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Production of Biodiesel by Enzymatic Transesterification using Immobilized Lipase

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Abstract - Biodiesel (monoalkyl esters of long-chain fatty acids) has great potential as an alternative to conventional diesel fuel. From environmental point of view it is a renewable, biodegradable and non-polluting source of energy. In this work, biodiesel was produced by enzymatic transesterification reaction. Transesterification is a three step consecutive reaction in which, three moles of biodiesel and one mole of glycerol are produced for every mole of triacylglycerol that undergoes complete conversion. Enzyme method of catalysis can act as a better substitute to the chemical ones as it is specific, thermo stable and environmental friendly. The high cost of liquid enzyme was mitigated by using immobilized lipase enzyme (CALB - DILBEADS^{TM} (10000)) due to its remarkable property of reusability. To further reduce the cost of production, restaurant waste cooking oil (WCO) was used as the feedstock. The effects of alcohol as solvent, enzyme concentration, alcohol to oil molar ratio and reaction time on production of biodiesel were investigated. The results indicated that methanol as solvent, 5% enzyme concentration, 3:1 methanol:WCO molar ratio and 8 hours reaction time was found to be optimum at 37°C and 150 rpm. Under these optimal conditions, the conversion of FFAs (Free Fatty Acids) to FAMEs (Fatty Acid Methyl Esters) was found to be 99.71%.

Keywords – Biodiesel, Waste Cooking Oil, Immobilized lipase, Batch production, Fuel Grade

INTRODUCTION

Fossil fuels have been a major source of energy since about 1850, the start of the industrial era. Presently, we are passing through the peak period of the fossil age. As the amount of fossil fuels is depleting, it is becoming costlier and thus its use is gradually declining. As a result new renewable energy reserves are continuously being discovered.[1]
Biomass is one of the most plentiful and well-utilized sources of renewable energy in the world. The term "biomass" refers to organic matter that has stored energy through the process of photosynthesis. Many of the biomass fuels (also referred to as *biofuels*) used today come in the form of wood products, dried vegetation, crop residues, and aquatic plants. It is a widely utilized source of energy, due to its low cost and indigenous nature. The different forms in which we use biofuels include bioethanol (bioalcohol), biogas, syngas (CH_4+CO_2), bioethers, and biodiesel.[2] Rapid growth has been witnessed in the production and consumption of biofuels for powering combustion engines for the transportation economic sector. Also, biofuels have increased in popularity because of rising oil prices and need for energy security.

Biodiesel

The term *biodiesel* is widely used to represent alkyl esters produced by the transesterification reaction of plant oils and animal fats by using alcohols and appropriate catalyst.[3] Biodiesel (monoalkyl esters of long-chain fatty acids) has a great potential as an alternative diesel fuel.[1] It has shown its ability to meet the energy demand of the world in the transportation, agriculture, commercial and industrial sector of the economy.[4] It can be used in vehicles as a substitute to diesel, for generating electricity, in compression ignition engines with no or minor modifications for heat production. Biodiesel has thus recently come up as a superlative alternative fuel as a green, renewable and potentially unlimited resource.

The striking properties of biodiesel which makes it an important fuel source are:
i) Its colour ranges from golden to dark brown depending upon the feedstock being used.
ii) It has high boiling points (315-350ºC)[5]
iii) It is immiscible with water.
iv) It has low vapour pressure.

There are several possible processes for biodiesel synthesis which include,
i) Pyrolysis: It is a type of thermolysis, and is most commonly observed in organic materials exposed to high temperatures. The biodiesel produced by this process has good quality due to the reduced viscosity. However it still produces more biogasoline than biodiesel.[6]

ii) Use of Microemulsions: A microemulsion is defined as colloidal equilibrium dispersion of optically isotropic fluid microstructures formed simultaneously from two normally immiscible liquids and one or more ionic or non-ionic amphiphiles.

Microemulsions using solvents like ethanol, methanol has been studied for biodiesel production. However, these biofuels have shown engine performance problems.[7]

iii) Tranesterification: It is also called as alcoholysis. It is a three-step consecutive reaction, in which diglycerides and monoglycerides are formed as intermediate compounds. Three moles of biodiesel and one mole of glycerol are produced for every mole of triacylglycerol (TAG) that undergoes complete conversion in the presence of suitable catalyst and alcohol (Fig.1).[4]

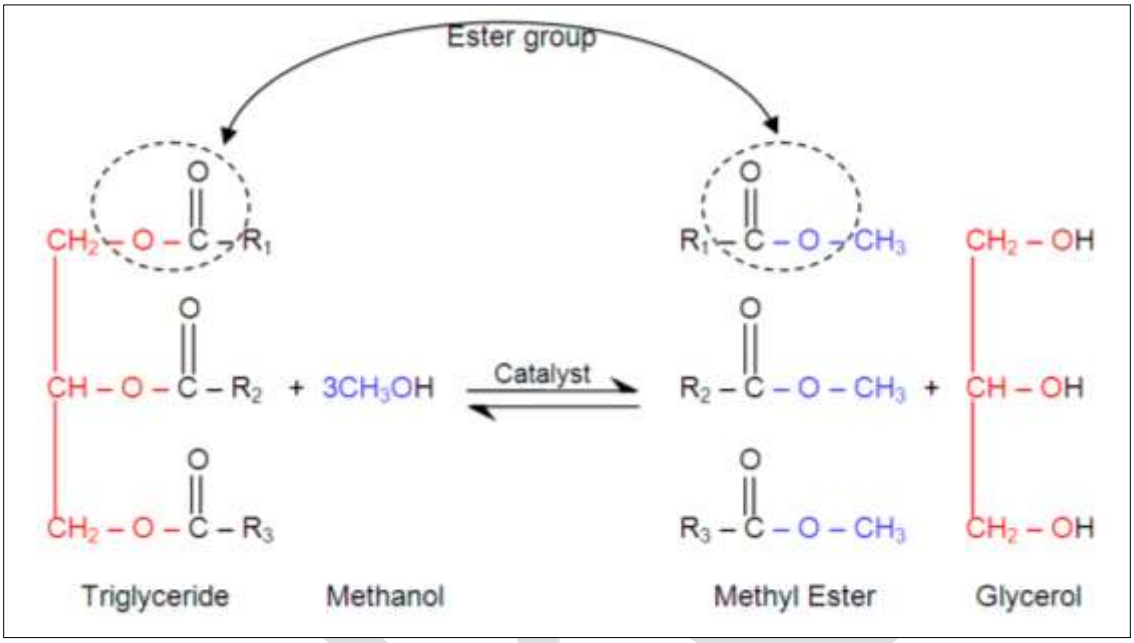

Fig 1: Transesterification reaction

Different types of catalysts that can be used for transesterification reaction include:

i) Acid: This requires heating to accelerate the reaction and the reaction time may vary from few minutes to several hours.[8]

ii) Alkali: This reacts with the free fatty acids present in the waste oil by producing soaps that inhibit the separation of the ester, glycerin and wash water.[8]

iii) Enzymes: This method has proven to be advantageous due its reusability, specificity, abilityto accept new substrates, thermo stability, mild reaction conditions in terms of low temperature and its environmental friendliness. However, the high cost of enzyme particularly lipase, makes enzyme driven processes economically unattractive.[1] Hence, the use of immobilized lipase is a possible solution to this problem because the enzyme can be recovered from the product and reused.[9][10]

Current instability of oil supplies and the continuous fluctuation of prices have further ignited widespread interest in alternative energy sources. However, biomass has the potential to offer diverse supplies of reliable, affordable, and environmentally sound biofuels to replace fossil fuels.

Therefore the major advantages of Biodiesel as a biofuel are:

i) Over conventional diesel:

✓ It comes from renewable sources, and hence does not contribute to new carbon dioxide emission.

✓ It is biodegradable.

✓ Its combustion products have reduced levels of particulates, sulphur oxides, nitrogen oxides, and therefore, significantly reduces pollution.

ii) Over other Biofuels:

✓ It can be pumped, stored and handled using the same infrastructure employed for conventional diesel fuel.

Biodiesel can be used for several applications including:

i) Transportation: biodiesel can be used in vehicles as a substitute to diesel.

ii) Power generation: biodiesel can be used for electricity generation.

iii) Heat production: biodiesel can be used in compression ignition engines with no or minor modifications.

MATERIALS AND METHODS

- **Procurement of Substrate and Enzyme**

The raw material used for the production of biodiesel was Waste Cooking Oil (WCO). WCO was obtained from a nearby fast food restaurant. The immobilized *Candida antartica* lipase B (CALB) enzyme DILBEADS^{TM} (10000) was procured from Fermenta Biotech Ltd, Thane, India and was stored at 4°C.

- **Pretreatment of WCO**

The WCO was first passed through a cloth to remove all the solid impurities. Then it was heated at 60°C for 20 minutes so as to homogenize the oil.[11]After heating, it was cooled to room temperature and stored in a clean container.

- **Production of Biodiesel by Batch Process**

The oil used was WCO (sunflower oil) and the alcohols selected were ethanol and methanol. The amount of alcohol added was according to the alcohol to WCO molar ratio and also the enzyme added was in percentage with respect to the quantity of WCO.
Enzyme concentration, type of alcohol (ethanol or methanol), molar ratio and reaction time were optimized for obtaining maximum yield and to fulfil the criteria for fuel grade biodiesel.

➢ **Enzyme Concentration**

The various enzyme concentrations tried were 4%, 5%, 10% and 15% (weight % of WCO) of enzyme in 50 gm of WCO. The alcohol to WCO molar ratio was kept as 3:1 since this was reported to be the least required stoichiometric ratio. The reaction was carried out at 37°C and 150 rpm.

➢ **Selection of Alcohol**

The alcohols like ethanol and methanol were tried for the production of biodiesel. The alcohol to WCO molar ratio was selected as 3:1 for both methanol and ethanol.50g of WCO was used and to which 5% (% of WCO)enzyme (2.5g) was added and all the other conditions were kept constant.

➢ **Molar Ratio**

After optimizing the enzyme concentration and alcohol to be used, the ratios of methanol to WCO were varied. Three molar ratios were selected i.e. 3:1, 6:1 and 9:1 methanol to WCO. The quantity of oil (50g) was kept constant and the optimized enzyme concentration (5%)was used for each of the molar ratios and the reaction was carried out by keeping all the other conditions same.

➢ **Reaction Time**

The production of biodiesel for 4, 6 and 8 hours were carried out with 50g WCO, 5% enzyme concentration, 3:1 methanol to WCO molar ratio.

- **Purification of product**

After the reaction time, the enzymes were separated from the reaction mixture by filtration using filter paper. The enzymes were first washed with distilled water several times and then finally with n-hexane which helped to remove the reaction mixture if present on it and also the rate of drying became faster. The dried immobilized enzymes were stored at 4°C in a clean container for further reuse. The reaction mixture was first washed with n-hexane and distilled water in the ratio 1:1(mixture: n-hexane) and 1:2(mixture: distilled water) in a separating funnel. The bottom layer of water was removed and the top layer was again washed with 1:1 (mixture: n-hexane) and 1:1(mixture: distilled water). After the second wash, the top layerconsists of FAME and n-hexane as the most of the methanol got removed with the bottom layer. The n-hexane and traces of methanol were separated from FAME using distillation process carried out at 80°C.[12][13]

- **Analysis of product**

1. TLC

The biodiesel produced after every batch process was analyzed qualitatively as well as quantitatively. The qualitative estimation of FAME was done using Thin Layer Chromatography (TLC). TLC Silica gel 60 F254, 25 Aluminum sheets 20×20 cm (Merck) were used, the developing solvent used was a mixture of toluene-chloroform-acetone (7:2:1, v/v/v) and for visual detection of FAME iodine chamber was used.[14]

2. Acid value

The quantitative estimation of unreacted FFA or the acid value of biodiesel was performed using titration method. The acid value was calculated using the following formula:[15]

$$\text{Acid value} = \frac{56.1 \times \text{Normality of KOH} \times \text{Burette Reading}}{\text{Weight of sample}}$$

Fig. 2: Biodiesel produced with optimized parameters

The percent conversion of FFA to FAME was calculated using the formula:[16]

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$$\%\text{conversion} = \left(1 - \frac{\text{Acid value of biodiesel}}{\text{Acid value of WCO}}\right) \times 100$$

3. GC-MS

The identification of FAME in the biodiesel produced was done using GC-MS analysis.[17] The column of GC-MS was Elite wax 30m × 0.3 mm (ID) and film thickness was 0.25 micrometer. Helium was used as a carrier which had a flow rate of 1ml/min. The injector and oven temperature was set as 250°C and 50°C to 250°C for 10 mins respectively. The run time was set as 25 mins. The sample used for the detection was diluted with n-hexane and out of which 0.1µl sample was injected into the system with a split ratio of 5:1.

- **Analysis for Fuel Grade Biodiesel**

The biodiesel produced under optimized condition was further analyzed for the fuel properties. The American Society for Testing and Materials (ASTM) has laid down standards for biodiesel fuel quality test specifications which was taken as a reference for comparing with the produced biodiesel.[18] Some of the properties such as acid number, viscosity, specific gravity, density, flash point, pour point and cloud point were estimated for the optimized batch of biodiesel.[15][19]

Table 1: Comparison of B100 Biodiesel fuel versus conventional petroleum based diesel fuel

Fuel Property	**Diesel**	**Biodiesel**	**Units**
Fuel standard	ASTM D975	ASTM D6751	
Lower Heating Value	129,050	118,170	Btu/gal
Kinematic viscosity at 40ºC	1.3 to 4.1	1.9 to 6.0	mm^2/s
Specific gravity at 60ºC	0.85	0.88	
Density	7.079	7.328	Lb/gal
Water and sediment	0.05 max	0.05 max	% volume
Carbon	87	77	wt %
Hydrogen	13	12	wt %
Oxygen	0	11	
Sulphur	0.0015	0.0 to 0.0024	wt %
Boiling point	180 to 340	215 to 170	ºC
Flash point	60 to 80	130 to 170	ºC
Cloud point	-15 to 5	-3 to 12	ºC
Pour point	-35 to -15	-15 to 10	ºC
Cetane number	40 to 55	47 to 65	

RESULTS AND DISCUSSIONS

In this study, all the parameters were optimized on the basis of Acid value of the product.

1. Effect of Enzyme Concentration

Biodiesel production is greatly affected by the concentration of enzyme. In optimization of batch mode, 5% enzyme concentration (by weight of WCO) gave the highest conversion with both methanol and ethanol. Effect of enzyme concentration on conversion of FFA to FAME is illustrated in Fig.3. It can be inferred that to get higher conversion rates with higher enzyme concentrations the reaction has to be carried out either for longer duration or with higher molar ratios of alcohol to WCO.

2. Effect of Alcohol

Short chain alcohols are widely preferred in biodiesel production. Thus in this study, batch mode was optimized for choice of alcohol using methanol and ethanol. The effect of alcohol was studied using different molar ratios of alcohol to WCO. Effect of alcohol on conversion of FFA to FAME is illustrated in Fig.3. It was found that in all molar ratios choice of methanol gave higher conversions than that with ethanol. Thus for all later studies, methanol was selected as an acyl acceptor.

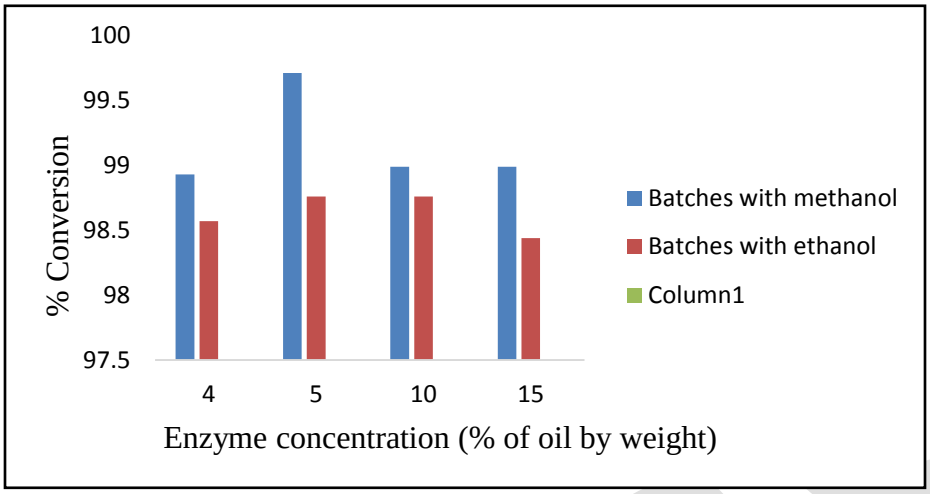

Fig. 3: Optimization of enzyme concentration and choice of alcohol

3. Effect of Molar ratio of alcohol to WCO

Methanol was used in different molar ratios with WCO for studying the effect molar ratio. Effect of molar ratio on conversion of FFA to FAME is illustrated in Fig.4. It was found that 3:1 molar ratio gave best conversion (99.71% conversion).

4. Effect of Reaction time

The optimum production of biodiesel with optimized enzyme concentration, choice of alcohol and molar ratio was reported at the reaction time of 8h. Also this reaction time was found optimal for every molar ratio with both methanol and ethanol. Although the difference between the %conversion with shorter reaction times and optimum reaction time is small, but on the industrial level maximum conversion is desired.

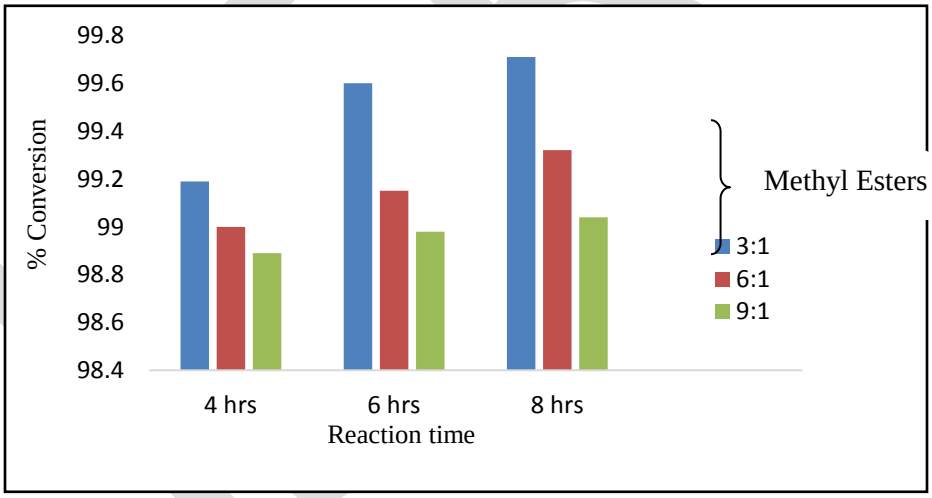

Fig. 4: Optimization of reaction time and molar ratio using methanol

5. Analysis of Biodiesel

5.1 Analytical study

5.1.1 TLC analysis was carried out as the primary confirmation of ester formation. The retention time for the band were found as 0.83 for the biodiesel produced under optimum conditions (Fig.5). Rf value for this was found to be similar which confirmed the presence of esters in the sample.

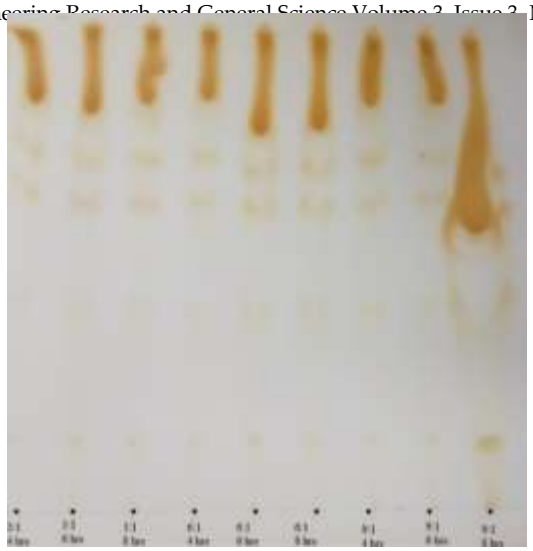

Fig. 5: TLC for samples in batch mode

5.1.2 GC-MS was performed to identify the chemical components of the biodiesel thus produced. Seven major peaks were obtained during analysis (Fig 6) and the highest peak was found to be for palmitic acid methyl ester. The identified FAMEs along with their retention times are summarized below in Table 2.

Table 2: FAME composition of optimized batch sample

Sr. No.	Retention time	Compound	Molecular formula
1.	9.922	Dodecanoic acid methyl ester (Lauric acid methyl esters)	$C_{13}H_{26}O_2$
2.	13.14	Methyl tetradecanoate (Myristic acid methyl ester)	$C_{15}H_{30}O_2$
3.	16.24	Hexadecanoic acid methyl ester (Palmitic acid methyl ester)	$C_{17}H_{34}O_2$
4.	19.13	8-octadecenoic acid methyl ester	$C_{19}H_{36}O_2$
5.	19.61	9,12-octadecadienoic acid (z,z) methyl ester (Linoleic acid methyl esters)	$C_{19}H_{34}O_2$
6.	24.23	N-Hexadecanoic acid (Palmitic acid)	$C_{16}H_{32}O_2$
7.	26.799	Cis-vaccenic acid (Omega-7 fatty acid)	$C_{18}H_{34}O_2$

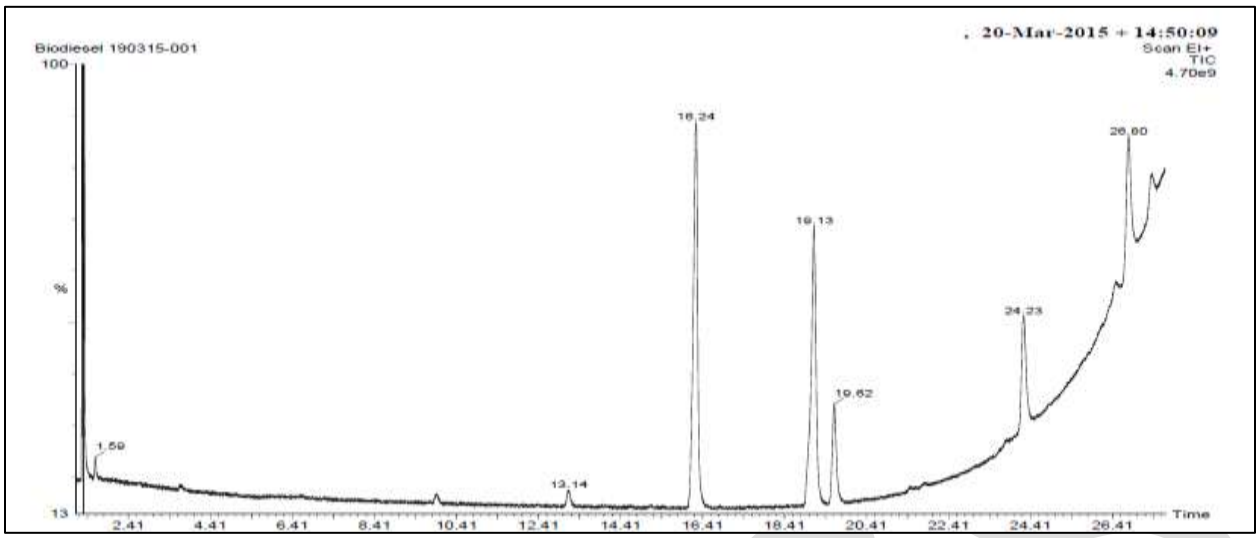

Fig. 6: GC-MS chromatogram for FAME composition

5.2 Characteristics of Product

In order to meet the fuel grade specifications set by ASTM, various specifications were reported for the biodiesel obtained in the optimum conditions which are summarized in Table 3.

Table 3: Comparison between biodiesel produced and the standard biodiesel in terms of fuel properties

Table 2: FAME composition of optimized batch sample

Acid number (mg KOH/gm of sample)	0.122	0.5 (max)
Kinematic viscosity (mm^2/s)	5.58	1.9 to 6.0
Specific gravity (kg/l)	0.891	0.88
Density (lb/gal)	7.458	7.328
Flash point(˚C)	142	130 to 170
Cloud point(˚C)	10	-3 to 12
Pour point(˚C)	7	-15 to 10
Moisture content(mg/kg)	13.5	300 (max)
pH	6.7	~7

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CONCLUSION

The objective of this study was to utilize low cost feedstock like waste cooking oil(WCO) for biodiesel production. The use of immobilized lipase enzyme further decreased the cost of production due to its remarkable property of reusability. The enzymatic reaction carried out throughout the study made the process eco-friendly as compared to the acid catalysis and economically beneficial as compared to the alkaline catalysis as it eliminated the soap formation.

The optimal conditions for the transesterification reaction were found to be: 5% enzyme concentration, 3:1 methanol:WCO molar ratio, 8 hours reaction time at 37ºC reaction temperature and 150rpm agitation speed. Under these optimal conditions, the conversion of FFAs to FAMEs was found to be 99.71%.

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