time and mixed well and allowed to stand in dark for exactly 30 min with occasional shaking.

iii. To the above, 10 ml of 15% potassium iodide was added and mixed thoroughly by adding 100 ml of freshly boiled and cooled water, washing down any free iodine on the stopper and titrated against 0.1N sodium thiosulfate until yellow solution turned almost colourless.

iv. Added a few drops of starch as indicator and titrated until the blue colour completely disappeared.

v. Towards the end of titration, the flask was stoppered and shaked vigorously so that any iodine remaining in solution in chloroform was taken up by potassium iodide solution.

vi. A blank without the sample was also maintained separately.

Calculation

The quantity of thiosulfate solution required for blank minus the quantity required for sample gives thiosulfate equivalent of iodine absorbed by the fat or oil taken for analysis.

$$\text{Iodine number} = \frac{(B - S) \times N \times 12.69}{\text{g sample}}$$

Where, B = mL thiosulfate for blank, S = mL thiosulfate for sample, N = Normality of thiosulfate solution

Amount of fat/oil taken should be adjusted such that the excess iodine in the added 25 ml of Hanus iodine solution has about 60% of excess iodine of the amount added i.e., if B –S is greater than B / 2, the procedure has been repeated with small sample.

b) Determination of Saponification Value of the Oil (William Horowitz, 1975)

Saponification is the process by which the fatty acid in the glycerides of the oil is hydrolyzed by an alkali.

Materials

- Accurately standardized hydrochloric acid 0.5 N.
- Alcoholic KOH – 40 g of KOH was dissolved in one litre of distilled alcohol, keeping the temperature below 15.5^0C until the alkali gets dissolved.
- Phenolphthalein indicator – 1% in 95% alcohol.
- Air condenser

Procedure

i. The sample was melted if it was not in the liquid state and filtered through paper to remove any impurities and the last traces of moisture. The sample was made completely free from moisture.

ii. 4 -5 g sample was weighed and taken in a flask. 50 ml of alcoholic potassium hydroxide was added from burette by allowing it to drain for a definite period of time.

iii. A blank was also prepared by taking only 50 ml of alcoholic potassium hydroxide allowing it to drain at the same duration of time.

iv. Air condenser was connected to the flask and the contents boiled gently for about 1 h.

v. After the flask and condenser were cooled, the inside of the condenser was rinsed with the little distilled water and the condenser was removed.

vi. Then 1ml of phenolphthalein indicator was added and titrated against 0.5N hydrochloric acid until the pink colour just disappeared.

Calculation

$$\text{Saponification value} = \frac{28.5 \times (\text{titre value of blank - titre value of sample})}{\text{Weight of sample (g)}}$$

c) Kinematic Viscosity (Sudharani, 1998)

The Viscosity of oil was determined by Redwood viscometer. The absolute viscosity of a fluid oil can be determined by measuring the rate of flow of the oil through a capillary tube kept at a uniform temperature, but in the case of lubricating oils, specific viscosity was generally determined by measuring the time taken for a given quantity of oil to flow through an orifice or jet of standard dimension under standard conditions.

Procedure

i. The viscometer cup was cleaned with the help of a suitable solvent and dried properly to remove any trace of the solvent.

ii. The instrument was leveled with the help of the leveling screws on the tripod.

iii. The bath was filled with water for determining viscosity at 80^0C and below.

iv. The brass ball was kept in position so as to seal the orifice.

v. The oil was poured carefully into the oil cup upto the tip of the indicator.

vi. The 50 ml flask was kept in position, below the jet.

vii. A thermometer and a stirrer was inserted and inserted and covered with lid. Water in the bath and oil in the oil cup were continuously stirred until the oil attains the described constant temperature.

viii. When the temperature of the oil has become quite steady in the oil cup and showed a constant reading, the ball valve was lifted and simultaneously the stopwatch was started and oil was allowed to fill in the flask upto 50 ml mark. The stop watch was stopped when the oil reached 50 ml mark and the time was noted in seconds.

ix. The ball valve was replaced in position to seal the cup to prevent over flow of the oil.

x. The oil was refilled up to the indicator tip of the oil cup. The experiment was repeated to get nearly reproducible results. The mean value was reported as "Red wood 1 viscosity at T°C = t seconds". This is the viscosity at room temperature, T°C.

xi. The experiment was repeated at five elevated temperature, 45, 55, 65, 75 and 85°C and the respective times of efflux was noted.

The Redwood viscosity obtained can be converted into Kinematic viscosity by using the formula

$$V = At - \frac{B}{t}$$

Where, V = Kinematic of the oil in centistokes, t = Time of flow in seconds, A and B are instrument constant.

The value of (A) = 0.264 and (B) = 190, when t = 40 to 85 seconds.

(A) = 0.247 and (B) = 65, when t = 85 to 2000 seconds.

100 Centistokes = 1 stoke

Unit conversions: Stoke x Density = poise (Absolute density)

1 / poise = centipoises.

Cloud Point and Pour Point (Sudharani, 1998)

The Cloud and Pour point apparatus was used to find out the cloud point and pour point of testing samples.

The Cloud point is the temperature at which the oil will just cease to flow or pour when cooled under definite prescribed conditions.

The Pour point is the lowest temperature at which the oil will just cease to flow or pour when cooled under definite prescribed conditions.

d) Flash Point and Fire Point (Sudharani, 1998)

The Penskymartens apparatus was used for Flash point and Fire point determination. The Flash point is the lowest temperature at which oil on vaporization gives sufficient quantity of vapours that will flash when brought into contact with flame.

The Fire point of oil is the lowest temperature at which it will give enough vapour, which on rising will begin to produce a continuous flame above the oil. After the flash point has been reached the oil is heated continuously at the rate of 1°C / per minute and the application of test flame is done after every 1°C rise in temperature of oil, at certain temperaturethe oil will ignite and continue to burn for a period of at least 5 seconds. The temperature at which such thing happens, is noted, that will give the fire point of the oil.

e) Ash Value (Sudharani, 1998)

The test was carried out by taking a known amount of the oil in a crucible and burning all the combustible products and then again weighing the residue.

f) Calorific Value

The Caloric value was determined using Bomb calorimeter.

g) Performance Characteristics of Engine and its Emission

The test was conducted on a single cylinder 499cc four – stroke, naturally aspirated, direct injection and water cooled diesel engine test setup. The engine develops a rated power of 9.7 hp / 7.2 kW at rated speed range of 2800 – 3000 rpm. This type of engine is being used by M/s. Force Motor in Minidor pickup van.

In this research work in order to know the performance of biodiesel in various blends a set of experiments were conducted in an eddy current dynamometer test

setup at a designed speed of 1900 rpm. The engine was started by self starter with diesel fuel supply, and it is allowed to steady state (for about 10 minutes). Water to engine cooling jacket is maintained by circulating water. The experiments were conducted at 25% of full load, 50% of full load, 75% of full load and full load condition (100%) of engine power, with neat diesel and blends of methyl ester of Jatropha/Pongamia oil seed oil extraction of both chemical and enzyme process.

The experiments were conducted at the design injection pressure of 200 bar. The engine was run with the neat biodiesel and blends of methyl ester of *Jatropha/Pongamia* oil seed oil varying from 10 to 50% by volume with diesel for the same above conditions, the performance and emissions tests were carried out.

The procedure for testing in the engine is given below.

i. The engine is started by starter motor with diesel fuel and is allowed to run for about 10 minutes to reach steady state conditions.

ii. After attaining the steady state, readings of exhaust gas temperature and the amount of diesel fuel consumed is noted down in idling condition (i.e. without load) along with emission level readings.

iii. The whole set of experiments were conducted at the constant engine speed of 1900 rpm at the designed injection pressure of 200 bar for 0%, 25%, 50%, 75% and 100% of full load using eddy current dynamometer

for diesel, *Jatropha/Pongamia* oil seed oil methyl ester and its blends viz., B10%, B20%, B30%, B40%, and B50%.

iv. Engine emissions are found out by using AVL meter(CO, HC, and Nox)

VII) GC – MS ANALYSIS

Analysis of Samples

The oil sample was methylated and analyzed in GC- MS for its fatty acid components.

i. GC Programme

Experiment	: Thermo GC - Trace Ultra VER : 5.0, Thermo MS DSQ II
Column	: DB 35 - MS Capillary Standard Non - Polar Column
Dimension	: 30 Mts, ID: 0.25 mm, Film: 0.25 µm
Carrier gas	: He, Flow: 1.0 ml/min
Temperature program	: Oven temperature 70^0C raised to 260^0C at 6^0C/min
Injection Volume	: 1 Micro liter
Software	: Xcalibur

ii. MS Programme

Library used	: NIST
Inlet line temperature	: No left inlet
Source temperature	: 220^0C
Mass scan: (m/z)	: 500.00
Total MS running time	: 37.49 mins

Statistical Analysis

The results were statistically analyzed by ANOVA and DMRT In all the results means followed by common letter were not significantly different at 5% level by DMRT (Duncan, 1955).

CD ($p < 0.05$), values are mean of triplicates, A means followed by a common superscript letter are not significantly at 5% level by using DMRT.