NJBAS

Available online at http://www.ajol.info/index.php/njbas/index
Nigerian Journal of Basic and Applied Science (June, 2018), 26(1): 23-30
DOI: http://dx.doi.org/10.4314/njbas.v26i1.3

ISSN 0794-5698

Biodiesel Production by Lipase Mediated Transesterification of *Acacia Nilotica* Seed Oil

[*1]A. Garba, [1]A. B. Sallau, [1]S. Ibrahim, [1]M. M. Abarshi, [1]A. Muhammad, [2]M. S. Galadima and [1]S. Babangida

[1]Department of Biochemistry Ahmadu Bello University, Zaria
[2]Department of Chemical Engineering, Ahmadu Bello University, Zaria
[3]Department of Nuclear Technology Centre, Nigerian Atomic Energy Commission, Abuja
[*Corresponding Author: E-mail: garbaauwalu2011@gmail.com]

ABSTRACT

Biodiesel is becoming prominent among the alternative sources of energy due to its economic, environmental and social values. This work investigated the possibility of using calcium alginate immobilized lipase from *Pseudomonas aeruginosa* for the production of biodiesel from *Acacia nilotica* seed oil. The physico-chemical properties of *Acacia nilotica* biodiesel were assessed. Methyl esters composition indicated the presence of octadecenoic (69.14%), hexadecanoic (15.47%) and (7.92%) octadecanoic acid as dominant fatty acid methyl esters. The biodiesel produced had a specific gravity of 0.84, refractive index of 1.34±0.04 and viscosity 2.73 ± 0.15 Pa.S. The cetane number recorded was 32.81 ±0.10, calorific value 29.0 $mJkg^{-1}$, flash point 113^{o}C, while the cloud and pour points were 1.06^{o}C and 4.0^{o}C respectively. The iodine value was 187.6±0.60 gI_2/100 g, acid value 0.61±0.01/g, saponification value 189±1.00mgKOH/g and peroxide value 6.60±0.05 meq/kg. The observed characteristics of the biodiesel produced were largely in conformity with the ASTM and EN biodiesel standards.

Key words: Acacia nilotica: seed oil: transesterification: immobilized lipase

INTRODUCTION

The increase in the demand of energy and fast depletion of fossil fuel reserves has led to the search for alternative energy sources. In recent years biodiesel, a renewable and biodegradable fuel, has generated considerable interest as a substitute to petro-diesel (Hoda *et al*., 2014). The main focus is to explore the non-edible oil resources like *Acacia nilotica*, Jatropha, Pongamia, Mahua, and Neem as potential sources for biodiesel production (Prerna *et al*., 2012). Biodiesel considered a safe alternative for internal combustion engines is attributed probably to the mono alkyl esters of fatty acids averagely (C_{14}–C_{22}) chain length, produced from sources such as triacylglycerols present in vegetable oils. Transesterification is a reversible reaction where triacylglycerols are converted to fatty acid alkyl esters and glycerol (Atapour *et al*., 2014). Due to the apparently insoluble nature of the two main reactants (oil and alcohol), the use of a catalyst is necessary as it improves the solubility and accelerates the reaction rate (Atabani *et al*., 2012; Balakrishnan *et al*., 2013). A number of processes have been developed for biodiesel production involving chemical, enzyme catalysis and supercritical alcohol treatment (Kusdiana and saka, 2004). Enzymatic transesterification of triacylglycerol has been demonstrated to be a good alternative to chemical processes due to its eco-friendly, selectivity and low temperature requirements (Du *et al*., 2004). Enzymatic methanolysis using lipases has become more attractive in biodiesel production, since it is considered to be an effective way to overcoming drawbacks associated with chemical processes (Ha *et al*., 2007). The main problem of enzyme catalyzed process however is the high cost of the lipases used as catalyst and the cost of raw material which accounts for about 70% of the total cost (Wang *et al*., 2006). Therefore, researchers are always looking cost effective means of biodiesel production.

Lipases (triacylglycerol acylhydrolase, EC3.1.1.3) are carboxylesterases that catalyze the

hydrolysis, esterification and transesterification of acylglycerols with acyl chains having more than ten carbon atoms at an oil-water interface (Treichel *et al.,* 2010). Low-cost production could be achieved by using low-cost feedstock like *Acacia nilotica* (L.) seed oil. The plant is a small to medium tree, 7 to 13 m tall, with a stem diameter of 20 to 30 cm and its seed are rich in oil for biodiesel production. In this work, lipase was immobilized and used for biodiesel production

MATERIALS AND METHODS

Materials

Acacia nilotica seeds, soil sample (for isolation of *P. aeruginosa*)

Methodology

Collection and Preparation of *Acacia nilotica* Seed

Acacia nilotica seeds were collected from the botanical garden, Ahmadu Bello University, Zaria and authenticated at the Herbarium of the Department of botany, Ahmadu Bello University Zaria, Nigeria with the voucher number G01457. The seeds were cracked, washed, the shell removed and the kernels were pulverized for oil extraction. The oil was extracted by solvent extraction. Exactly 100g of A. nilotica seed kernel powder was placed in the thimble which was inserted in the centre of the extractor. The extraction was conducted for two hours. The pre-weighed flask containing the oil was cooled in the desiccator and the resulting mixture containing the oil was heated at 70oC to recover solvent from the oil and the percentage of oil extracted was determined.

Collection, Screening and Isolation of Lipase Producing *P.aeruginosa.*

Soil sample was collected from groundnut oil spilled area in Samaru market and the isolates were grown on cetrimide agar. Colony capable of utilizing olive oil as sole source of carbon was isolated and streaked on modified lipase assay media containing peptone (1.5% w/v), agar (1.5% w/v), NaCl (0.1%w/v), $CaCl_2$ (0.1% w/v), olive oil (1% v/v) as described by Shukla *et al.,* (2007) with modification. The plates were incubated at room temperature for 72 hours.

Production of extracellular lipase by Submerged fermentation

This was carried outaccording the method of Shukla *et al*., (2007) in 250ml flasks containing 50 mL of media composed of peptone (0.5%), yeast extract (0.3%), NaCl (0.25%), $MgSO_4$ (0.05%) and olive oil 3.0% v/v .The medium was inoculated with a single colony and incubated for 60 hours. At the end of the incubation period, the fermentation media was centrifuged at 6000Xg for 10 minutes, mixed with acetone: ethanol (1:4 v/v) for 1 hour at 4°C and centrifuged at 6000Xg for 10 minutes. The precipitate was dissolved in 50mM phosphate buffer (pH 8.0) and used as crude lipase preparation for further purification.

Purification of lipase

The crude lipase was precipitated with ammonium sulphate to 85% saturation. The precipitate was dissolved in 0.05M tris-HCl buffer pH 8 and dialyzed against same buffer. The enzyme mixture was loaded onto the DEAE-cellulose column 50×2.5 cm pre-equilibrated with tris-HCl and eluted with a linear gradient of 0.1-1 mM NaCl. Active lipase fraction (2 mL) from DEAE column was loaded onto sephadex G-75 column and with 0.05M tris-HCl buffer pH 8.0. All the fractions were assayed for lipase activity and protein concentration was determined.

Preparation of lipase immobilized in calcium alginate gel microspheres

This was carried out according to the method of Bhushan *et al*., (2008) with modification. Exactly 5 g of sodium alginate was dissolved in 30ml distilled water and autoclaved at 121^{o}C for 15 minutes. After cooling 20 ml of sodium alginate was mixed with 5 ml of purified lipase and then allowed to stand for 10 minutes. The enzyme/alginate mixture was pumped drop wise in 0.4 M $CaCl_2$ and kept for 1 hour to ensure its complete hardening. The beads were washed with distilled water. The lipase / calcium alginate

beads were kept in 0.05M tris-HCl buffer of pH 8.0 at 4^{o}C until used.

Enzymatic transesterification

Transesterification reaction was carried out a in screw-capped vials according to the method of Kumari *et al.,* (2007).The initial reaction mixture of 100ml consisted of oil: methanol ratio 1:10 and four beads each containing 0.13mg protein/bead (64.0 U/ml calcium alginate immobilized lipase) was added and incubated at 45^{o}C for 195minutes.The impeller speed was fixed at 150 rpm for continuous agitation.At the end of the reaction period, 5ml was taken from the reaction mixture and centrifuged in order to obtain the upper layer` that was analysed.

Recovery and Purification of the Biodiesel Produced

Crude biodiesel was purified by gravity separation according to the method of Gerpen (2005).The resulting mixture was allowed to stand for 4 hours in a separating funnel for phase separation to occur. The glycerol and the beads were run off first retaining the biodiesel in the separating funnel. Further purification of the biodiesel was carried out using distilled water at and the biodiesel was heated at 70^{o}C to remove *n*-hexane and residual methanol.

Analysis of reaction products

The percentage conversion of methyl ester was measured by determining the remaining unreacted fatty acid in the reaction mixture by titration with 0.1 M NaOH.The percentage conversion of methyl ester was calculated using the formula below:

$$Conversion\ of\ methylester(\%) = \frac{(Vol.of NaOHused\ in\ enzyme\ untreated\ mixture\ - \ Vol.of\ NaOH\ used\ in\ enzyme\ treated\ mixture)}{(Vol.of NaOH\ used\ in\ enzyme\ un-treated\ mixture)} \times 100$$

Analysis of methyl ester

The fatty acid profile of *A. nilotica* was determined by GCMS. Twenty microlitre of the sample was diluted in a 1:1 hexane/ethyl acetate mixture of which 2 µL was injected into the GC column fitted with a 30 m × 0.25mm × 0.25 µm CP WAX 52 CB in a split injection system with ratio of 1:20. The injector temperature was set at 280^{o}. The column temperature was set at180^{o}C and then programmed to rise at 20^{o}C/ minute up to 220^{o}C, where it was maintained constant for 6 minutes. Hydrogen was used as the carrier gas at a flow rate of 2 ml/minute and the column pressure was maintained at 20 psi. Heptadecanoate was used as internal standard according to Vieira *et al., (*2006) with modification

Characterization of biodiesel

The fatty acid methyl ester was characterized for specific gravity using specific gravity bottle, viscosity was determined using viscometer, refractive index using Abbe's refractometer, The cetane number of the produced biodiesel was determined according to Krisnangkura (1989) using the following equation

$$CN = 46.3 + \frac{5458}{SV} - 0.225\ x\ IV$$

Calorific value of the biodiesel produced was measured according to the method of Gerpen (2005). Flash point was determined according to ASTM D 93 (2000).The cloud points was determined according to ASTM D2500 (2000). Pour point was determined according to the ASTM D 97 (1997).Other physicochemical properties such as acid value, iodine number, saponification and peroxide values were determined by titrimetric method according to AOAC (2000).

RESULTS AND DISCUSSION

The profile of fatty acids methyl esters in *A. nilotica* biodiesel shown in Table 1 indicates that methyl ester- octadecenoate as the dominant ester with 69.14%. The methyl ester

hexadecanoate was found to be the second most abundant ester in the biodiesel produced with 14.57%. Octadecanoic acid methyl ester had 7.90%. Others include pentaflouropropionic, eicosanoic, tridecanoic docosanoic, heptadecanoic and pentadecanoic acid methyl esters (Table 1). Generally transesterification process does not change fatty acid composition of the primary oil (Salvi and Panwar, 2012). As shown in (Table 1) the most abundant ester in the *A. nilotica* biodiesel is the monounsaturated methyl octadecenoic acid. The implication of having monounsaturated fatty acids methyl ester is the overall improvement on the stability of the biodiesel as higher degree of unsaturation in the fatty acid methyl esters affects its suitability for use as a fuel for combustion engines due to high polymerization tendency as a result of peroxidation (Gaby and Peter, 1997).

Elevated temperature experienced in combustion engines accelerates peroxidation which eventually may cause gumming of the engine when polymerized fatty methyl esters predominate (Mohibbe *et al.,* 2005).The result obtained agrees with earlier findings of Adhikesavan *et al*., (2015) reported 68.33% octadecenoic acid methyl esther from *A. nilotica* biodiesel.

The fuel property of the produced biodiesel is shown in Table 2. The specific gravity was 0.84 ± 0.005 and refractive index was 1.34±0.04. The biodiesel had viscosity of 2.73 ±0.15 Pa.S while the cetane number was 32.81±0.05.The calorific value and flash point were estimated as 29.0 ± 0.10 mJ/kg and 113±0.10^{o}C. The biodiesel had the cloud and pour point of 1.06±0.01^{o}C and 4.20±0.10 respectively.

The specific gravity of biodiesel produced comforms with ASTM D 6751 standards for biodiesel which has been reported to be 0.85–0.90. It is an important parameter for diesel fuel injection systems. For complete combustion, the values must be maintained within tolerable limits to allow for optimal air to fuel ratios as high specific gravity of biodiesel can lead to incomplete combustion and particulate matter emissions (Galadima *et al*., 2008).

The molecular weight, fatty acid chain length, degree of unsaturation, and degree of conjugation determine the refractive index of biodiesel (Sadrolhossein *et al*., (2011). The low refractive index is an indication that the oil is not viscous evident by high proportion of monounsaturated fatty acids.

Table 1: Fatty Acid Methyl Esters (approximate wt %) of Biodiesel Produced from Seeds Oil

Fatty acid methyl ester type	Percentage composition	Similarity index
Hexadecanoic acid methyl ester	5.47	93
Octadecenoic acid methyl ester	69.14	92
Octadecanoic acid methyl ester	7.9	92
Pentaflouropropionic acid methyl ester	1.9	83
Eicosanoic acid methyl ester	1.02	90
Tridecanoic acid methyl ester	2.46	93
Docosanoic acid methyl ester	1.11	91
Heptadecanoic acid methyl ester	0.34	94
Pentadecanoic acid methyl ester	0.64	94

Viscosity is the measure of internal friction of the fuel to its flow. It influences the fuel injection process and atomization of fuel (Demirbas, 2008). The decrease in the viscosity of the produced biodiesel might be due to low glycerol content of the reaction product. Low viscosity can cause leaks in the fuel system, while high viscosity leads to incomplete combustion, increased deposits in the engine and the need for more power to pump fuel (Encinar *et al*., 2005). The viscosity of *A. nilotica* fatty acid methyl esters falls within acceptable limits for biodiesel specification for both ASTM and European Standard limits, Kinematic viscosity b (mm^2/s).

Table 2: Some physical properties of *A nilotica* biodiesel

Fuel Properties	*A. nilotica*	ASTM D 6751*	EN 14214 **
Specific gravity	0.84 ± 0.005	0.85-0.90	-
Refractive index	1.34 ± 0.04	1.20-1.80	-
Viscosity (P.as)	2.73 ± 0.15	1.9–6.0	-
Cetane number	32.81±0.05	42.5	-
Calorific value mJ/kg	29.0 ± 0.10	45–50	-
Flash point ^oC	113 ± 0.10	>130	>120
Cloud point ^oC	1.06 ± 0.01	NS	NS
Pour point ^oC	4.20 ± 0.10	NS	NS

The values were expressed as Mean ±SD of three replicates Source: *Schinas, *et. al* (2009) and ** Van-Gerpen *et al .,* (2005); NS= Not specified

The cetane number of the biodiesel produced as shown in (Table 2) however was below ASTM specification. The cetane number of a fuel reflects its ignition delay. Fuel with lower cetane index will result in difficulty in starting, noise and exhaust smoke. Generally, higher cetane index gives a good ignition performance (Da-Silva, 2013). The implication of low cetane number from our findings is the possibility of ignition delay when used.

The lower calorific value of *A. nilotica* fatty acid alkyl esters were found to be in agreement with ASTM 6751 and EN 14214 and could be attributed to the presence of chemically bound oxygen in the fatty acid chains (Srivastava and Prasad, 2000). Aldo *et a*l., (2012) reported the calorific value of 30.4 $mJkg^{-1}$ in castor oil biodiesel. Results obtained suggest *A. nilotica* biodiesel is non-inflammable and safer for handling in wide range of environment. This means the produced biodiesel is not likely to ignite accidentally and could be safely handled for storage. Adhikesavan *et al*., (2015) reported flash point of 160^{o}C from *A. nilotica* biodiesel

Cloud point refers to the temperature at which the biodiesel begins to form cloud. The observed cloud point of *A. nilotica* biodiesel is lower than the values reported from lard methyl ester (13ºC), edible tallow methyl ester (16ºC) and inedible tallow methyl ester (15ºC) and higher than soybean methyl ester (1ºC) and canola oil 0ºC (Vyas *et al*., 2009). Cloud point within the observed range suggests that the biodiesel can be used effectively in the tropics like Nigeria where temperature does not go below 1ºC as such there is less possibility of clogging injectors, filters and fuel pipelines.

Pour point refers to the temperature at which the biodiesel in solid form starts to melt or pour. The pour point as shown in Figure 2 suggest less likelihood of heating fuel tanks, filters and pipelines to achieve adequate flow. Table 3 presents some physico-chemical properties of *Acacia nilotica* biodiesel. The iodine value was 187.6±0.40 $gI_2/100$ g, acid value was 0.61±0.01mgKOH/g, saponification value was 189±1.00 mgKOH/g and peroxide value was 6.60±0.05 meq/kg respectively. The iodine

number measures degree of unsaturation of oil. A higher iodine number may lead to deposit formation in diesel engine injectors (Samniang *et al*., 2014). From our finding, the produced biodiesel is expected to be stable owing to the insignificant amount of polyunsaturatred fatty acids, as the increased polyunsaturated fatty acid methyl esters content decreases its oxidation stability Park *et al*., (2008).

Acid value of biodiesel indicates its quality as it determines the level of free fatty acids present (Schinas *et al.,* 2009). Similarly the observed acid value of the biodiesel suggest good quality

Table 3: Physicochemical properties of *A nilotica* biodiesel

Fuel Properties	*A.nilotica*	ASTM D 6751*	EN 14214 **
Iodine value (g I_2/100 g)	167.6 ±0.40	125-150	<120
Acid value (mgKOH/g)	0.61±0.01	0.5	0.5
Saponification value (mgKOH/g)	189.0 ± 1.00	185-196	-
Peroxide value (meq/kg)	6.60 ± 0.05	4.0-7.5	-

The values were expressned as Mean ±SD of three replicates

Source: *Schinas *et. al* ,(2009) and ** Van-Gerpen *et al .,* (2005); NS= Not specified

Since the possibility of causing wear in fuel systems and storage tanks is minimal (Schinas *et al.,* 2009). Zahira *et al. (*2013) reported acid value of 3.6 mgKOH/g oil in biodiesel produced from waste cooking oil.

The low peroxide value obtained from of *A. nilotica* oil methyl ester is an indication the oil is less liable to rancidity at room temperature.

CONCLUSION

The fuel properties of biodiesel produced under optimum conditions including viscosity, specific gravity, cloud point, pour point, flash point, calorific value, saponification value and iodine value were in conformity with ASTM 6751 and EN 14214 biodiesel standards.

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