

**PRODUCTION AND ANALYSIS OF BIODIESEL FROM WASTE COOKING OILS
USING ASPERGILLUS SP**

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ABSTRACT:

The present study was carried out to produce and analyse biodiesel from waste cooking oils using fungal lipase enzyme produced by the isolated organisms. The waste cooking oils were analysed qualitatively and also by Thin Layer Chromatography (TLC). Soil samples were collected from Madukkur, Thanjavur (Dt), Tamil Nadu and physico chemical characteristics of the soil sample were analysed. The soil samples were subjected to serial dilution agar plating method for the isolation and identification of fungi *Aspergillus Sp* confirmed according to *The Manual of Soil Fungi*. The maximum yield of 58.6ml of biodiesel (COB) was obtained in 100 ml of oil with methanol, NaOH and *Aspergillus niger* lipase mediated transesterification process. This was followed by *Aspergillus flavus* mediated transesterification process. The methanol, NaOH and fungal lipase process in all the reactions produced lesser quantity of 51ml of biodiesel (POB).

KEYWORDS: Waste cooking oils, Physico chemical analyse, TLC, Transesterification, Biodiesel Production

INTRODUCTION

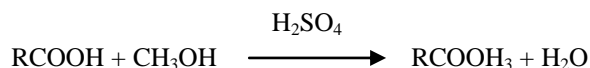
Biodiesel has gained importance in the recent past for its ability to replace fossil fuels, which are likely to run out within a century. The environmental issues concerned with the exhaust gases emission by the usage of fossil fuels also encourage the usage of biodiesel. This has proved to be eco-friendly for more than fossil fuels. Biodiesel is known as a carbon neutral fuel because the carbon present in the exhaust was originally fixed from the atmosphere and a mixture of mono-alkyl esters obtained from vegetable oils like soybean, jatropha,

rapeseed, palm, sunflower, corn, peanut and cotton seed oil¹.

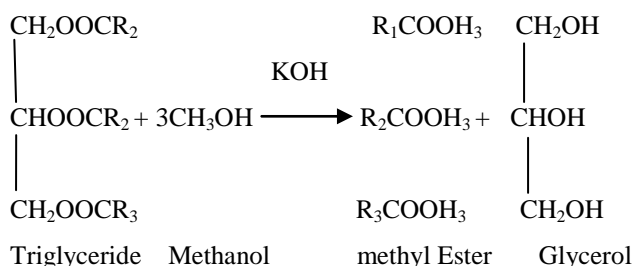
Biodiesel, mean while, is an alternative or additive to standard diesel fuel that is made from biological ingredients instead of petroleum. Biodiesel is usually made of bio oils through a series of chemical reactions but is non-toxic and renewable.

The transesterification process was widely used in biodiesel production from different biomass materials. The process consists of two steps namely, acid esterification and alkali transesterification².

Step 1: Acid esterification reduces the FFA value of unrefined oil using an acid catalyst.



Step 2: Alkali transesterification : After removing the impurities of the product from the Step 1, it is transesterified to monoesters of fatty acids using an alkali catalyst.



Transesterification is a process of displacement of an alcohol group from an ester by another alcohol. In vegetable oil almost 90-95% is glycerides, which are basically esters of glycerol and fatty acids. The use of methyl, ethyl, and butyl alcohols for the transesterifications of rape oil, sunflower oil, cotton seed oil, peanut oil, soybean oil, and palm oil to produce methyl, ethyl, and butyl esters. The transesterification were enhanced by the use of potassium hydroxide, sodium hydroxide, sodium methoxide, or sodium ethoxide as catalysts. Important reaction parameters for the transesterification are: (a) ratio of alcohol to vegetable oil, (b) temperature, (c) rate of agitation, and (d) amount of water present in reaction mixture. Ethanol rather than methanol to produce ethyl ester of vegetable oils. Although use of different alcohols present few differences with regards to the kinetics of reaction, the final yield of esters remains more or less unchanged. Therefore, selection of the alcohol is based on cost and performance consideration. Ethanol can be produced³. The waste cooking oil is generated from the fried food, which need large amounts of oil because it requires the full immersion of food at temperature greater than 180° C. Accordingly to the high temperature are generated changes in its chemical and physical composition, as^{a)} well as in its organoleptic properties which affect both the food and oil quality. Used cooking oil is normally black, a strong odor and does not have large amount of^{b)} solids because its collection is passed through a fine mesh⁴. The potential of isolated filamentous fungi, *Aspergillus sp*, as whole cell acts as biocatalyst for biodiesel production using Potato Dextrose Broth. The traditional feed stocks for biodiesel production as vegetable oils and animal fats results in competition with the feed industry. Single Cell Oil (SCO) from microbes is considered as an alternative oil source due to the high productivity and low land requirement.⁵ The biggest advantage of biodiesel is environmentally friendliness that is has over gasoline and petroleum

diesel. The biodiesel as a diesel fuel are its portability, ready availability, renewability, higher combustion efficiency lower sulfur, aromatic content higher and higher biodegradability. It's potential for reducing a given economy's dependency on imported petroleum, biodegradability high flash point and inherent lubricity in the neat form⁶. Hence, the present study was made to produce biodiesel from waste cooking oil under laboratory condition by *Aspergillus sp*.

MATERIALS AND METHODS

OIL COLLECTION

The waste cooking oils were collected from local restaurants in Madukkur, Thanjavur (Dt), Tamil Nadu. The oils used were Palm oil, Coconut oil, Sunflower oil and mixed oil.

QUALITATIVE ANALYSIS OF OILS

Solubility test

Solubility of the oils were tested in

- (a) Water (2ml): Oil floated on the surface of water forming a layer
- (b) Ethanol (2ml): Sparingly soluble
- (c) Chloroform (2ml): Completely soluble

Emulsification test

1ml of oil was shaken vigorously with 2ml of water. An emulsion was formed as two separate layers, on standing.

1ml of oil, 2ml of soap solution was added and shaken well. An emulsion was formed which does not separate into two layers.

Acrolein test

A pinch of Potassium bisulphate was taken in a dry test tube and 4 drops of oil were added and then heated.

Saponification test

Two drops of oil and 4ml of 2% Sodium carbonate solution was added and shaken vigorously. The mixture of oil and sodium carbonate solution were boiled and then cooled. A soap solution was formed and is divided into three part, To the first part, few drops of concentrated hydrochloric acid was added. To the second

part, equal volume of Sodium chloride was added. To the last part, few drops of Calcium chloride was added.

Test for Unsaturation

To 3 drops of oil in 2ml of ethanol, 0.5% of alcoholic bromine solution was added in drops.

Test for Cholesterol

a) Libedmann- Burchard test

To 2 or 3 drops of oil, 2ml of chloroform and few drops of acetic anhydride were added and mixed well. And then 2 drops of concentrated H_2SO_4 were added along with sides of the test tube mixed well till the appearance of dark yellow colour.

b) Saikaushi reaction

To a little of the oil, 2ml of chloroform and equal volume of concentrated nitric acid were added drop by drop along the sides of the test tubes.

ANALYSIS OF OILS BY TLC

30g of silica gel was added and flask containing 60ml distilled water (1:2). Slurry was prepared by stirring for 1-2 minutes and poured into the applicator positioned on the head glass plate. The slurry coated over the glass plates at a thickness of 0.25mm for chemical analysis by moving the applicator at a uniform speed from one end to the other. The plates were left to dry at room temperature for 15-30 minjtes. The plates were headed in an oven at 100-120°C for 1-2 hours and to remove the moisture and then to activate the absorbent on the plate. Left 2.5 cm from one end of the glass plate and at least an equal distance from the edges. The sample spotted the silica gel coated plate and respective standards onto the plate. Poured the appropriate developing solvent [toluene: ethyl acetate: formic acid (6:3:1)] into a glass jar at least one hour before use. It was saturated the jar with the runing solvent vapours. The plate was dipped in the running solvent just below the sample load. The solvent allowed to run due to capillary action till it reached nearly the end of the plate. The plate removed from the jar and left it dry. and iodine vapour sprayed for the visualization of the compound. The distance of the

solvent was measured and the compound travelled to obtain the RF values.⁷

SAMPLE COLLECTION (SOIL)

Soil sample were collected randomly from the restaurants in Madukkur, Thanjavur (Dt). The samples were mixed to prepare a composition mixture for fungal isolation.

PHYSICO-CHEMICAL ANALYSIS

Determination of soil pH⁸

The pH was determined using pH paper.

Estimation of alkalinity⁹

10ml of filtered sample and 2 drops of phenolphthalein indicator was added and titrated against 0.1N HCL. The sample was used to detect both phenolphthalein alkalinity and total alkalinity. The end point was appearance of pink colour.

Estimation of Ammonia⁹

10 ml of filtered sample and 0.4 ml phenol reagent were added and stirred well, and then 0.4 ml of nitro prusside reagent was added and mixed well. Finally, 1ml of the oxidizing reagent was added and mixed thoroughly and then incubated at room temperature for an hour. Absorbance was measured at 630 nm in spectronic 20. The amount of ammonia was calculated by using a standard graph prepared by using NH_4 chloride.

Estimation of Nitrate¹⁰

50 ml of sample and 1 ml of EDTA solution were taken and 1.0 ml of sulphanilic acid was added after 10 minutes. Then 1.0 ml of α naphthylamine hydrochloride solution was added. And then the optical density of the solution was read at 520 nm after 10 minutes.

Estimation of calcium¹¹

10 ml of filtered sample, 0.4 ml of 0.1 N NaOH and 50mg of muraxide indicator were added. This titrated against 0.01 N and EDTA solution until the pink colour solution changed to purple.

Estimation of chloride¹²

10 ml of filtered sample in a Erlenmeyer flask and few drops of K_2PO_4 were added and titrated against 0.0142 N

Silver nitrate solution until a persistent red colour appearance.

Estimation of BOD¹³

20 ml of sample was taken in the flask. Then 10 ml of potassium dichromate solution, pinch of silver sulphate and mercuric sulphate were added. And then 30 ml of sulphuric acid were added. The contents were refluxed for two hours. Then the flasks were cooled. After 2-3 drops of ferroin indicator solution was added and titrated against with NH₄ SO₄ solution. At the end point blue green colour of contents changed to reddish blue in colour.

Isolation of fungi¹⁴

One gram of soil sample was serially diluted upto 10⁻¹ to 10⁻⁷ dilution. Approximately diluted samples were plated initially on Potato Dextrose Agar (PDA) medium. The plates were incubated at 28°C for 3 days.

MICROSCOPIC OBSERVATION

Wet mount technique¹⁵

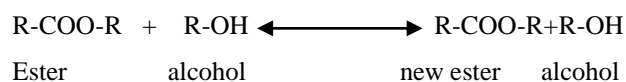
A small portion of fungal colony was placed on a slide. A drop of lactophenol cotton blue mounting fluid was placed on fungal culture. Cover slip was positioned on the culture and gently pressed the slide to disperse the sample. The prepared slides were examined under low power and high power objectives.

FUNGAL LIPASE EXTRACTION¹⁶

The liquid medium was centrifuged at 10,000 rpm for 10 minutes and then the supernatant was discarded. The pellet was taken in 5 ml of methanol:chloroform in 2:1 ratio and kept in shaker for 20 minutes, then centrifuged at 10,000 rpm for 10 minutes. The organic phase was washed with one ml of water and again centrifuged at 20,000 rpm for 5 minutes. The upper aqueous phase was removed and the lower organic phase was rinsed twice with 5 ml of methanol and water in 1:1 ratio. Finally, the extracted lipid with lipase was collected from the solvent phase and stored for further experimental work.

TRANSESTERIFICATION REACTION

Tranesterification reaction process also called alcoholysis, is the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis except than an alcohol is used instead of water. This has been widely used to reduce the viscosity of the triglycerides. The transesterification is represented as:



This transesterification reaction was performed by waste cooking oil with alcohol or methanol in the presence of a catalyst Sodium hydroxide or Hydrochloric acid and fungal enzymes. The esterification mixture consisted of 100 ml of oil, 20 ml of ethanol or methanol, 3 g of NaOH or 3 ml HCL and 5 ml of fungal enzyme. The experiment was performed at 40°C and the reaction time was kept constant for 3 hours for all the experiments.

After the transesterification reaction, the produced biodiesel was separated from glycerol with the help of separating funnel washed with 5% water followed by Magnesium sulphate anhydrous to remove the water. The biodiesel and glycerol ratio was recorded.

PURIFICATION OF BIODIESEL

After separation of the two layers, the upper layer of biodiesel was purified by distilling the residual methanol at 60°C. The remaining catalyst was removed by successive rinsing with distilled water by adding 1-2 drops of acetic acid to neutralize the catalyst. The residual can be eliminated by treatment with anhydrous Sodium sulphate (NaSO₄) followed by filtration. Transparent blackish liquid was obtained as the final product.

BIODIESEL ANALYSIS

Biodiesel analysis such as viscosity, density and flash point were analysed¹⁷. High viscosity is the major problem preventing the use of vegetable oil directly in diesel engines as it affects the flow of fuel and spray characteristics¹⁸.

STATISTICAL ANALYSIS ¹⁹

The results obtained in the present investigation were subjected to statistical analysis like mean (X) and standard deviation (SD).

RESULTS AND DISCUSSION

The present study was carried out to produce and analyse biodiesel from waste cooking oils using fungal lipase enzyme, analysis of physico chemical characteristics of the soil sample and to investigate the biodiesel production potency of the test organisms.

Qualitative analysis of oils

Waste cooking oils were subjected to different qualitative analysis such as, solubility test,

emulsification test, acrolein test, saponification test, unsaturation and cholesterol test. In solubility test, (A) all oils were insoluble in water, (B) all oils were sparingly soluble in ethanol, (C) all oils were soluble in chloroform. In emulsification test, (D) all oils formed temporary separate layer, (E) all oils formed permanent separate layer. In acrolein test, (F) triglycerides were present in all oils. In saponification test, (G) soluble fatty acid was present in all oils, (H) sodium salt of fatty acid present in all oils, (I) calcium salt of fatty acid was present in all oils. In Unsaturation test, (J) unsaturated fatty acid was present in all oils. In cholesterol test, (K) cholesterol was present in all oils, The performance was evaluated according to Mangold *et al.*, 1961.(Table-2).

Qualitative analysis of waste cooking oils

Experiment	Palm	coconut	Sun flower	Mixed oil
Solubility	A Insoluble	Insoluble	Insoluble	Insoluble
	B Sparingly Soluble	Sparingly Soluble	Sparingly Soluble	Sparingly Soluble
	C Soluble	Soluble	Soluble	Soluble
Emulsification	D Temporary	Temporary	Temporary	Temporary
	E Permanent	Permanent	Permanent	Permanent
Acrolein	F Triglycerides present	Triglycerides present	Triglycerides present	Triglycerides present
Saponification	G Soluble fatty acid present	Soluble fatty acid present	Soluble fatty acid present	Soluble fatty acid present
	H Sodium salt of fatty acid present	Sodium salt of fatty acid present	Sodium salt of fatty acid present	Sodium salt of fatty acid present
	I Calcium salt of fatty acid present	Calcium salt of fatty acid present	Calcium salt of fatty acid present	Calcium salt of fatty acid present
Test for unsaturation	J Unsaturated fatty acid present	Unsaturated fatty acid present	Unsaturated fatty acid present	Unsaturated fatty acid present
Test for cholesterol	K Cholesterol present	Cholesterol present	Cholesterol present	Cholesterol present
	L Cholesterol present concentrated formed	-	Cholesterol present concentrated formed	Cholesterol present concentrated formed

TLC- studies of oils

The fatty acid composition of the waste cooking oils were used for biodiesel production showed the presence of butyric, caproic, caprylic, capric, lauric, myristic, palmitic, steric, oleic, linoleic, α -linoleic, erucic, EPA, eicosenic, riconoleic and dihydrosteric acids identified as per the peak position and the relative retention time of those standard methyl esters in TLC.

Physico chemical characteristics of soil sample

The physico chemical characteristics of the soil sample showed the pH of 6.5 , Carbon di oxide (30±1.5), Alkalinity (12.5±1), Nitrate (16±2), Ammonia (24±5.8), Phosphate (19.3±1.2), Calcium (12.9±0.5), Chloride (11.5±0.2) and BOD (46.4±1.7) were analyzed (Table-1).

Physico chemical characteristics of the soil sample

S.No	Properties	Values
1	pH	6.5
2	Carbondioxide	30±1.5
3	Alkalinity	12.5±1.0
4	Nitrate	16±2.0
5	Ammonia	24±5.8
6	Phosphate	19.3±1.2
7	Calcium	12.9±0.5
8	Chloride	11.5±0.2
9	BOD	46.4±1.7

BOD- Biological Oxygen Demand

The physico chemical properties of the soil sample namely, pH, BOD, Sodium, Potassium, Calcium, Magnesium, Chloride and sulphate were analysed by standard methods²⁰. This study was carried out to analyse the physico chemical characteristics of soil sample namely pH, Phosphate, alkalinity, Calcium, Nitrate, Magnesium, carbon dioxide and Chloride were analyzed by standard methods.

Isolation and identification of fungi

The potential of isolated filamentous fungi *Aspergillus sp* as whole cell acts as biocatalyst for biodiesel production using Sabourauds Dextrose Broth Medium (SDBM) and Corncob Waste Liquor (CWL) as substrates²¹. In the study, Madukkur restaurants soil samples were used for the isolation of the fungal species using serial dilution and agar plating methods. Serially diluted sample was poured into the Potato Dextrose Agar medium (PDA) showed the number of fungal species. The colonies were identified by lacto phenol cotton blue method using manual of soil fungi. The following species were isolated viz, *Aspergillus niger* and *Aspergillus flavus*.

Efficiency of the fungal lipase enzyme in biodiesel production

Waste cooking oils biodiesel such as, POB, COB, SOB and MOB yield 41.6, 39, 58.6 and 40.3ml respectively were obtained in 100 ml of cooking oil with methanol, NaOH and *Aspergillus niger* lipase mediated transesterification process. This was followed by *Aspergillus flavus* mediated transesterification process. The methanol, NaOH and fungal lipase transesterification process in all the reactions produced biodiesel such as POB, COB, SOB and MOB yield i.e., 51, 34.6, 49 and 36 ml respectively (Table-4).

The fungal lipase has a vital role in catalytic activity of esterification process and generated high quality and quantity of biodiesel²². This study was carried out to produce biodiesel from waste cooking oil and to use micro emulsion with solvents like ethanol and methanol following acid, alkali and fungal enzyme catalysis methods. The best suited method of biodiesel production was methanolic and alkali mediated transesterification process rather than methanolic and acidic transesterification. The maximum yield of biodiesel was obtained from *Aspergillus niger* lipase enzyme, methanolic and alkali mediated transesterification reaction produce high amount of biodiesel.

Biodiesel yield of waste cooking oils

S.No	Cooking oil biodiesel	Concentration of catalyst NaOH (g)	Amount of catalyst NaOH (g)	<i>Aspergillus niger</i>		<i>Aspergillus flavus</i>	
				Biodiesel (%)	Glycerine (%)	Biodiesel (%)	Glycerine (%)
1.	POB	Normal	0.7	41.6	57.40	51	49
		Double	1.4	39	61	56	44
		Half	0.35	28	72	34.6	65.40
2	SOB	Normal	0.7	39	61	49	51
		Double	1.4	34	66	30	70
		Half	0.35	28	72	25	75
3	COB	Normal	0.7	58.6	41.50	49	51
		Double	1.4	41	59	44	56
		Half	0.35	40	60	35	65
4	MOB	Normal	0.7	40.30	59.70	36	64
		Double	1.4	38	62	33	67
		Half	0.35	26	74	21	79

Waste cooking oil can be an important source for biodiesel production in Canada as there is large quantity of waste cooking oil available. Use of waste cooking oil helps improve the biodiesel economics²³. In this study was carried out maximum yield (58.6ml) of biodiesel (Coconut Oil Biodiesel) was obtained in 100 ml of oil with methanol, NaOH and *Aspergillus niger* lipase mediated transesterification process. This was followed by *Aspergillus flavus* mediated transesterification

process. The methanol, NaOH and fungal lipase transesterification process in all the reactions produced lesser quantity (51ml) of biodiesel (Palm Oil Biodiesel).

Fuel properties analysis

The experimental investigation was carried out for different fuel properties and the performance was evaluated according to ASTM D-445, D-1298, D-93, D-1500, D-4294 and compared with diesel (Table-5)

Comparative analysis for fuel properties of waste cooking oils biodiesel

S.No	Type of diesel	Viscosity	Density	Flash point
1	POB	5.09	0.83	120
2	SOB	5.36	0.82	123
3	COB	5.13	0.84	128
4	MOB	5.0	0.80	122
5	HSD	1.3-4.1	0.83	60-80

CONCLUSION

The present study was carried out to produce and analyse biodiesel from waste cooking oils like using fungal lipase enzyme produced by the isolated organisms. The advantages of utilizing biodiesel replacing conventional fuel are that biodiesel is non-toxic, biodegradable,

renewable and less pollutant in emissions. Engine life can be prolonged by reducing the frequency of engine part replacement and increasing the lubricity. Due to the high cost of feed stock used for biodiesel synthesis, it is not yet commercialized globally. Commercial utilization of biodiesel will lead to many advantages like green

cover for waste land, greater support for agriculture and rural economies, thereby reduced dependence on imported crude oil and a smaller rate of increase in air pollution.

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