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Research Article
Production and Characterization of Biodiesel from the Microalga, *Chlorella vulgaris* (Beijerinck 1890)

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Abstract

Background and Objective: Due to the declining fossil fuel resources as well as the need for an alternative source of biodegradable source of energy, biodiesel has a great advantage over conventional fuel due to its low content of particulates. This study investigated the production and characterization of biofuels produced from the fresh water microalgae; *Chlorella vulgaris*. **Materials and Methods:** Water was sampled from Sharada industrial run-off within Kano metropolis for isolation and identification of algal species. *Chlorella vulgaris* was identified under a light microscope and isolated by repeated sub-culturing on BG-11 media. Scale-up of the culture was done using a designed photo-bioreactor for 23±2 days after which oil was extracted using solvent extraction method. This oil was converted to biodiesel through transesterification with KOH as a catalyst and the physico-chemical characterization of the fuel were achieved using special physico-chemical characterization methods to evaluate its quality. A FT-IR spectrum was used to determine if fatty acids were converted to fatty acid methyl esters. **Results:** Microalgal species isolated were; *Spirogyra*, *Scenedesmus*, *Nitzschia*, *Anabaena*, *Chlamydomonas*, *Oscillatoria*, *Zygnema*, *Volvox* and *Chlorella* sp. A 50 g of biomass of *C. vulgaris* produced almost 5.092 mL of oil in 3 weeks. The physico-chemical properties obtained were in line with the American Society for Testing Materials (ASTM) standard values. The biodiesel properties of the oil indicated that the biodiesel meets the ASTM standard. FT-IR spectra analysis revealed the peak of biodiesel production at 1745 C=O produced. **Conclusion:** So, it was concluded that the biofuel produced by *Chlorella vulgaris* biomass have excellent qualities suitable for use in the transport industry and therefore; it can be recommended for use as an economically viable source of renewable energy.

Key words: *Chlorella vulgaris*, biodiesel, fossil fuel, biofuels, algal species, biodiesel production

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The growing demand for the use of fossil fuels in various industries have been reported as the major factor causing high CO_2 levels in the atmosphere resulting in global warming[1]. The world is presently confronted with the crisis of fossil fuels depletion and environmental degradation and it is estimated that in the next 50 years, fossil fuels must have been exhausted[2]. Therefore, biofuels becomes an alternative fuel option for industries and transportation.

Microalgae are photosynthetic micro-organisms that require light energy and carbon dioxide for the production of high value compounds ranging from carotenoids to polyunsaturated fatty acids[3,4]. Microalgae may be destined to different applications, such as; biofuel production and wastewater purifications[5]. Biofuel production can take place either under autotrophic or mixotrophic conditions. Microalgae are known as "efficient solar energy converters" that can produce a great variety of metabolites[6]. Also, the high productivity of microalgae justified by its shorter generation time and higher oil content compared to crops is a clear sign of how significantly microalgae can contribute to the large scale production of biofuel[7].

Microalgae have been studied as alternative fuel sources that is renewable, economical and environmentally friendly[8]. Biodiesel production from microalgae is an emerging technology considered by many as a very promising source of energy, mainly because of its competition for land in the generation of biofuels, as some species contain up to 60% of overall mass by fatty acids or lipids. This oil from algae can be extracted, processed and converted into biodiesel used as transportation fuel using current available technology. Biodiesel from algae lipids is highly degradable and non-toxic[9]. Microalgae like higher plants produced storage lipids in the form of triacylglycerols (TAGs), although triacylglycerols could be utilized in producing a wide range of chemicals. Biodiesel can be synthesized from triacylglycerols through a simple transesterification reaction in the presence of methanol. Biodiesel can be used in unmodified diesel engines and it has series of advantages over the conventional diesel fuel, because it is biodegradable, renewable and produces less sulphur compounds and particulate emissions when burned[10]. This study was carried out to produce a biodegradable oil that will serve as an alternative source to replace the fossil fuels which are known for their negative impact on the environment. This study was aimed at producing an affordable biodiesel from *Chlorella vulgaris* as well as analyzing the qualities of the biodiesel produced. Fuel produced from this alga will serve as alternative fuel that can replace the fossil fuels due to its less negative impact on the atmosphere.

MATERIALS AND METHODS

Sampling site: The study was carried out from June-November, 2015. Sampling of water was carried out in Kano Metropolis from Sharada industrial run-off waste waters. This pond is located at 8.521°N and 11.981°E. Water in this pond was traced to have resulted from the Sharada Industrial Estate, where industrial wastes were deposited into the water. The pond measures about 220 m long and 50 m wide.

Sample collection: Water samples were collected using phytoplankton sample bottles as described by Indabawa[11].

Isolation and identification of microalga: The study was carried out in the Laboratory of the Department of Botany, Bayero University, Kano, Nigeria. The identification of the microalga was done as described by Anaga and Abu[12] using standard psychological keys, charts and illustrations by Hilary and Erica[13] and Marvin[14]. *Chlorella vulgaris* was isolated from the water samples, this was achieved by repeated sub-culturing of the microalga on the culture media as described by Agwa *et al.*[15] and Makoto[16]. In this method, single-cell isolation by micropipette was employed, where glass capillary micropipettes was used for the isolation. A light microscope equipped with a binocular lenses and a graduated stage was used for the identification and isolation procedures.

Preparation of the culture media: The culture media was used for culturing process of the microalga BG-11 media. The media was prepared in the Plant Biology Department laboratory of Bayero University Kano.

Purification of microalga: The two paths of purification methods adopted in this research were physical separation of the *Chlorella vulgaris* microalga from contaminants and the use of antibiotics to kill contaminants such as; bacteria and viruses.

Antibiotic treatment: The antibiotic solution was prepared as described by Guillard[17]. In this method, 100 mg of penicillin, 25 mg of dihydrostreptomycin sulfate and 25 mg of chloramphenicol was dissolved in 10 mL of distilled water and sterilized by filtration.

Culturing and sub-culture of the microalga: *Chlorella vulgaris* was cultured, revived and maintained in BG-11 media using a 1000 mL capacity flask and a designated 10 L capacity photo-bioreactor as described by Ripka *et al.*[18] and Anderson[19] employed by Indabawa[11]. The sub-culture of

the microalgal sample was achieved by serial dilution, where the initial 1 mL of the sample was enriched with 9 mL of BG-11 media and was allowed to stand for 14 days. The cell was in the log phase of its growth during these days. The cells were exposed to a light period of about 8 h as described and employed by Indabawa[11].

Scaling up of microalgal culture: The microalgal cells were pre-cultivated in 100 mL liquid medium in a 250 mL Erlenmeyer flask with BG-11 media on a shaking platform at a rotation speed of 100 rpm. The pre-cultivated microalgal cells were used as inoculums for 10 L capacity photo-bioreactor cultivation. All cultivations were illuminated under a light intensity of 35 μ mol^{-1} m^{-2} sec^{-1} at a cycle of 8 h light and dark cycle at 23±2 for 20 days. The culture was sub-cultured again into 1000 mL Erlenmeyer flask, where 100 mL of the culture was placed into 900 mL. At this juncture, the culture apparatus was designed in order to introduce aeration of 8 h as at the same time it was exposed to 8 h photoperiods. The cell density of the microalgal growth rate was taken daily by measuring the optical density of the algal culture at 540 nm using a spectrophotometer (Spectronics D20) as described by Anaga and Abu[12].

Measuring of microalgal growth: The growth pattern or growth rate of the microalgal culture was measured by optical density (OD) at 540 nm, during the cultivation period with visible ultraviolet spectrophotometer as employed by Indabawa[11].

Harvesting of microalgal biomass: *Chlorella vulgaris* was harvested by flocculation method as described by Vandamme *et al.*[20].

Lipid extraction from the microalga: Lipid was extracted from the microalgal biomass by solvent extraction method[21].

Purification of the microalgal oil: Lipid extracted from the microalga was purified by distillation method as employed by Chen *et al.*[22].

Production of fatty acid methyl ester (FAME): Transesterification of the lipid was carried out according to the method employed by Indhumathi *et al.*[23].

Washing and drying of biodiesel: It was noticed that the biodiesel obtained as a result of the transesterification of the biodiesel process of the microalgal oil contained some small amount of methanol, glycerin, soap, catalyst and other impurities. In order to remove the unwanted materials from the oil, water was warmed to about 45°C and was passed through the esters to allow all the soluble materials to stick to the bottom of the vessel. Water was then removed from the vessel periodically until the pH of the biodiesel became relatively neutral. The biodiesel was afterwards still looking cloudy which was an indication of the presence of water. To get rid of the water, it was then heated to a temperature of 100°C until all moisture was completely removed[23].

FT-IR spectrum analysis of the biodiesel: The NEAR FT-IR machine was used for the analysis of the oil extracted from the microalgal *Chlorella vulgaris*. The stage of the FTIR machine was thoroughly cleaned using a cotton bud that had been dipped in petroleum ether. The machine was then test run to ensure no impurities remained on the stage. The sample was then loaded onto the stage when the machine had finished the test running process and was declared ready for use as displayed on the computer screen. A drop of the sample was loaded on the equipment by the use of a clinical syringe. The peak values of the analysis were marked and the reading was recorded accordingly[11].

Physico-chemical characterization of the microalgal oil: Physico-chemical properties of *Chlorella vulgaris* biodiesel (Iodine value, acid value, saponification value, flash point, fire point, cloud point, pour point, density, refractive index and pH) were determined in order to compare the biodiesel produced with standards.

Determination of iodine value [ASTM D974(01)]: Iodine value [ASTM D974 (01)] was achieved using the method employed by Indabawa[11].

Determination of acid value and free fatty acid value (ASTM D974): About 2 g of oil was measured and poured in a beaker. About 50 mL of a neutral solvent prepared by the mixture of ethanol and petroleum ether was taken and poured into the beaker containing the oil sample. The resulting mixture was stirred vigorously for 30 min. About 0.56 g of potassium hydroxide pellet was measured and placed in a conical flask and 0.1 M potassium hydroxide was prepared, 3 drops of phenolphthalein indicator was added to the sample and was titrated against 0.1 M potassium hydroxide until a pink coloration was observed. The acid value (AV) was calculated using the following relations as employed by Indhumathi *et al.*[23]:

$$AV = \frac{(56.1 \times A \times N)}{Woil}$$

Where:

A = Volume of standard alkali used
N = Normality of standard alkali used
Woil = Weight of oil used

Determination of saponification value (ASTM 5558-95): A freshly prepared solution of alcoholic potassium hydroxide was made by dissolving potassium hydroxide pellet in ethanol. About 2 g of oil was measured and poured into a conical flask. About 25 mL of the ethanolic potassium hydroxide was then added to it. Blank solution was used as well. The sample was covered and placed in a steam water bath and was allowed for 30 min, while shaking the sample periodically, 1 mL of phenolphthalein indicator was added to the mixture.

The mixture was titrated against 0.5 M Hydrogen chloride (HCL) and the titer value was noted. The saponification value (SV) was then obtained using the following relations according to Indhumathi *et al.*[23]:

$$SV = \frac{(B-A) \times 28.92}{Woil}$$

Where:

B = Volume of HCL used in titration with the blank
A = Volume of HCL used in titration with the oil
Woil = Weight of oil used
B = 18.4
A = 5.10
Woil = 2 g
SV = 192.61 mg g^{-1}

Determination of flash point and fire point (ASTM D6751): Flash point is the temperature at which a combustible mixture can be formed above the liquid fuel. An ignition source is required to determine a flash point. An ignition was placed on measured microalgal oil in an open flame. A thermometer was placed in order to record the temperature changes. When the source of ignition was removed, the vapor ceased to burn and this temperature was recorded as the flash point. The flash point was measured as the temperature for which the vapor continued to burn for at least 5 sec after the source of ignition was removed at an open flame[11]. The fire point was assumed to be almost 10°C higher than the flash point. The standard flash point is between 130-205°C.

Determination of cloud point (ASTM D2500): A sample of the biodiesel was placed in a test tube and then placed in a cooling bath after it had been heated to about 40°C. When the biodiesel started to form cloud below the test tube, its temperature was quickly measured and taken as the cloud point. This procedure was repeated 3 times and the mean value was recorded[23].

Determination of pour point (ASTM D97): A sample of the biodiesel was placed in the freezer for 24 h. The biodiesel was removed after the period and placed in a beaker containing warm water of about 10°C to melt. The temperature at the bottom of the test tube at which the biodiesel starts to pour is recorded as the pour point of the biodiesel[11].

Determination of specific gravity/density (ASTM D1298) by hydrometer method: This procedure was used by Indhumathi *et al.*[23] to evaluate the specific gravity of the biodiesel.

Determination of refractive index: This was done using an Abti refractometer as employed by Indabawa[11].

Determination of pH: A portable pH meter was used to determine the acidity or basicity of the oil.

Statistical analysis: Percentage abundance was calculated for the alga species, mean was calculated for the weight of algal biomass and volume of biodiesel obtained. Plot of the optical density was done using Microsoft Excel version 2013.

RESULTS

Species of alga isolated from sharada industrial wastewater: Microalgae sampled from the water include members from three classes of microalgae; Class Chlorophyceae: *Spirogyra* sp., *Scenedesmus* sp., *Chlorella* sp., *Chlamydomonas* sp., *Zygnema* and *Volvox* sp. Class Diatoms: *Nitzschia* sp. and Class Cyanophyceae: *Anabaena* sp. and *Oscillatoria* sp. (Table 1). The frequency of the algae species varies with *Oscillatoria* sp. and *Spirogyra* sp. having the highest occurrence of 20%.

Alga biomass production: There was no significant difference ($p>0.05$) in the weight of the algal biomass produced from *C. vulgaris* for a period of 21 days of culturing, the highest biomass obtained for the period was 50 g while the least was 49 g. Also, no significant difference

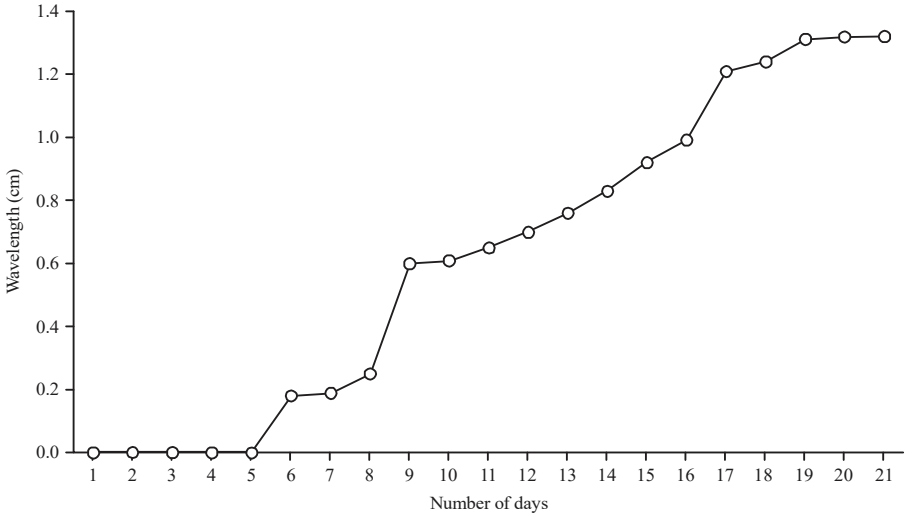

Fig. 1: *Chlorella vulgaris* growth curve in BG-11 media over 21 day period

Table 1: Algae species present in the water in order of abundance (%)

Alga species	Class	Abundance (%)
Spirogyra sp.	Chlorophyceae	20
Oscillatoria sp.	Cyanophyceae	20
Volvox sp.	Chlorophyceae	15
Anabaena sp.	Cyanophyceae	10
Chlorella sp.	Chlorophyceae	10
Chlamydomonas	Chlorophyceae	10
Scenedesmus sp.	Chlorophyceae	5
Zygnema	Chlorophyceae	5
Nitzschia sp.	Diatom	5

Table 2: Weight of algal biomass and the volume of biodiesel obtained from *C. vulgaris* in 21 days

No. of sampling	Weight of algal biomass (g)	Days of culture	Vol. of biodiesel obtained (mL)
1	49.00	21	5.211
2	50.00	21	5.013
3	50.00	21	5.131
4	50.00	21	5.011
Mean	49.75		5.092

Table 3: Physico-chemical characterization of *Chlorella vulgaris* biodiesel, test limit and ASTM standards

Physicochemical properties	Biodiesel	ASTM standard
Saponification value (mg KOH g^{-1})	192.61mg g^{-1}	191-202 mg g^{-1}
Acid value (mg KOH g^{-1})	0.64 g g^{-1} of oil	$\leq$0.8 g g^{-1} oil
Iodine value (mg I_2 g^{-1})	85	82-98 mgI_2 g^{-1}
Refractive index	10	
Fire point (°C)	105°C	140-215°C
Pour point (°C)	4°C	-15 to +10°C
pH	7.2	7.0
Density at 40°C	848 g mL^{-1}	$\leq$920 kg m m^{-3}
Cloud point	+2	+2
Specific gravity at 40°C	0.855 g cm^{-3}	0.9
Flash point (°C)	115°C	100-170°C

(p>0.05) was observed in the volume of biodiesel produced from *C. vulgaris* for the period of 21 days which ranges from 5.011-5.211 mL from approximately 50 g of *C. vulgaris* biomass (Table 2).

Optical density: The optical density of the microalgal culture was estimated using the spectrophotometer (Spectronics D20) for 25 days and the measurement was initiated from the 5th day after inoculation. The growth curve of the optical density (absorbance) was plotted against the number of days as indicators of the growth (Fig. 1). Rapid growth rate was observed from day 8 through day 9 from 0.25-0.6 wavelengths, respectively with the highest growth rate on day 20 and 21 (1.32 cm).

FT-IR characterization of biodiesel: The NEAR FT-IR characterization of the biodiesel indicated highest peak region of 1745 wavelength (cm^{-1}) at 50.922 absorbance followed by 2924 cm^{-1} at 55.190 absorbance which was as a result of C-H stretch. Carbonyl stretch C=O of esters appears however; as aliphatic from 1750-1735 cm^{-1}. The peak band of 3010 cm^{-1} was olefinic =C-H stretch. The bands between 600-1200 cm^{-1} indicated the presence of cis-alkene. The peak 2855 was formed as a result of C-H stretch. C–H bend or scissoring from 1470-1450 cm^{-1} indicated the presence of saturated hydrocarbons (alkanes). The peak 1380 indicated C-H rock methyl. The band 724 indicated the presence of long chain methyl bonds while the peak 1100 indicated the presence of unsaturated C–O bond (Fig. 2).

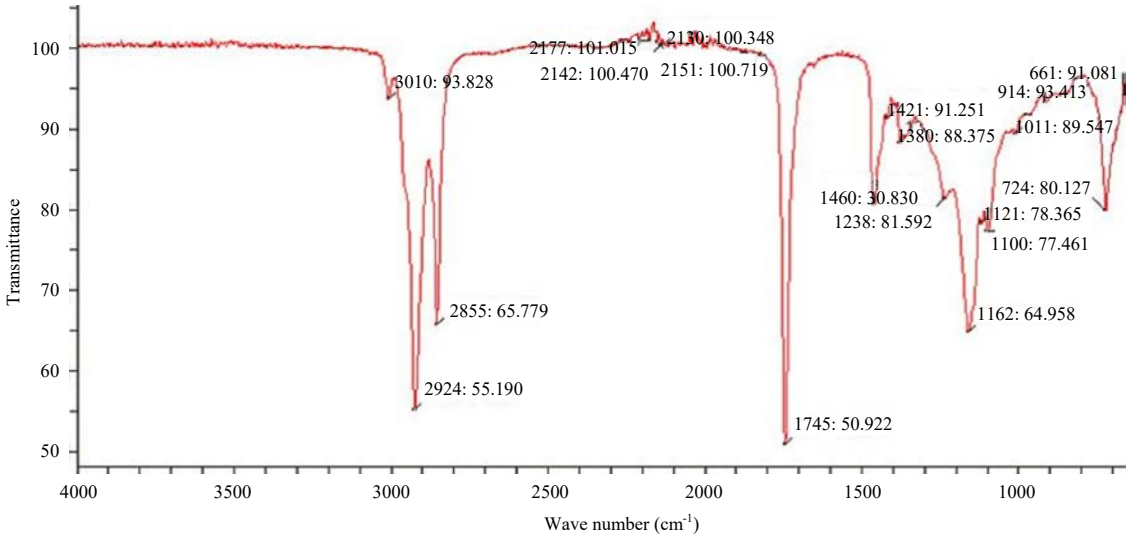

Fig. 2: FT-IR spectrum analysis of the *Chlorella vulgaris* biodiesel

Physico-chemical qualities of biodiesel: The 11 physico-chemical qualities of biodiesel produced from *C. vulgaris* all fell within the ASTM acceptable limit with saponification value of 192 mg KOH g^{-1}, an acid value of 0.64 mg KOH mg^{-1} and iodine value of 85 mg g^{-1} ml_2. Also, the biodiesel had a refractive index of 10, fire point of 105°C and pour point of 4°C. A pH of 7.2 was obtained while ASTM value is 7.0 and density at 40°C was obtained to be 848 g mL^{-1}. Cloud point of +2°C was observed while specific gravity at 40°C was measured to be 0.855g cm^{-3} with a flash point of 115°C (Table 3).

DISCUSSION

About 9 species of algae were found in water samples collected from Sharada Industrial wastewater, Kano. These species are *Spirogyra* sp., *Scenedesmus* sp., *Chlorella* sp., *Chlamydomonas* sp., *Zygnema* sp., *Volvox* sp. *Nitzschia* sp. *Anabaena* sp. and *Oscillatoria* sp.

A checklist of the optical density of the *C. vulgaris* growth obtained which showed that the lowest absorbance observed at day 4 and 5, respectively was as result of the initiation stage for which the algal cells starts to adapt the media environment in which they were introduced. Optimum culture temperature used in this study yielded maximum biomass and this was in agreement with findings of Converti *et al.*[24], who stated that *Chlorella vulgaris* showed a promising growth in the designed photo-bioreactor from 5-23 days with tangible biomass obtained from the batch cultures, this biomass was higher than the 41.64 g observed by Al-lwayzy *et al.*[9], who produced biodiesel in laboratory scale from *C. vulgaris* in freshwater body for diesel engine. This indicated that *C. vulgaris* from non-sterile ponds contain high amount of lipids. The high amount of lipids suggested that *C. vulgaris* has good potential to be used as an economically viable sources of renewable oil and biodiesel production. This was in conformity with the findings of Al-lwayzy *et al.*[9] and Senthil *et al.*[25], while Converti *et al.*[24] declared that high lipid productivity of *C. vulgaris* depends on the culture conditions such as; optimum temperature of 25-30°C and increased CO_2 concentration.

The presence of biodiesel was detecting using the Fourier transform infrared spectroscopy (FT-IR). The NEAR FT-IR characterization of the biodiesel indicated peak region at 1745 cm^{-1}, this was due to the presence of carbonyl ester. This indicated the presence of aliphatic aldehydes which however was in accordance with the findings of Cortes[26]. Ester was however present in the biodiesel, the ester present is palmitoleic acid ($C_{17}H_{32}O_2$). Another peak value of 2924 cm^{-1}, which was as a result of C-H stretch at 2924, alkyl was therefore suspected to be present. The values ranging from 2000-2500 cm^{-1} indicated the presence of unsymmetrical internal alkynes (4-octyne). The peak band of 3010 cm^{-1} was due to olefinic = C – H stretch. This indicated the presence of an aromatic compound or alkene while the bands between 600 and 1200 cm^{-1} indicated the presence of cis-alkene. The peak 2855 was formed as a result of C-H stretch. This was suspected to be a carbonyl compound (ketone, H–C=O stretch 2830-2695 cm^{-1}) as a result of unsaturated C-H bond. This was also confirmed by Barbara[27]. The 1162 band was due to C-O stretch and this indicated the presence of unsaturated fatty ester oil[27].

The saponification value was in agreement with standard value for biodiesel set by the American System for Testing Materials (ASTM D). The acid value of the biodiesel obtained was within the ASTM range, but higher than an acid value of 0.4 g obtained by Vijayaraghavan and Hemanathan[28] from freshwater algae and 0.37 mg KOH g^{-1} from *Chlorella protothecoides* in the study by Xu *et al.*[29]. Iodine value also fell within the ASTM range while the fire point obtained was lower than the ASTM D7651 value of 140-215°C. The pour point in this study was in agreement with the ASTM D97, but higher than the pour point observed by Xu *et al.*[29] in biodiesel produced from *Chlorella protothecoides*. The pH observed in this study was slightly higher than the ASTM standard of 7.0 for biodiesel. Density and specific gravity were in conformity with ASTM D6751 standard. The flash point obtained in this study was similar to the previous findings observed by Xu *et al.*[29] with a flash point of 115°C for *Chlorella protothecoides*, but higher than flash point of 98°C obtained by Vijayaraghavan and Hemanathan[28]. The flash point in this study was lower than the ASTM standard of 130°C. The result indicated that majority of the physico-chemical properties of biodiesel produced from *Chlorella vulgaris* were in line with the ASTM standard except for the flash point that was 10% lower and the pH which was slightly higher. This indicated that the transesterification process was approximately complete and the quality of the *C. vulgaris* biodiesel obtained from this work was up to standard. Studies on the cost implication of producing biodiesel from *Chlorella vulgaris* will help provide information for policy makers in the oil industry as this study did not capture that aspect.

CONCLUSION AND RECOMMENDATION

Chlorella vulgaris was isolated from industrial run-off waste waters. *C. vulgaris* produced biodiesel with high yield per algae biomass as about 50 g of *C. vulgaris* biomass generated 5.1 g of lipid which was converted to biodiesel from simple transesterification reaction. FT-IR spectrum revealed that *C. vulgaris* lipids were converted to fatty acid methyl ester (FAME) at 1745, while the standard value for ester bond formation was obtained at 1744. BG 11 media is recommended for biomass production of *Chlorella vulgaris*. FTIR is recommended for easy and accurate characterization of biodiesel. *Chlorella vulgaris* is recommended for use as it is economical and viable sources of renewable oil and biodiesel production.

SIGNIFICANCE STATEMENT

This study discovered that *Chlorella vulgaris* biomass can be used to produce an economical and biodegradable quality biodiesel. BG 11 media is the best media for the production of *Chlorella vulgaris* biomass. This study will help researchers in the transport industry uncover the critical areas for mass production of biodiesel from *Chlorella vulgaris*.

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