Available online at www.worldscientificnews.com

World Scientific News

An International Scientific Journal

WSN 154 (2021) 76-88 EISSN 2392-2192

Neem (*Azadirachta indica* A. Juss.) oil: A source of alternative fuel through enzyme technology

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ABSTRACT

Production of alternative energy sources from eco-friendly raw materials is supposed to be the most challenging job among researchers and academicians in the present world. Biodiesel has been considered as most useful green energy sources which is produced from Crude Neem (*Azadirachta indica*) oil (CNO) in the present study. The main advantages of CNO is that it is non-edible and is obtained abundantly in nature from neem seed. Initially, after bleaching of CNO, it is transesterified with methanol in the presence of non-specific enzyme catalyst Novozyme 40013 (*Candida antarctica*) maintaining certain reaction conditions. The parameters of the transesterification reaction have been identified as 8:1 molar ratio of methanol to CNO, 60 °C temperature in the presence of 6% enzyme Novozyme 40013 and 600 rpm of mixing intensity for 9 hrs of reaction duration. The product obtained, after completion of reaction, has been estimated of 91.24% biodiesel along with minor amounts of triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG). Finally, the physico-chemical properties of CNO biodiesel have been analysed and it showed good conformity with diesel fuel and biodiesel standard.

***Keywords*:** Crude Neem oil, *Candida antarctica*, triacylglycerol, diacylglycerol, monoacylglycerol, *Azadirachta indica*

(Received 21 January 2021; Accepted 09 February 2021; Date of Publication 10 February 2021)

1. INTRODUCTION

Identification of renewable sources for the production of energy is one of the important judgments in the present world. The depleting nature of non-renewable resources and the present environmental tribulations also push the scientists and academicians to search for alternative environmental-friendly renewable sources of energy. Among the alternative sources of energy, biodiesel has gained considerable attention during the last few decades. Biodiesel is a non-toxic, biodegradable, environment-friendly renewable fuel that can be produced from a wide variety of raw materials like fresh or used edible or non-edible vegetable oils, animal fats, microalgae and oilseed plants [1-5].

Biodiesel has also the advantages of significantly lower emissions than diesel fuel when it is used either in pure form or blended with diesel fuel. It does not enhance the level of carbon dioxide in the atmosphere which ultimately helps to reduce the intensity of greenhouse effect [6, 7]. In addition, biodiesel shows better properties with respect to sulfur content, flash point, aromatic content and biodegradability than diesel fuel [8] which encourages the researchers to use it as an alternative fuel.

Preparation of biodiesel along with property analysis has been considered from various sources including different edible and non-edible vegetable oils like sunflower oil, canola oil, soyabean oil, jatropha curcas oil, palm oil, rapeseed oil, peanut oil, cotton seed oil and also waste cooking oils using different process technology with the help of chemical or biochemical catalysts [9-13].

Among the non-edible oils, jatropha curcas oil, karanja oil, neem oil and rubber seed oil attracted considerable attention for the production of alternative fuel, biodiesel. The present author also successfully produced biodiesel from non-edible oils like karanja oil [14] and jatropha curcas oil [15, 16] in the presence of non-specific enzyme. Waste cooking oil [17] and canola oil deodorizer distillate [18] were also utilized by the present author for the production of biodiesel. Neem (*Azadirachta indica*) oil also comes into reality for the production of biodiesel as the alternative source of energy for the last few years using different process technology [19-22].

Neem oil is obtained from the seeds of neem tree which is considered under the mahogany family, Meliaceae. This tree grows abundantly in various parts of India and on almost all types of soils like clayey, saline and alkaline conditions. After collecting the neem seeds from this tree, they are de-pulped, sun dried and crushed for getting the oil. The seeds contain 40-45% oil which has high potential for the production of biodiesel [23]. Banik *et al.* [24] successfully extracted neem oil from its seeds and produced biodiesel using chemical catalyst through transesterification method.

Chemical catalytic method was also used by many researchers for biodiesel production from neem oil [25-31] but a few studies [32-35] have been made on its production from non-edible oils using biocatalyst. Use of biocatalyst has several advantages as the process is clean, energy saving (high temperatures may be avoided), no byproduct formation, easier separation of product and furthermore biocatalyst can be recycled many times which ultimately reduces the process cost.

So the present study aims at identifying a suitable bioprocess technology for biodiesel production from neem oil by analyzing different reaction parameters and components in the product. The obtained results showed a good conformity with conventional diesel fuels.

2. EXPERIMENTAL

2. 1. Materials

Crude Neem oil (CNO) was collected from Lila Agrotech Pvt. Ltd. Madhyamgram, Kolkata, West Bengal. Novozyme 40013, an immobilized nonspecific lipase from *Candida antarctica* was used as the catalyst in the reaction with ester synthesis activity of 10000 propyl laurate unit/g. The chemicals diacylglycerol (DAG) and monoacylglycerol (MAG) were purchased from Scientific and Laboratory Instrument Co., Kolkata. Except otherwise specified, all other chemicals were of A.R. Grade.

2. 2. Methods

2. 2. 1. Bleaching of CNO

About 500 g of CNO was filtered and taken in an Erlenmeyer flask and heated under vacuum (2-4 mm Hg pressure) in a boiling water bath with shaking for 30 min. Then 4% Tonsil earth (Sud Chemical Company, Germany) and 0.5% activated charcoal (E. Merch, India Ltd.) were added and shaken vigorously for 20 min under vacuum. After that it was cooled to 50 °C and filtered under vacuum. The bleached CNO was then stored in a refrigerator for further use.

2. 2. 2. Transesterification of CNO

500 g bleached CNO was taken in an Erlenmeyer flask and heated up to 80 °C to drive off moisture by continuous stirring for about 1 h. Henceforth, methanol was added to it for transesterification reaction through stepwise manner in an appropriate proportion using solvent hexane at a specified temperature for 9 hours. Enzyme Novozyme 40013 was added in definite proportion (w/w) to the reaction mixture. The progress of reaction or production of biodiesel was monitored by thin layer chromatographic (TLC) method and the typical yield of each reaction product was determined separately by column chromatography using silicic acid as an adsorbent and 160 mL of hexane diethyl ether (99:1) as eluting solvent. TLC was done by spotting the lipid mixture on a silica-gel G plate (0.2 mm thick) using hexane-diethyl ether-acetic acid (90:10:1) as a developing solvent. After completion of reaction, the enzyme was washed with hexane, dried and reused for the next experiment. Biodiesel characterization was done according to the American Standard Testing Method (ASTM). Values are reported as mean ± s.d., where n=3 (n=no. of observations).

2. 2. 3. Gas chromatographic analysis

Fatty acid composition of CNO biodiesel was determined by a gas liquid chromatographic (GLC) method after converting into methyl ester. The HP 5890A GLC was connected with a HP 3390A data integrator. The GLC was fitted with a glass column (1.83 m × 3.175 mm id) packed with 10% DEGS supported on Chromosorb – WHP (100/200 mesh) of HP make. The oven temperature was programmed from 100 to 190 °C at 5° per min. The injector and detector block temperatures were maintained at 230 and 240 °C, respectively. IOLAR-2 nitrogen was used as the carrier gas (flow rate 30 mL/min). The fatty acid esters peak was identified and calibrated with standard methyl esters. Data were represented an averages of three determinations.

3. RESULTS AND DISCUSSIONS

3. 1. Analysis of CNO

The analytical characteristics of CNO is shown in **Table 1**. It was observed from Table 1 that the acid value of CNO is quite high due to hydrolysis of oil in the presence of moisture. Physical state showed its golden yellow color. Iodine value, dynamic viscosity, and saponification value were 83.16, 41.82 mm^2/s, and 201.27, respectively. Due to higher flash point (234 °C), it could be used as fuel safely as such but the higher viscosity restricted its direct use as a fuel. The calorific value of CNO showed to be 39.129 MJ/kg while the water content was negligible. Before enzymatic transesterification, CNO was thoroughly bleached to remove peroxides.

Table 1. The physicochemical properties of CNO

Properties	Value	Test method
Acid Value (mg KOH/g)	28.172 ±0.091	ISO 660-2009
Iodine Value	83.16 ±0.156	ISO 3961- 2009
Dynamic viscosity (mm^2/s)@ 40 °C	41.82 ±0.087	ASTM-D445
Saponification value	201.27 ±0.184	ASTM D558
Physical state at room temperature	Liquid (Golden yellow)	-------
Cloud point (°C)	12.7 ±0.023	ASTM D-2500
Density at room temperature (kg/m^3 @30 °C)	921.25 ±0.264	ASTMD 1298
Flash point (°C)	234 ±0.175	ASTMD-93
Calorific Value, MJ/kg	39.129 ±0.061	ASTM-D 240
Water content, (%v/v)	0.0123	IP-74/57
Color index	4.2 ±0.005	ASTM-D 1500

Table 2. Fatty acid composition of CNO

Fatty acid	Per cent composition
Oleic acid (C18:1)	20.78 ±0.012
Linoleic acid (C18:2)	56.76±0.076
Linolenic acid (C18:3)	0.63±0.009

Palmitic acid (C16:0)	9.06 ±0.011
Stearic acid (C18:0)	8.75±0.016
Arachidic acid (C20:0)	3.59 ±0.017
Behenic acid (C22:0)	0.80 ±0.002
Lignoceric acid (C24:0)	0.55 ±0.006

Table 2 shows the fatty acid composition of CNO. It has been observed from the table that CNO contains mostly oleic acid, linoleic acid, palmitic acid and stearic acid along with minor amounts of arachidic acid. Apart from that, CNO contains very small amounts of linolenic acid, behenic acid and lignoceric acid.

3. 2. Analysis of reaction parameters for biodiesel conversion

The enzymatic conversion of CNO to biodiesel has been done by optimization of reaction parameters. The parameters identified for the bioprocess technology were 8:1 molar ratio of methanol to CNO (**Figure 1**), 60 °C temperature (**Figure 2**) in the presence of 6% enzyme Novozyme 40013 (**Figure 3**) and 600 rpm of mixing intensity (**Figure 4**) for 9 hrs of reaction duration. The conversion of biodiesel was 91% along with minor percentage of TAG, DAG and MAG. The identified parameters were optimized by using a range of values of each parameters like molar ratio from 4:1 to 8:1, temperature from 30 to 70 °C, 3 to 7% concentration of enzyme and a mixing intensity of 300 to 700 rpm with 9 hrs duration of reaction.

3. 2. 1. Optimization of molar ratio of methanol and CNO

Enhancement of methanol in the reaction mixture increases the possibility of interaction with CNO in the presence of biocatalyst which ultimately helps faster conversion of desired product. So enhancing molar ratio (methanol : CNO) creates a great positive impact on the overall completion of reaction. Here, molar ratio of methanol and CNO has been optimized by varying the ratio from 4:1 to 8:1 (methanol : CNO) as indicated in Figure 1. It has been observed from Figure 1 that conversion of biodiesel increases by increasing the amount of methanol. Initial rate of conversion is also high up to ratio 7:1 and nearly 90% conversion has been achieved at this ratio. After that, by enhancing the amount of methanol, conversion has been increased to some extent but at lower rate. So 8:1 is the optimum ratio for this transesterification reaction and maximum biodiesel has been obtained by maintaining this ratio for 9 hrs.

3. 2. 2. Optimization of reaction temperature

Temperature plays an important role for transestrification reaction in the presence of enzyme. Increasing temperature enhances the activation energy of the reaction which helps reaction progression. Here, analysis has been done by increasing the temperature from 30 °C to 70 °C, as shown in **Figure 2**. It has been observed from the Figure that initially reaction conversion is high and maximum conversion has been achieved at 60 °C temperature. After that enhancing temperature did not increase the production of biodiesel rather opposite effect has been observed (decrement of conversion).

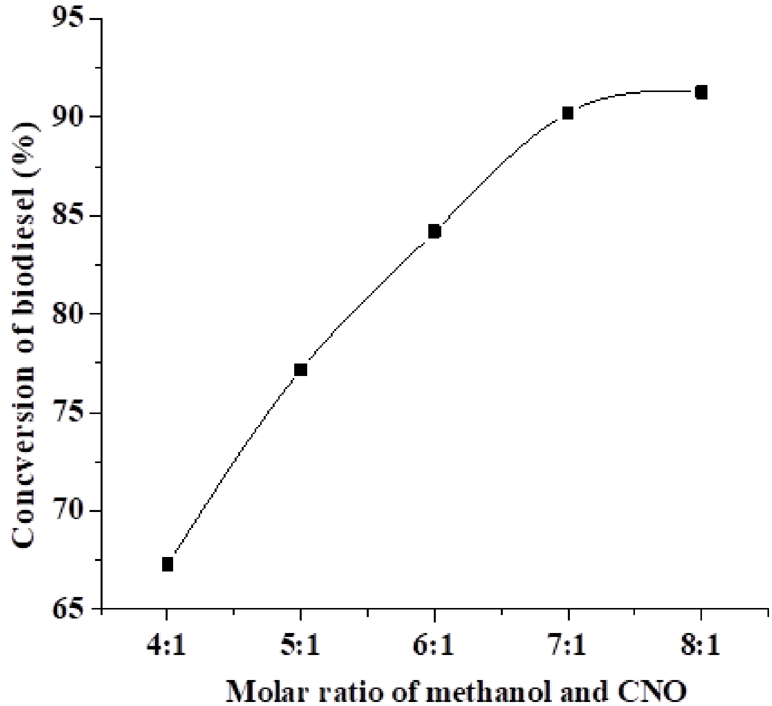

Figure 1. Analysis of molar ratio of reactants for biodiesel production from CNO

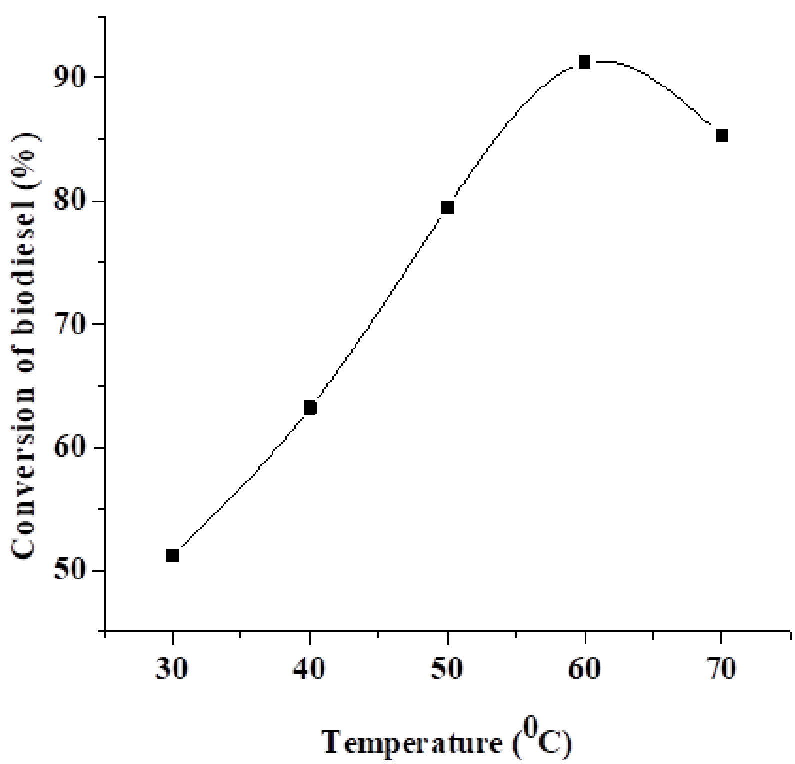

Figure 2. Analysis of temperature for biodiesel production from CNO

This may be due to the fact that any enzyme is active up to certain temperature beyond that deactivation occurs and opposite effects have been obtained. So, here 60 °C is the optimum temperature for the maximum conversion.

3. 2. 3. Optimization of enzyme concentration

Enzyme acts as biocatalyst and catalytic activity smoothly enhances the conversion of reaction for biodiesel production. Here, analysis has been done by varying the enzyme percentage from 3-7 and it has been observed from **Figure 3** that increasing the concentration of enzyme up to 6% increases the conversion of biodiesel but after that no positive results have been obtained by enhancing the enzyme concentration. This may be due to the fact that by increasing the amount of enzyme actually increases the agglomeration of enzyme and beyond 6% concentration, agglomeration is so high that active sites may be covered and hence conversion decreases. So 6% is the optimum concentration for transesterification reaction.

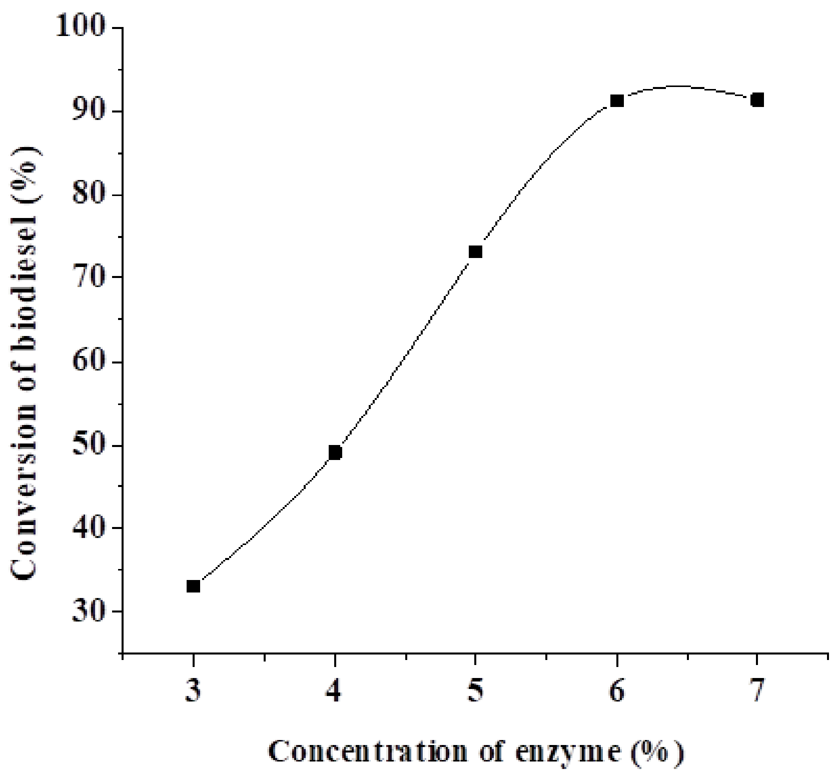

Figure 3. Analysis of enzyme concentration for biodiesel production from CNO

3. 2. 4. Optimization of mixing intensity

Mixing intensity helps to enhance the collision between the reactants and catalysts which is essential for the successful conversion of any reaction. Here, mixing intensity has been analysed from 300 to 700 rpm, as shown in **Figure 4**. It has been observed from Figure 4 that increasing the mixing intensity enhances the conversion from 300 to 600 rpm but beyond that conversion decreases to some extent. This may be due to the fact that higher mixing intensity (beyond 600 rpm) decreases the contact time between reactants and catalysts and hence conversion decreases. So 600 rpm is the optimum mixing intensity for this transesterification reaction.

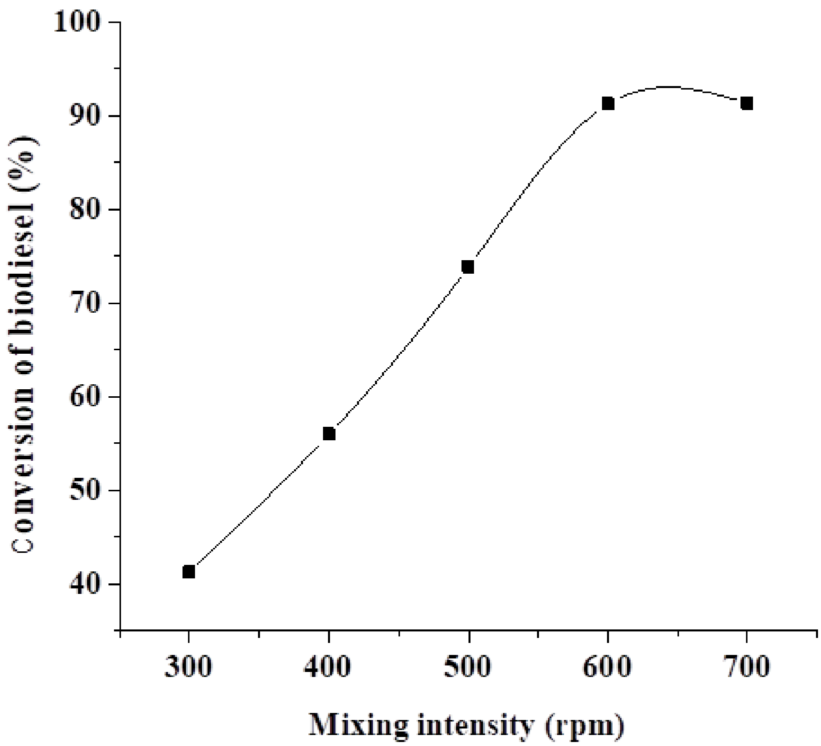

Figure 4. Analysis of mixing intensity for biodiesel production from CNO

3. 3. Analysis of reaction components during biodiesel production

At the commencement of the reaction, the compositional analysis of CNO showed that it contained 70.12% TAG, 10.83% DAG, and 4.67% MAG along with free fatty acids and other minor components. The rate of transesterification reaction was faster initially, as evidenced from **Figure 5**. From Figure 5, it was observed that rate of production of biodiesel and rate of breakdown of TAG was almost inversely related with time. Within first three hours of reaction, nearly 60% conversion was achieved and finally 91.24% production was obtained after the completion of reaction within 9 hrs. Similarly, TAG content decreased from 70.12% to nearly 30% within the first three hours of reaction and finally it reached an almost negligible amount after 9 hrs.

Initially, DAG and MAG contents were 10.83% and 4.67%, respectively, which decreased during the course of reaction and finally reached to 3.11% and 2.78%, respectively. So it is understood from the analysis of reaction components that breakdown of TAG is faster than DAG and MAG and conversion of TAG to DAG or MAG is also poor. Rather, TAG directly converts to biodiesel in the presence of enzyme with very unstable intermediate.

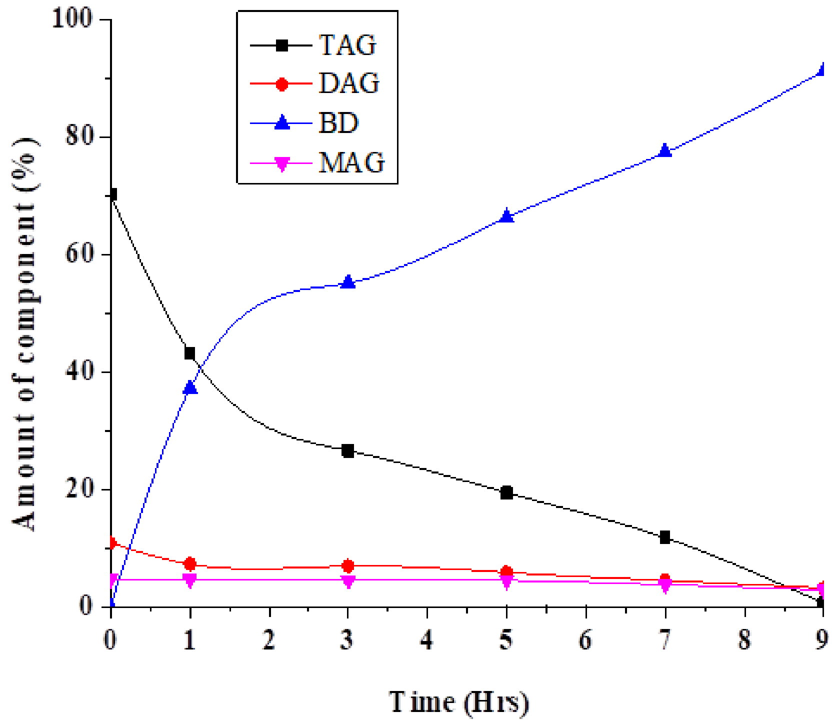

Figure 5. Analysis of reaction components with time during the reaction

3. 4. Characteristics of biodiesel

Biodiesel prepared from CNO and methanol has been characterized and compared with the biodiesel standards on the basis of physico-chemical properties like specific gravity, kinematic viscosity, density, acid value, calorific value, flash point, cloud point, etc., as shown in **Table 3**. It has been observed from Table 3 that the characteristics of biodiesel are quite comparable with biodiesel standards in almost all the properties. Higher flash point of biodiesel compared to diesel fuel proves that it can handled and used safely than diesel fuel. The calorific value of diesel fuel is somewhat higher than biodiesel but with regard to other characteristics, CNO biodiesel is analogous to diesel fuel and could be used safely without modification of engines.

Table 3. Characteristics of CNO biodiesel

Properties	Biodiesel	BD standard	Diesel fuel	Test method
Specific gravity (15 °C)	0.88±0.007	0.86 to 0.90	0.82- 0.95	ASTM D 6751-02
Kinematic viscosity (mm^2/s) at 40 °C	4.88±0.008	1.96 to 6.0	1.3-4.1	ASTMD-445

Density at 15 °C (kg/m^3)	857.2±0.157	865-900	820-860	ASTMD- 4052-96
Acid value (mg KOH/g)	0.567	0.8 max	--	ASTM –D 664
Calorific value (MJ/kg)	39.45±0.181	33 to 40	45	ASTM- 6751
Cloud point (°C)	2.71±0.011	5	--	ASTM D-2500
Flash point (°C)	134±0.109	>120	60-80	ASTMD-93
Cetane number	61±0.108	40 min	50	ASTMD-6751

Table 4 shows the fatty acid profile of CNO biodiesel. It has been observed from the Table that CNO biodiesel contained nearly 80% unsaturated fatty acids and 20% saturated fatty acids. Among the unsaturated part, linolenic acid had a major share while saturated part contained nearly equal amount of palmitic and stearic acids.

Table 4. Fatty acid profile of CNO biodiesel

Fatty acid	**Percent composition**
Oleic acid (C18:1)	19.38±0.087
Linoleic acid (C18:2)	56.03±0.114
Linolenic acid (C18:3)	0.42±0.004
Palmitic acid (C16:0)	10.72±0.009
Stearic acid (C18:0)	8.88±0.007
Arachidic acid (C20:0)	3.27 ±0.021

4. CONCLUSIONS

Bio-catalytic method has been identified in the present study for the conversion of biodiesel from non-edible crude neem oil. The reaction parameters have been identified and the reaction components were analyzed in the reaction duration. The physico-chemical properties of the product have been compared with biodiesel standard and diesel fuel which established the correctness of the present bioprocess technology. The present study paved a new way for the bio-process utilizing the crude neem oil as a source of alternative fuel in the near future.

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