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Bio-waste Transesterification Alternative for Biodiesel Production: A Combined Manipulation of Lipase Enzyme Action and Lignocellulosic Fermented Ethanol

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Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

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ABSTRACT

Petroleum hydrocarbon oil contaminated soils were excavated from a gas station from Nicosia Cyprus, Turkey and subjected to fungal isolation/analysis. During the fungal essay, *Aspergillus nigar, Penicellum notatum* and *Mucorales* were isolated and identified. *Aspergillus nigar* was selected and cultivated for lipase enzyme production due to its role in lipase production. The produced lipase enzyme was immobilized and the number of lipases recovered after purification was 85.1%. The purified enzymes that were supported by physical adsorption which resulted in high (85%) yield showed high stability than free lipase, encouraged transesterification in this study. Pretreatment evaluation of groundnut powder showed high cellulose yield (82.3% and 87%) and high lignin removal of 75%, 88% respectively for lipase enzyme accessibility and ethanol production. Transesterification involving purified lipases and groundnut shell fermented ethanol yielded Methyl ester which represents the organic biodiesel with a C_{18} number showing a minimum level of lubricity for friction reduction between surfaces when a car is on motion and has specific gravity 0.95 ul, density 15.5℃, and calorific value of 43.2 MJ. Kg.

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1. INTRODUCTION

As fossil fuels are depleting year after year, the emergence of new technologies should be paramount in order to produce fuel from waste and renewable biomass [1].

In early 1970's acute fuel shortage culminated diversifying fuel resources and thus biodiesel as fatty esters were developed as alternatives to petroleum diesel. After-words towards 1990's there was a kind of relieve on atmospheric pollution from fossil fuel-based diesel and interest started rising on the use of transesterification and esterification alternative. Nowadays, diesel engines require cleaner burning, a stable fuel that will operate under a variety of conditions [2]. Transesterification is a technique used in the soap industries for soap and detergent production in many industries, which employs similar chemical process as soap using base-catalyzed transesterification as it is the most economical process, requiring very low temperatures and pressures while producing 98% conversion yield [3].

During transesterification process, the fatty acid /glyceride is reacted with an alcohol in the presence of a catalyst, usually a very strong alkaline such as potassium hydroxide or sodium hydroxide with a high amount of waste product [4]. Developing a clean and renewable liquid alternative fuel as an alternative to crude oil has become an urgent mission [5]. The conception is that the new fossil fuel alternative would increase from 1.9 million barrels per day in 2010 to 5.9 million barrels per day in 2030, the danger is high waste production [6]. Methods that are currently used in the production of biodiesel include oil extraction, purification, esterification, and transesterification process that uses NaOH/KOH and ethanol/or methanol as catalyst [7]. Vegetable oils are used for transesterification reaction with alkali as a catalyst in the presence of reasonable ethanol to form esters and glycerol and this process produces a high quantity of biodiesel and waste products.

Unfortunately, biodiesel productions that use chemical processes are experiencing some difficulty in the area of bulk density resource, while preserving its energy content [8]. Scientist/researchers are brainstorming for most efficient and effective ways of producing biodiesel with no corresponding waste products. The resurgence of biodiesel from waste and animal fats has been affected by regulations and legislation in different countries in order to minimize pollution and to rule out competition with food industries. Many of the regulations and laws are centered on promoting nations Agro-economy, national security and reducing climate change. However, since transesterification and esterification involve use of chemicals for the production of biodiesel from animal fats or oil, development of new technology is needed in order to zero waste production that follows chemical based transesterification, hence this research project.

This study will produce bio-based ethanol and bio-based lipase catalyst as alternatives to a chemical-based catalyst that have been used over the years. İt will discourage waste production, issue of greenhouse gases and reduce to the barest minimum current global climate change. The illuminating potentiality will produce biodiesel biomaterial alternative that is carbon neutral with smoke-free engine.

2. MATERIALS AND METHODS

2.1 Materials

Groundnut shells were collected from a waste bin from Cyprus international university. The raw groundnut shell was washed with clean water and dried at 50℃ for 48 h to enable pore size reduction for effective pretreatment. Dried raw materials were grounded to powdery form and sieved to obtained a homogenous material (2 mm) particle size and stored at 4℃. *Aspergillus niger* was isolated from oil contaminated soil and kept at 28℃ for this study.

2.2 Fungi Subculture

Lipase producing strains of Aspergillus niger isolated from oil contaminated soils were subcultured on potato dextrose agar using groundnut paste as substrate and incubated for 72 h at 27℃. Materials samples were transferred into a sterile 50 ml beaker containing deionized water and centrifuged for 1 h, then filtered and the filtrate was used for lipase cultivation.

2.3 Enzyme Extration and Immobilization

Growth was enhanced in 250 mL in lipase growth medium containing 10 g/L $NaH_2SO_4.7H_2O$, 0.125

g/L $MgSO_4.7H_2O$, 0.125 g/L CaCL2, 1% NH_4SO_4, and 2% waste olive oil with 1% (w/v) and ampicillin antibiotic to prevent bacterial growth for 72 h.

Lipase activity was determined in a glass petri-dish containing 1 g/L agar, 2% (w/v) groundnut paste. A deep circle hole was observed on the material content of the plate and was used to determine lipase activity for 72 h. After 6 h a round circle object representing lipase activity was noticed, then measured (mm) and immobilized. Immobilized enzymes were kept at 25℃ for transesterification.

2.4 Pretreatment

Two pretreatment methods were used for fermentable sugar production in 20% (w/v) solid loading of groundnut shell raw material.

Alkaline pretreatment, involving 2% (w/v) NaOH and 2.5% (w/v) Ca $(OH)_2$ were used at 90℃ for 60 min in a water bath at 150 rpm. After pretreatment the material contents of the sample in 1000 mL beaker were neutralized and filtered. Liquid fractions were used for total reducing sugar (TRS) and solid recovery analysis. Equation (1) solid recovery [5].

$$\text{Solid recovery (\%)} = \frac{Sp}{Sup}\ X\ 100 \quad (1)$$

Where S_p is the dry weight of the pretreated biomass and S_{up} is the dry weight of the untreated biomass.

2.5 Enzymatic Hydrolysis and Fermentation

Enzymatic digestibility of the pretreated samples were determined at pH 5.0, 120 rpm, 50℃. 50 $FPU/_{graw\ material}$ cellulase loading with 20% solid loading in 1000 mL with citric acid buffer. After 72 h of incubation samples were taken for TRS analysis.

The total reducing sugar obtained after enzymatic hydrolysis were analysed by [9] method using DNS (3,5-dinitrosalicylic acid)

$$\text{TRS yield (\%)} = \frac{weight\ of\ TRS}{weight\ of\ total\ dry\ substrate}\ X\ solid\ recovery\ (\%) \quad (2)$$

Saccharomyces cerevisiae was used for fermentation in this study. Seeding was achieved in 15.0 g/L glucose, 1.5 g/L $MgSO_4.7H_2O$, 5.5 g/L yeast extract, 5.0 g/L $(NH_4)_2SO_4$, 0.5 g/L $CaCL_2$ at 30℃ and 150 rpm [5]. The pH of the medium was adjusted to 4.4 using HCl in 1000 ml at 30℃. After 72 h fermentation period, TRS and ethanol analysis was carried out to determine TRS to ethanol yield using equation (3) [10,5,9].

$$\text{TRS to ethanol conversion yield (\%)} = \left(\frac{\frac{g}{L}of\ ethanol\ obtained}{\frac{g}{L}of\ initial\ TRS\ X\ 0.511}\right) X\ 100 \quad (3)$$

2.6 Ethanol Analysis

Ethanol content was analyzed using HPLC (Shimadzu LC20), the mobile phase was equipped with Coregel-87H3(Transgenomic) column by using 5.0 Mm H_2SO_4 with 0.5 Ml/min flow rate at 65℃ and 10-micrometer sample volume for 45 min [11]. Shimadzu RID-10 refractive index detector was used in the sample analysis.

2.7 Transesterification

Biocatalytic processes involving lipases isolated from *Aspergillus niger* and fermented ethanol obtained from groundnut shell were used for transesterification in this study. Transesterification was carried out in molar ratio of 6:1 ethanol to waste olive oil in 250 mL beaker containing 100 mL of waste olive oil (% w/w), lipase enzyme activity of pH 5.0 with fermented ethanol 89% (v/v) at 70℃, 150 rpm in a water bath for 180 min. Thereafter, the contents were removed and poured into separating funnel and kept overnight to settle. The biodiesel from the upper layer was separated from the glyceride, then washed and tested for specific gravity using a hydrometer.

Amount of biodiesel yield and specific gravity were calculated using equation (4) and equation (5) [7,12] respectively.

$$\text{Biodiesel yield (\%)} = \frac{amount\ of\ biodiesel\ produced}{amount\ of\ waste\ olive\ oil\ used}\ X\ 100 \quad (4)$$

$$\text{Specific gravity} = \frac{W3-W}{W4-W} \quad (5)$$

Where:

W is the weight of the specific gravity bottle, W_3, the weight of the specific gravity bottle + sample, W_4 is the weight of the gravity bottle + water.

Heating value:

This is referred to as the energy content of diesel fuel and its rate of combustion. It is the heat released when a specific quantity of fuel is burned under specific conditions.

Calculated using brake specific fuel consumption (BSFC)

$$BSFC = \frac{m_r}{bp} \quad (6)$$

Where r is the fuel consumption rate in grams per second (g/s)
P is the power in watts, p= rw
B is the engine speed in radians per second (rad/s)
R is the engine torque in newtons per meters (N-m) [13].

2.8 GCMS Analysis and Spectroscopic Method for Fatty Acids and Methyl Esters

Chromatographic application and spectroscopic process were used to determine and categorize the minor components of biodiesel that may influence biodiesel fuel quality. Higher performance HPLC Shimadzu 2010 for environmental solutions with operational pressures (50-350) bar. Column dimensions are 2.1-4.6 mm diameter and 30-250 mm length was used for mixture separation.

2.8.1 Physical methods

The physical analysis was done by means of specific gravity, temperature, and pH using the acceptable standard of scientific methods in biodiesel production.

3. RESULTS AND DISCUSSION

3.1 Lipase Cultivation/Growth

Table 1 Summarizes the rate of lipase growth after cultivation for 72 h. Lipase activity started in 6 h of incubation and was on geometric progression until at the 48th h with the formation of round circler material that indicated lipase activity. However, at 54th h lipase activity started decreasing, this may be due to exhaustion of food required for normal lipase growth in a medium. Biomass growth rate evaluation was in the range of 85% to 90% from the 6th h until the 42nd h. noticeable decrease in lipase activity was observed after 48th h with reduced round circle formation indicating exhaustion of food supply as could be seen in Table 1.

Table 1 Summarizes enzyme immobilization using sodium alginate provided increased resistance in the changes to pH, which kept the pH at 5.5 and temperature of the medium at 25℃.

pH is one of the major parameters that can affect enzyme reaction in a medium. Since pH was maintained at 5.5 it is an indication of optimal lipase activity. Lipase activity in this study started sparingly at the 6^{th} h (arithmetically) and changes geometrically at the 18^{th} h until 48^{th} h with the formation of round circular object indicating lipase aspergillus induced growth. High lipase formation after 42 h explains the fact that groundnut shell is a good substrate for induced lipase growth. However, retardation in the growth was noticed as shown in Table 1 after 48 h till 72^{nd} h, this could be as the result of nutrient exhaustion/depletion in the culture media or enzyme activity is inhibited by enzyme saturation, that means the reaction rate was dependent on the substrate concentration as in Fig. 1. The values increased with substrate concentration and then leveled off when substrate concentration reached 50 mg/g, this could be due to enzyme demand/saturation for food.

Complete lipase separation and purification were achieved at pH 6 with 0.5 g potassium phosphate buffer solution containing 1% NH_4S0_4 as nitrogen source. The number of lipases recovered after purification was 85.1%. Purified enzymes that were supported by physical adsorption which resulted in high (85%) yield and high stability than free lipase. The number of free lipase yield in 2^{nd} cycle attached to NH_2 attachment/ physical adsorption material in Fig. 2 was large enough to support transesterification process.

Table 1. Enzyme immobilization

Time (h)	6	12	18	24	36	42	48	54	60	72
Growth (mm)	0.25	0.82	2.0	4.5	9.2	18.4	36.8	25	24.7	20.2

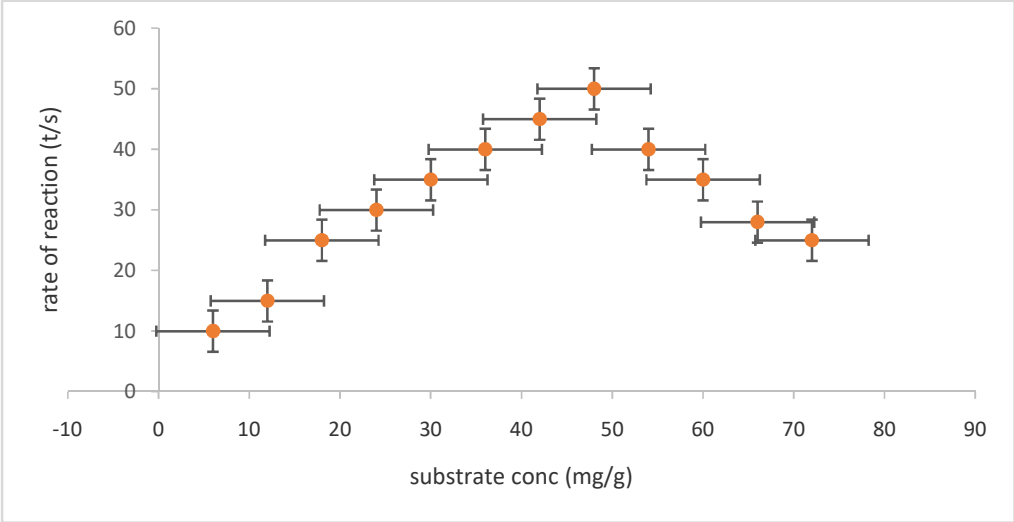

Fig. 1. Rate of lipase enzyme activity at substrate concentration

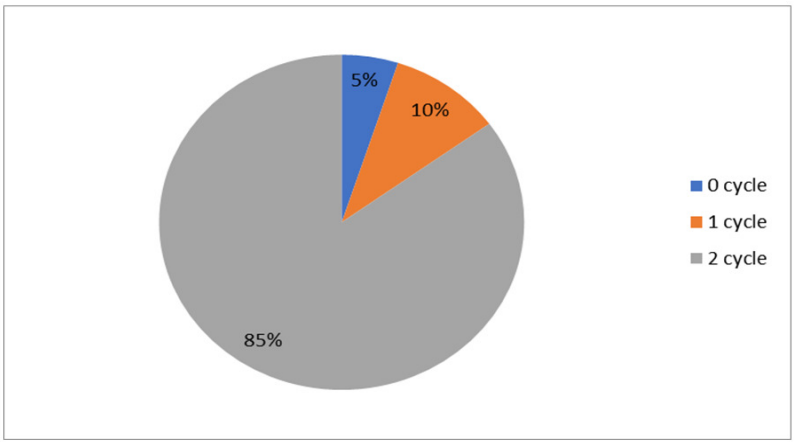

Fig. 2. Lipase recovery after purification

Table 2. The untreated and pretreated groundnut shell before and after pretreatment

Pretreatment method	Conditions with max. efficiency	Cellulose (%) content.	recovery	Hemicellulose (%) content.	removal	Lignin (%) Content.	removal
Untreated		37.4	-	26.6	-	36	-
Alkaline 3.0 % $Ca(OH)_2$	80^0C, 90 min	46.5	70	28.2	40	9.5	75
Alkaline 2.0% NaOH	90^0C, 60 min	47.6	82.3	7.2	52	4.2	87

Pretreated groundnut shell according to the composition in Table 3 comprised of 36% lignin and 26.6% hemicellulose which necessitated pretreatment to enable enzyme penetrability and accessibility of microorganism for conversion to ethanol. Both pretreatment conditions evaluated showed high cellulose recovery (70% & 82.3%) and high lignin removal (75% & 87%) respectively, however, high cellulose yield (82.3%) and lignin removal (87.0%) was obtained with 2% NaOH pretreated groundnut shell. The high lignin removal efficiency of both alkaline pretreated samples could be because alkaline (NaOH, $Ca(OH)_2$ may cause the lignocellulosic

cell wall to be swollen with the increase in the internal surface area [5]. The swollen cell wall caused an increase in the internal surface area and cleaving the linkages of lignin, pentoses that destroyed cellulose-hemicellulose-lignin matrix and accessibility of sugars for enzymatic hydrolysis. Yan [11], reported alkaline pretreatment of sweet sorghum bagasse with high lignin removal efficiency similar to this result.

Since both alkaline pretreated conditions showed high cellulose yield and high lignin removal, both samples were further enzymatically hydrolyzed for fermentation to ethanol.

3.2 Enzymatic Hydrolysis and Fermentation of Groundnut Shell

Enzymatic hydrolysis of NaOH and $Ca(OH)_2$ pretreated groundnut samples were carried out in 4.8 pH buffer solution at 200 rpm and 0.3 units/mg of cellulase from Aspergillus nigar. A high sugar yield of 45 g/L (NaOH pretreated) and 38.2 g/L ($Ca(OH)_2$ pretreated) was obtained at exactly 48 h after 72 h of incubation. The increase in yield from 88.9 mg/g and 67.2 mg/g to 45.0 g/L and 38.2 g/L in sugar content could be as the result of the conversion of cellulosic content of groundnut shell of the alkaline pretreated samples to sugars by cellulase. Since NaOH and $Ca(OH)_2$ pretreated samples had high cellulosic sugar conversion both were separately used for fermentation to ethanol.

Enzymatically hydrolyzed materials were carried out without detoxification due to the absence of furan derivatives in both samples. Ethanol production in the two alkaline pretreated samples is shown in Table 3. Highest ethanol production (89.7%) was obtained in NaOH pretreated groundnut shell samples compared to $Ca(OH)_2$ pretreated (79.6%) which was leveled off in 24 h after 72 hours of fermentation. This could due to possible inhibition of the fermentable microorganisms by high ethanol concentration. Udeh & Emrah, [5] had a similar report in a study with Phoenix caneriansis and Opuntia ficus-indica that showed inhibition of TRS consumption by the increasing ethanol concentration.

3.3 The Immobilized Lipase

Enzymes are easily denatured/inactivated in the presence of heat and organic solvents. In this study, immobilized and purified enzymes obtained from Aspergillus spp were found to be tolerant to organic solvent (bio-ethanol) from groundnut shell pretreated and fermented samples. Immobilization efficiency obtained using lipase isolated from *Aspergillus nigar* was 82.5%, this is comparable to 80% and 85% immobilization efficiency obtained from bead and powder from T. Lanuginose as reported by Dizge [14].

However, the novelty of this study is to produce biodiesel with high octane rating and maximum enzyme activity and to reduce the effect of inorganic chemicals including its pollutant effect in conventional biodiesel production that uses vegetable oil and methanol.

3.4 Temperature Effect on the Purified Lipase

Lipase is a water-soluble enzyme that can catalyze and enhance the hydrolysis of lipid substrates. Most importantly, in the processing, transportation of dietary lipid during digestion. However, during the transesterification process, as shown in Fig. 3, it was observed that lipase activity increased with increase in temperature from 10℃, 15℃, 20℃, 25℃, 30℃, 35℃, 40℃, 45℃, 50℃, and was optimum at 37℃. After 37℃, lipase activities were gradually decreasing, this could be as the result of the inhibition at high temperatures. It could also be explained that the rise in temperature caused the kinetic energy to rise and making more molecules to gain greater energy than kinetic energy. Again, as the temperature rise in temperature lead to greater activation energy resulting to higher enzyme activity, continuous increase in temperature caused the enzyme to denature because enzymes contain protein thereby leading to the reduction in enzyme activity.

Table 3. GC Analysis of the biodiesel lipase catalyzed fermented ethanol

Carbon compound	Carbon numbers	Observed (minutes)	Methyl esters
Methyl palmitrate	16	11	-
Methyl Oleate	18.1	13.5	Methyl ester
Methyl linoleate	18.3	14	Methyl ester
Methyl arachidonate	20.2	16	-
Methyl behenate	20.1	16.5	-
Methyl erucate	22	18	-

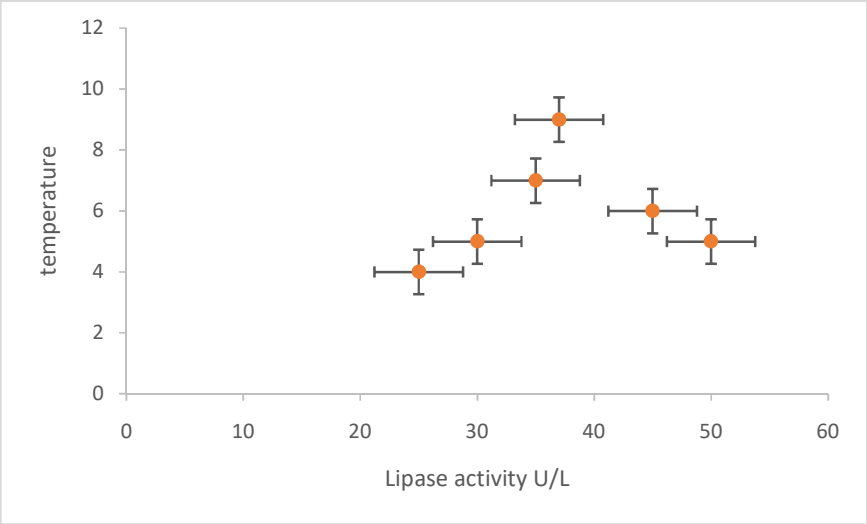

Fig. 3. Effect of temperature on the purified enzyme

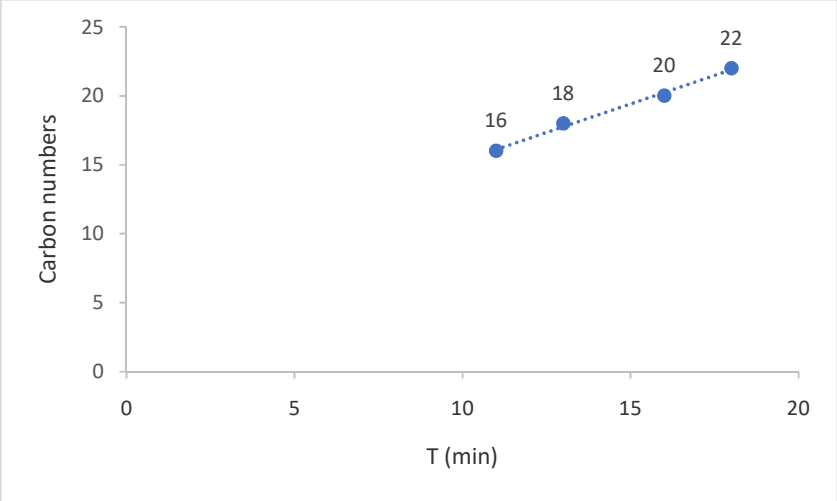

Fig. 4. Effect of time

3.5 GC Analysis of the Biodiesel Lipase Catalyzed Fermented Ethanol

Biotechnological manipulation of lipolytic enzyme and ethanol (from groundnut shell) biocatalytic process yielded 70% of biodiesel (Table 3). According to GC analysis, the rate of reaction and biodiesel yield was dependent on the lipase activity and temperature of the medium. 6 different samples of lipase-fermented ethanol biodiesel GC analysis in this study showed exciting results on the increasing carbon content which increased with lipase activity as the temperature tends towards optimum (37℃). Carbon peaks were observed after 2 min at 16 C to C22 in 18 min which was the highest peak. Methyl ester which represents the organic biodiesel was observed in C18 as shown in Table 3 and the methyl docosahexaenoate (C22) had long retention times because of high polarity of this compounds which is in accordance with literature during the analysis of FAMEs in soya-bean biodiesel sample by McCurry [15].

The lipase-catalyzed biodiesel product recorded viscosity is 2.88 cm^2s^{-1} which is slightly above 2.81 cm^2 obtained from waste sunflower vegetable oil earlier reported by. This will increase the cetane number of the fuel and by so doing the knocking intensity will be decreased

with shorting of ignition delay which will encourage reduced noise in the compression chamber, an indication for high engine runs with no carbon pollutant effect as lipolytic enzymes and fermented ethanol are environmentally friendly products. This will help to reduce waste, air pollution, and landfill depositions. Viscosity result from this study shows that frequent engine cracks and knocks will be reduced to the barest minimum by application of lipase-ethanol transesterification method.

GC analysis evaluation indicates that the lipase-catalyzed biodiesel product in this study has high volatility rate (density) of 15.5℃ which is in accordance with literature (Wikipedia, 2015) and specific gravity of 0.95 against fossil fuel (0.87) and waste sunflower oil based (0.914) as reported [16]. The calorific value was 43.2 MJ/Kg and since the heating value per volume is directly proportional to density when all things (fuel properties) remain unchanged, energy output will be high to enhance fuel economy even at a low temperature, which is expressed as volume used per unit distance usually in liters per 100 kilometers covered with no evidence of sulfur evolution through the waste pipe.

4. CONCLUSION

The immobilized lipase enzymes isolated from *Aspergillus nigar* were used for biodiesel production in this study and the number of lipases recovered after purification was 85.1% which is large enough for transesterification process. Purified enzymes that were supported by physical adsorption which resulted in high (85%) yield showed high stability than free lipase. Again, the fermented ethanol obtained from lignocellulosic material in combination with free lipases produced clean biodiesel with high cetane number, the high-cetane number diesel product obtained in this study can withstand cold climate condition. This will, however, encourage smooth engine runs, good ignition quality with improving cold start performance. Issues such as a misfire, smoke emissions, noise and cold start (unless the temperature is below freezing point) will be minimized. Since the amount of pressure needed during combustion in biodiesel is lower than that of fossil fuel and also engine crack/knocks may be avoided. Lipase-catalyzed diesel should be encouraged because this will help to minimize/reduce excessive wear with a minimum level of lubricity for friction reduction between surfaces when a car is in motion.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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