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Production of biodiesel by enzymatic transesterification of waste sardine oil and evaluation of its engine performance

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Abstract

Waste sardine oil, a byproduct of fish industry, was employed as a low cost feedstock for biodiesel production. It has relatively high free fatty acid (FFA) content (32 mg KOH/g of oil). Lipase enzyme immobilized on activated carbon was used as the catalyst for the transesterification reaction. Process variables viz. reaction temperature, water content and oil to methanol molar ratio were optimized. Optimum methanol to oil molar ratio, water content and temperature were found to be 9:1, 10 v/v% and 30 °C respectively. Reusability of immobilized lipase was studied and it was found after 5 cycles of reuse there was about 13% drop in FAME yield. Engine performance of the produced biodiesel was studied in a Variable Compression Engine and the results confirm that waste sardine oil is a potential alternate and low-cost feedstock for biodiesel production.

Keywords: Environmental science, Chemical engineering

1. Introduction

The increase in environmental awareness and depletion of petroleum based diesel had paved the way for the search of alternate fuels. Among several options available, biodiesel is highly attractive and scalable. Biodiesel is preferred over

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petroleum diesel as it can be derived from abundantly available vegetable/animal sources. Moreover, emissions from combustion of biodiesel are relatively clean, nontoxic and noncarcinogenic [1].

First generation biodiesels were mainly derived from edible plant and animal sources. With increasing awareness on food security, second generation biodiesels were produced from non-edible feedstock like non-edible vegetable oils, used vegetable oils, and animal fats. Some of the second generation biodiesel feedstocks, derived from plant sources, reported in the recent past include Castor oil [2], Jatropha [3], Calophyllum inophyllum [4], Karanja [5], cotton seed [6], neem [7], and used sun flower oil [8]. Similarly, animals wastes like beef tallow, lard, and fish oil are low-value byproducts of meat and fishery industry and were recently used as alternate feedstock for biodiesel production. Among them fish oil is least explored as a raw material for biodiesel production. An enormous quantity of waste is generated by fish industry, and indiscriminate disposal of these wastes poses a threat to the environment. In 2014, world fish production was estimated to be around 167 million tonnes [9]. Roughly about 50% of the total volume of fish processed is discarded as waste during fish processing. Oil content in the waste varies from 40% to 65% [10]. A recent survey reports that about 102,850 tons of wastes are generated by fish processing industry located in Newfoundland and Labrador alone, and this accounts for about 25% of the total fish waste generated in Canada [11].

Generally, these wastes are used in low-value applications like animal feed [10]. In some cases, where EicosaPentaenoic Acid (EPA) and DocosaHexaenoic Acid (DHA) content of the oil is greater than 20%, these waste can also be used for the recovery of a high-value product namely omega-3 concentrate which is used in pharmaceutical and functional food applications. Ethyl esters of of eicosapentaenoic acid and docosahexaenoic acid obtained by ethanolysis of sardine oil and tuna oil were effective for the treatment of arteriosclerosis obliterans and hyperlipemia [12, 13, 14]. However, EPA and DHA contents are usually very much lower than 20%, and therefore, recovery of omega-3 from fish oil is not economical. Thus, most of the fish wastes are only used in low-value applications as indicated earlier. In this scenario, use of fish oil waste for biodiesel production looks both economically and environmentally attractive.

In canning industry, the mostly used oily fishes are sardine, mackerel, and tuna. Processing of sardine, an important fish widely used in the commercial market, also leads to discharge of considerable amount of wastes that include head, liver, and intestine [15]. Waste sardine oil refers to the wastes which are collected during canning process. Annual production of sardine oil was about 571,639 tons in the year 2014 [16]. Reports on conversion of sardine oil into biodiesel are very much limited as mentioned earlier. Therefore, the present work aims at conversion of

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sardine oil into fatty acid methyl esters with potential application as biodiesel through enzymatic transesterification.

Costa *et al.,* 2013 utilized waste oil extracted from fish canning industry for biodiesel production. The initial acid pre-treatment was done with sulfuric acid. Biodiesel yield of 73.9 wt% was obtained by alkali catalyzed transesterification with 1 wt. % catalyst and 60 vol. % of the methanolic solution [17].

Biodiesel is obtained from transesterification reaction involving a vegetable oil or animal fat and a short-chain alcohol like methanol or ethanol in the presence of a suitable catalyst (acid, base or biochemical catalyst). Compared to chemical catalysts, enzymes require relatively low reaction temperature and less alcohol to oil molar ratio. Also, chemical catalysts require multi-stage pretreatment if the feedstock contains free fatty acids (FFA), particularly when FFA content is more than 0.5 wt % of feedstock. Moreover, drawbacks such as product contamination, wastewater generation, free fatty acids and water interferences and glycerol recovery can be easily circumvented if enzyme catalysts are used. While using enzyme catalysts, it is necessary to recover and reuse enzymes to reduce processing cost. Immobilization of enzymes facilitates recovery and enables reuse of expensive enzymes for several cycles [18]. Polyethylene gylated polyurathane nanoparticles had been shown to be effective for immobilization of enzymes used for esterification of fish oil [13, 14]. Immobilized lipase enzyme had been used to catalyse ethanolysis of sardine oil and tuna oil [12, 13, 14].

This work is the first of its kind to report biodiesel production from waste sardine oil catalyzed by lipase immobilized on activated carbon support matrix. Effect of methanol to oil ratio (6:1–12:1), temperature (30 to 50 °C) and water content (5% v/v – 15% v/v) were studied and optimized. Reusability of immobilized enzyme was studied at 30 °C. Produced diesel was blended with petroleum diesel (10:90 ratio), and its performance and emission characteristics were evaluated using a Variable compression ratio (VCR) engine.

2. Materials and methods

Waste sardine oil was collected from a canning industry located in Nagapattinam District, Tamilnadu, India. The fatty acid profile of the collected sardine oil was as follows: Octadecanoic acid ($C_{18}H_{36}O_2$): 7.39%, Tridecanoic acid ($C_{13}H_{26}O_2$): 15.54%, 9-Hexadecanoic acid ($C_{16}H_{30}O_2$): 13.47%, Hexadecanoic acid ($C_{16}H_{32}O_2$):19.43%, 10-methyl Octadecanoic acid ($C_{19}H_{38}O_2$):19.58%, 5,8,11,14,17-Eicosapentaenoic acid ($C_{20}H_{30}O_2$):16.08%, and Others: 8.5%. *Aspergillus niger* Lipase (99%, 16 U/mg) and Methanol (99%) were purchased from Himedia Laboratories Pvt. Ltd., India.

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2.1. Immobilization of lipase on activated carbon

The enzyme solution was prepared by in phosphate buffer at pH 7 to prevent the deactivation of enzyme during immobilization [19]. Immobilized lipase was obtained by mixing 150 ml of enzyme solution (protein content = 1 mg/ml; activity = 16 U/ml) and 0.5 g of activated carbon. The above mixture was kept in a shaker for 24 h at room temperature. After equilibration of the mixture, the solution was filtered using Whatman filter paper, and immobilized lipase was collected separately [20]. Percentage immobilization was estimated as follows (Eq. (1)):

$$\text{Percentage immobilization} = \frac{S_0 - S_t}{S_0} \times 100 \quad (1)$$

Where S_0 and S_t refer to protein concentration in the solution before and after immobilization. Protein concentration was determined using Lowry's method. The specific enzyme activity of immobilized lipase was determined using olive oil emulsion method [21].

2.2. Biodiesel production

Biodiesel was produced as described by Arumugam et al., 2014 [4]. Briefly, waste sardine oil was mixed with methanol (methanol to oil ratio 6:1, 9:1 and 12:1) and water (5, 10 and 15% v/v). Immobilized *Aspergillus niger* lipase (100 mg) was added to the mixture, and the reaction mixture was maintained at a constant temperature (30 to 50 °C) in an orbital shaker at 200 RPM for 10 h [22]. Reaction time also affects the yield of biodiesel. Typical reaction time for enzymatic transesterification of various oils range from 4 h to 72 h [23, 24]. Our preliminary experiments on transesterifcation of sardine oil, with *Aspergillus niger* lipase (free enzyme) as catalysts, suggested that maximum conversion could be achieved in less than 10 h reaction time. Thus, in this study with immobilized lipase enzyme, we have chosen 10 h reaction time.

2.3. Properties of biodiesel

The properties of biodiesel- such as Cetane number, Cloud point, Flash point, Density, Kinematic viscosity were determined using ASTM methods [25]. The composition of the biodiesel produced was estimated by Gas Chromatography – Mass spectroscopy (GC – MS) (Clarus 500, PerkinElmer, USA).

2.4. Experimental setup for engine testing

Specification of the Variable Compression Ratio (VCR) engine used in this study is given in Table 1. For engine test, diesel – biodiesel (90:10) blended fuel (B10) was used. Fuel injection pressure and compression ratio (CR) were maintained at 200 bar and 18:1 respectively during the test run. The flue gas from engine exhaust

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Table 1. VCR engine specifications.

Engine type	Kirloskar/PS 234
Number of cylinder	One
Stroke length (mm)	110
Bore (mm)	87.5
Swept volume (cc)	661.45
Compression ratio	12:1 to 18:1
Engine speed (rpm)	1500
Injection timing (°CA)	20–40
Injection opening pressure (bar)	210

was analyzed with DELTA 1600-L (specification is shown in Table 2). The engine was started with no load, and the required speed and steady state condition were attained by adjusting the feed control. Data acquisition system was used to measure power output, rpm, exhaust gas temperature, and fuel consumption.

3. Results and discussion

3.1. Percentage immobilization and specific enzyme activity

The percentage lipase immobilization and specific enzyme activity were found to be 57% and 13 U/mg of support matrix, respectively. The specific enzyme activity of the immobilized enzyme was similar to the free enzyme (16 U/mg), showing that there was no reduction in specific enzyme activity due to the matrix – enzyme interaction during the immobilization process.

3.2. Biodiesel production

As mentioned in materials and methods section, immobilized lipase enzyme was used to produce biodiesel from sardine oil. Methanol to oil ratio, percentage water content and temperature are the most important process variables that affect

Table 2. Gas analyzer specifications.

Measured quantity	Measuring Range	Resolution
O	0-15.00%	0.01%
CO_2	0-20.00%	0.01%
Hydrocarbons	0–20000 ppm	1 ppm
NO_X	0–2000 ppm	1 ppm

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biodiesel yield. Optimum values of these variables in turn depend on the biodiesel feedstock used, source of lipase and type of immobilization. Therefore, effect of these variables of biodiesel yield was studied.

3.2.1. Effect of methanol-to-oil ratio

It is well known that both esterification and transesterification reactions are equilibrium-limited. To shift the reaction towards ester formation, excess methanol is usually used [26]. Also, excess alcohol minimizes diffusion limitations by reducing the viscosity of reaction mixture and improves mass transfer rate and in turn enhances overall reaction rate. In the present study, we used 6:1, 9:1, and 12:1 methanol to oil molar ratios to study the effect of methanol to oil ratio on the yield of biodiesel. The results are shown in Fig. 1(a). Biodiesel yield increased from 85.73% to 94.5% ($p < 0.001$) when methanol to oil molar ratio was increased from 6:1 to 9:1. Further increase in the molar ratio did not promote ester formation. Thus, for further studies methanol to oil molar ratio 9:1 was used. Optimum alcohol to oil ratio reported in previous literature widely varies. It varies from 3.5 to 12, depending upon biodiesel feedstock, microbial source of enzyme and molecular weight of alcohol used in the reaction [27]. Too much of excess methanol could result in emulsification of glycerol and reduce biodiesel yield

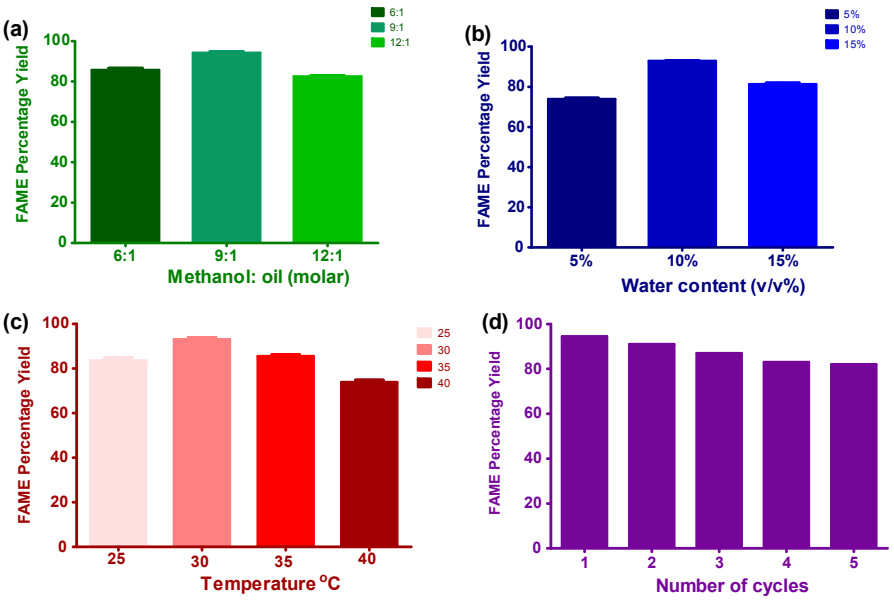

Fig. 1. (a) Effect of methanol to oil molar ratio (6:1, 9:1, 12:1) on methanolysis of waste sardine oil for temperature 30 °C and reaction time of 10 h (b) Effect of percentage water content (5%, 10%, 15%) on methanolysis of waste sardine oil for 9:1 molar ratio of methanol to oil, temperature 30 °C, and reaction time of 10 h (c) Effect of temperature (T = 25, 30, 35 and 40 °C) on methanolysis of waste sardine oil for methanol to oil molar ratio of 9:1 and reaction time of 10 h (d) Reusability studies Immobilized lipase on methanolysis of waste sardine oil at optimum conditions.

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through recombination of ester and glycerol [28]. Also use of excess methanol, a polar short chain linear alcohol, beyond a certain level may lead to structural changes in lipase and cause enzyme deactivation [29]. Various approaches had been followed by previous researchers to reduce enzyme denaturation. This includes: (i) stepwise addition of methanol (ii) use of other acyl acceptors like ethanol, 2-butanol, 2-propanol, methyl acetate, ethyl acetate, etc. as alternate to methanol and (iii) use of organic solvents. But, all of these approaches have their own advantages and disadvantages [18]. Overall, simple single step addition of methanol has been most commonly accepted by researchers as best option.

3.2.2. Effect of water percentage on methanolysis

In transesterification reaction catalyzed by enzymes water plays a vital role in the conversion of triglycerides to Fatty Acid Methyl Esters (FAME) [30] as the stability and catalytic activity of the lipase enzyme are affected by the presence of water/any other solvent. Lipase requires an oil-water interface for their action. Addition of water favors formation of oil-water droplets and hence increase interfacial area available for the catalytic action. Also, dispersing the lipase in water/solvent prevents deactivation of lipase by methanol [31]. The effect of water content on the enzyme-catalyzed transesterification reaction of waste sardine oil was investigated with different percentage of water (5%, 10%, and 15%) and the results are shown in Fig. 1(b). The highest conversion of approximately 92.5% was obtained at a water content of 10% (v/v). Apart from transesterification reaction, lipase also catalyzes other reactions like hydrolysis. Presence of excess water favors hydrolysis and hence lowers biodiesel results [32].

3.2.3. Effect of temperature

Temperature is another important factor influencing reaction rate. Rate of reaction increases with increase in temperature. However, for an enzymatic reaction at higher temperatures reaction rate falls beyond some point due to enzyme deactivation. Optimum temperature for an enzyme catalyzed transesterification reaction depends on enzyme as well as alcohol used. For most of the lipase catalyzed transesterification reactions, optimum temperature falls in the range of 30 to 50 °C [30]. We have studied the effect of temperature on methyl ester yield by increasing reaction temperature from 25 at an interval of 5 °C, until there was a noticeable drop in the FAME yield. The results are shown in Fig. 1(c). The FAME yield was significantly higher ($p < 0.001$) at 30 °C and further experiments were conducted at 30 °C.

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3.2.4. Reusability

The reusability for the lipase immobilized on activated carbon was studied for five cycles. From Fig. 1(d), it can be seen that FAME yield decreased very slowly upon each successive transesterification cycle. After five successive cycles, there was about 13% reduction in FAME yield. The percentage reduction in biodiesel yield was likely due to leaching of the lipase from the support matrix [32].

Enzymes can be immobilized on support matrix by chemical or/and physical methods. Chemical methods include adsorption, ionic binding and covalent bonding. Physical methods include entrapment and encapsulation. Each method has their own advantage and disadvantage. Adsorption involves only weak forces, enzyme leaching is a common problem associated with this method. Inspite of this shortfall, adsorption is still one of the most popular methods of enzyme immobilization owing its inherent advantages over other immobilization methods. Some of the advantages of this method include (i) simple, (ii) does not require toxic chemical treatment, and (ii) mass transfer resistances (particularly internal) are neglible. Ionic binding and covalent bonding methods employ stronger chemical bonding between enzyme and support matrix. As the binding between the enzyme and matrix is stronger enzyme leaching can be either completely prevented or minimized. However, it may cause a drop in the enzyme activity if the some of the active sites of the enzymes are blocked in the process. Moreover, this method requires chemical treatment of the support matrix/enzyme prior to immobilization. Physical methods like entrapment and encapsulation can also prevent leaching of the enzyme. However, strong internal mass transfer resistances becomes a limitation in these methods and result in lower reaction rate. Most recently, hybrid methods involving combination of two or more immobilization methods had been shown to be effective in certain cases. In this study, we have used only adsorption for enzyme immobilization and the results are in favor of the method. However, the optimum/best method of immobilization for any enzyme-substrate system can be established only after examing all methods with careful consideration of synthesis cost, enzyme reusability, enzyme stability, ease of product separation, etc. [23].

3.2.5. Properties of biodiesel

The fuel properties of biodiesel produced from waste sardine oil are listed in Table 3. All these properties except for cloud point were well within the ASTM standards for diesel fuel. A comparison some of the enzymes catalyzed transesterification processes for the biodiesel production, reported in recent literature, is given in Table 4.

Cloud point of biodiesel (16 °C) was higher than for a diesel fuel. This is a cause for concern, particularly, if the biodiesel is to be used in countries where the ambient temperature is below its operability limits, leading to potential cold start

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Table 3. The comparison of biodiesel production from various sources using immobilized lipase on different support matrix in the literature with the present work.

Properties of biodiesel	*Sardine oil biodiesel*	ASTM methods and of biodiesel
Density (kg/m^3) at 15 °C	891	D5002-94 (860–900)
Flash point (°C)	161	D93 (130)
Cloud point (°C)	16	D91 (-3 to 12)
Kinematic viscosity at 40 °C (cSt)	3.9	D445 (1.9 to 6)
Cetane number	56	D613 (49)

problems. However, use of additives, preheating and filtration before injection can be useful to overcome this issue and to improve low-temperature operation of biodiesel. Thus, sardine oil could be an alternative feedstock for biodiesel production. Ester content of the biodiesel produced was found to be: 9-Hexadecenoic acid, methyl ester, (Z)- ($C_{17}H_{32}O_2$) – 13.2%; Hexadecanoic acid, methyl ester ($C_{17}H_{34}O_2$) – 19.41%; Tridecanoic acid, methyl ester ($C_{14}H_{28}O_2$) – 14.95%; 5,8,11,14,17-Eicosapentaenoic acid, methyl ester ($C_{21}H_{34}O_2$) – 16.25%; 10-methyl octadecenoic acid, methyl ester: ($C_{20}H_{40}O_2$) – 19.13%; Octadecanoic acid, methyl ester: ($C_{19}H_{38}O_2$) – 10.49%.

3.3. Performance and emission characteristics

3.3.1. Brake thermal efficiency

Brake Thermal Efficiency is defined as the ratio of the heat equivalent of the break output to the heat supplied to the engine. It is used to evaluate the effectiveness of an engine in converting the heat from combustion of fuel to mechanical energy [47]. About 10–12% of oxygen present in vegetable oil based fuel can favorably affect combustion efficiency of biodiesel as compared to petroleum diesel [48]. That is, the ester molecules present in the biodiesel favors complete combustion. The biodiesel blend has brake thermal efficiency (BTE) lower than that of diesel fuel in all percentage load conditions (Fig. 2(a)). The BTE values for B10 and diesel fuel were 27% and 33% respectively indicating that the diesel fuel has a higher BTE value at 100% load [49]. Lower BTE of the biodiesel blend compared to diesel fuel is mainly due to high fuel density, high viscosity and low heating value of the blends [3].

3.3.2. Specific fuel consumption (SFC)

The Specific Fuel Consumptions (SFC) of B10 blend was compared with petroleum diesel, and the results are shown in Fig. 2(b). Biodiesel SFC was higher

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Table 4. The comparison of biodiesel production from various sources using immobilized lipase on different support matrix in the literature with the present work.

Reference	Support	Source	Catalyst/Enzyme	Reaction time	% yield
Jegannathan et al. 2010 [33]	κ-carrageenan	Palm oil	lipase PS from *Burkholderia cepacia*	72 h	100.0
Juan Camilo Naranjoa et al. 2010 [34]	Activated carbon	Palm oil	*Candida antarctica* B lipase	12 h	100.0
Jingjing Zheng et al. 2012 [35]	K_2SO_4 micro-crystals	Soybean oil, sunflower seed oil, olive oil, camellia oil, corn oil and rapeseed oil	*Pseudomonas cepacia lipase*	12 h	99.8
Yao Wang 2014 [36]	Acrylic resin	crude algal oils	Novozym 435	4 h	99.1
Huang Y et al. 2010 [37]	Resin	Lard oil	Novozym 435 and Lipozyme TLIM	20 h	97.6
Kaili Nie et al. 2011 [38]	Textile fibre	Salad oil and Waste oil	*Candida* sp. 99–125 lipase	30 h	96.0
Li et al. 2010 [39]	Micro-aqueous phase	*Sapium sebiferum* oil	Lipase from *Pseudomonas cepacia G63*	12 h	97.1
Present work	Activated carbon	Sardine oil	Lipase from *Aspergillus niger*	10 h	94.0
Yucel, 2011 [40]	Olive pomace	Pomace oil	Lipase from *Thermomyces lanuginosus*	24 h	93.0
Kawakami et al, 2011 [41]	Silica monolith	Jatropha oil	*Burkholderia cepacia* lipase	12 h	90.0
Huang et al, 2015 [42]	Crystalline PVA microspheres	Microalgae Oil	*Rhizomucor miehei* lipase	24 h	90.0
Yan et al. 2010 [43]	Cross-linking and lipase coating with K_2SO_4	Waste cooking oil	*Geotrichum* sp. Lipase	4 h	85.0
Kalantari et al. 2013 [44]	Magnetic silica nanocomposite	Soybean oil	lipase from *Pseudomonas cepacia*	24 h	55.0
Roman et al. 2017 [45]	Chitosan	Coconut oil	*Pseudomonas fluorescens* lipase	24 h	12.0
Ramani Kandasamy et al. 2010 [46]	Activated carbon	Olive oil	*Pseudomonas gessardii*	2 h	-

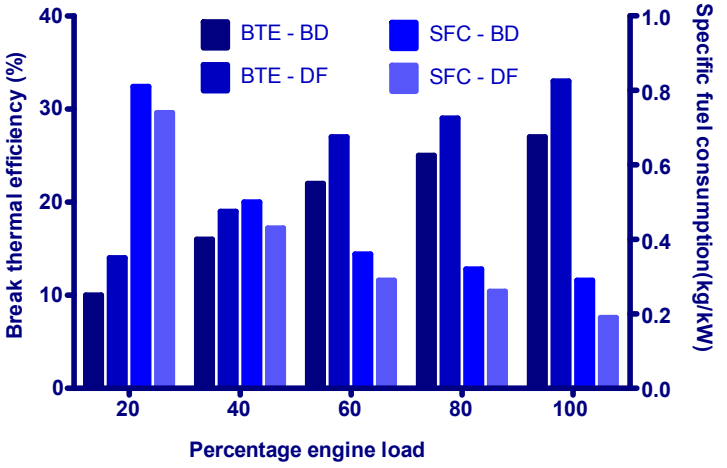

Fig. 2. Performance characteristics of sardine oil biodiesel. (BTE - Break thermal efficiency; SFC - Specific fuel consumption; BD - 10% Biodiesel blend; DF - Petroleum based diesel fuel) **(a)** Variation in specific fuel consumption with load for diesel fuel and B10. **(b)** Variation in brake thermal efficiency with load for diesel fuel and B10.

compared to petroleum diesel. High viscosity, density and low heat content of biodiesel blend when compared with that of diesel attribute to the high SFC. The presence of additional oxygen molecules in the fuel ensures better/complete combustion of the fuel. This is considered to be the major reason for higher SFC and higher power generation [50]. The ignition performance of the fuel is also improved due to oxygenated nature of the biodiesel. Cetane number was greater than that of diesel fuel [51].

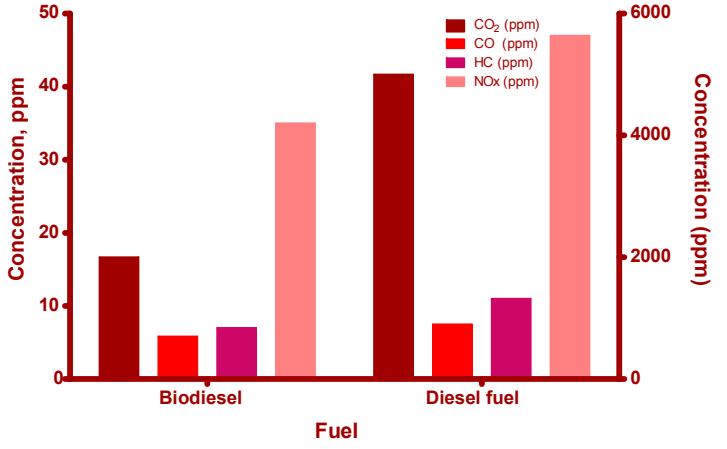

Fig. 3. Emission characteristics of biodiesel compared with petroleum diesel. **(a)** Variation in CO_2 and CO emission at 100% load for diesel fuel and biodiesel. **(b)** Variation in HC and NO_X emission with 100% load for diesel fuel and biodiesel.

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3.3.3. Exhaust gas emission

A five gas analyzer (AVL Di Gas – 4000 model) was used to analyze exhaust discharges such as carbon monoxide, nitrogen oxides, and unburned hydrocarbon (Fig. 3). Carbon emission is based on the efficiency of combustion and the fuel's carbon content, which will undergo a series of oxidation reactions during combustion. The complete oxidation of carbon leads to the formation of CO_2. If the oxidation is not complete, the exhaust will contain more of CO and hydrocarbons [52].

The formation of CO was 22.2% lower at 100% load in biodiesel due to the presence of oxygen atoms as mentioned earlier [53]. However, higher oxygen content of the fuel results in a reduction in the calorific value in biodiesels. Fig. 3 shows that biodiesel (B10) emits less amount of hydrocarbon compared to diesel fuel (36% reduction). The partially or completely burnt hydrocarbons are found in the emissions from the engine [54]. Once again this is attributed the presence of oxygen molecules in the fuel [55].

NO_X are generated in a diesel engine because of high flame temperature, peak pressure prevailing inside the cylinder, nitrogen content of the fuel and the residence time of the fuel inside the cylinder [56]. Biodiesel shows 25% reduction in NO_x emissions compared to diesel fuel, which is essentially due to the reduction of temperature in the combustion chamber. Higher the temperature, greater is the amount of NO_x emission. The residence time or ignition delay may be less in ester-based fuel because of its high cetane number [55]. The delay period for the ester is reduced because of the presence of oxygen which increases the ignition quality.

4. Conclusion

Biodiesel was produced from waste sardine oil, a non-edible renewable source, by transesterification catalyzed by lipase immobilized on activated carbon. The optimal conditions for methanolysis are 9:1 (mol/mol) methanol to oil ratio, 10% water content and 30 °C. Under these optimal conditions, about 94.55% of methyl ester yield was obtained. Immobilized enzyme could be used for 5 cycles without appreciable loss in activity. Specific energy consumption and exhaust emissions also saw substantial advantages in biodiesel as compared with diesel fuel. The performance analysis of 10% blend of waste sardine biodiesel with diesel fuel showed that sardine oil biodiesel used along with petroleum fuel as a blend improves the engine performance and minimizes emission of air pollutants in the flue gas. Use of such blended fuel does not require any key hardware alteration to the structure.

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Declarations

Author contribution statement

A. Arumugam: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

V. Ponnusami: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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