

Physiochemical Characterization of Oil Extracted from Microalgae *Chlorella Pyrenoidosa*

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Abstract

*On the basis of technological projection and processing scientific knowledge, the biofuel derived from the microalgae, that is a third generation biofuel could be one of the promising answer to different issues related with food crops biofuel and lignocellulose material biofuel. In this study, autotrophic cultivation of Chlorella pyrenoidosa in 250ml of Fogg's media using urea as nitrogen source was done to obtain oil rich biomass. After 5-6 days of lag phase, algal growth increased exponentially and extensive biomass i.e. 5.467 ± 0.034 g/l with lipid content of 27.2 ± 0.26 (% Dry Cell Weight) was obtained. The lipid enriched fraction in form of ORF was obtained using solvent extraction process. This ORF was used to study its various parameters like moisture content, iodine value, peroxide value and acid value. UV-Visible Spectroscopy, Fourier Transform Infrared Spectroscopy (FTIR) and thin layer chromatography (TLC) studies of ORF was done to detect relevant hydrocarbons in our ORF sample. Low moisture content ($0.21\% \pm 0.089$), high iodine value (102.45 ± 0.12) and low acid value (1.67 ± 0.059) indicted that our ORF sample has long chain of fatty acids with no free fatty acids. UV-Vis spectrogram of our oil sample showed broad region absorption peaks at the range of 450-520nm; it shows a high concentration of long chain fatty acids in our ORF sample. The results obtained indicated that oil obtained from *C. pyrenoidosa* meets the ASTM standards and has excellent qualities to convert it to biodiesel to use it viable renewable energy source in the future.*

Keywords: Acid value, Chlorella, FTIR, Oil rich fraction, Peroxide value, UV Visible Spectroscopy

I Introduction

India is a fastest growing country with an average 7.3% increase in Gross Domestic Product (GDP) every year and it is expected to continue in coming years also [1]. At present, natural gas, coal, nuclear power and petroleum which are the non-renewable energy resources have been used extensively. As in past decade, the use of liquid fuel comprised of 96.3% and out of this less than 1% share was contributed by biofuels [2, 3]. It has been reported by International Energy Agency that in 2035 around 29% of energy will be obtained from the conventional liquid sources, whereas 57% will be from non-conventional oil resources and about only 6% will be from biomass in the form of biofuels [4, 5]. It is estimated that at present, approximately 92% of the total energy demand in a year is fulfilled by using the non-renewable resources and rest of the energy comes from renewable energy resources such as biomass, hydropower, solar and geothermal energy [6]. Fossil fuels are increasing the carbon dioxide (CO₂) concentration in the environment, which is detrimental for the people if they would not adopt “going green” in their lives [7]. Microalgal CO₂ sequestration is a sustainable process which also helps in environmental cleaning processes such as wastewater treatment and bio-sorption of heavy metals [8, 9]. The microalgae have 20-30 times faster growth rate and 10-50 times higher CO₂ fixation ability as compared to the terrestrial plants [10]. Biofuels are one of the reliable energy resources which are gaining attention by various researchers, scientists and environmentalist as these fuels are produced from biological matter. Biomass of crops, algae and even the organic waste of human as well as animal activities can be used for biofuel production. Microalgae have 20-60% dry weight basis oil content and even some of the strains contain more than 80% of oil content. In microalgal cell, the oils get accumulated in form of the triacylglycerols (TAGs) usually under stress conditions and are rich in C16 and C18 fatty acids which get used in biodiesel preparation [11, 12].

Taking into account the high potential of microalgae to produce neutral lipids in the form of triacylglycerols (TAGs), this paper aims to investigate some physico-chemical properties of microalgae oil extracted from the specie *Chlorella pyrenoidosa* and its suitability to convert into biodiesel. *Chlorella pyrenoidosa* is a freshwater microalgae occurs worldwide belongs to division Chlorophyta. The cells are spherical having a parietal and mantle-shaped chromatophore with a prominent pyrenoid body. *C. pyrenoidosa* has medicinal uses, as it acts as a chelator agent to extract dioxins and its compounds from the body. It is widely used as one of the major constituent in traditional medicines in China and Japan to cure fibromyalgia, ulcerative colitis and hypertension [13].

II. Material and Methods

Isolation and culture of algae from the sewage water sample

In order to isolate algal strains, the water sample was collected from the sewage treatment plant, Pholriwal, Jalandhar, Punjab. The collected sample was filtered using Whatman filter paper with pore size 0.50 μ m to remove any particulate matter. The methodology of serial dilution was applied to isolate various microalgal strains from the sewage sample. Using the streaking technique, each dilution was poured in petri plates containing solidified Fogg's media [14]. *Chlorella pyrenoidosa* was identified with the help of AlgaeBase and was selected for further experimental work.

Harvesting and drying of algal biomass

Biomass harvesting was done by flocculation method using ferric alum as flocculant [15]. 40mg alum was added to culture flasks and for complete extraction it was kept undisturbed for 4-5 hours. Algal biomass was settled down at the bottom of the flasks due to coagulation process. This wet algal slurry was separated and washed 3-4 times with distilled water to remove traces of salts. The biomass was dried in oven at 80°C for 2 hours till constant weight was attained. The dry powdered biomass was used to extract lipids.

Extraction of lipids from dry biomass

Extraction of lipids was carried out using chloroform: methanol, (1): (2) solvent system [16]. 1g of dried algal biomass was weighed and placed in a solvent resistant tube. In this tube, 4ml methanol and 2ml of chloroform was added. 1ml of 1M NaCl was also added as it prevents binding of denatured lipids to acidic lipids [18]. It was shaken vigorously for 3-4 min. and was kept undisturbed for 24 hours. Then 0.4ml of distilled water and 2ml chloroform was added to the above solvent mixture and the whole of the content was poured into the separating funnel. The funnel was vortexed for 30 seconds and was centrifuged at 3500rpm for 10min. It resulted in the formation of two layers. The lower chloroform layer with lipids dissolved in it was separated and transferred to a clear tube. The lipids extracted from dry algal biomass was concentrated by removing water and solvent if any using rotary evaporator at 100°C for 1 hour and a dark brown layer the oil rich fraction (ORF) was obtained.

Analysis of physical parameters of ORF

The ORF of *C.pyrenoidosa* was used to study its different physico-chemical properties viz. moisture content (MC), iodine value (IV), peroxide value (PV) and acid value (AV) according to ASTM

(American Society for Testing and Materials) and AOAC (Association of Analytical Chemists) standard methodology [19].

Calculation of Moisture content (MC) ORF: The MC of ORF was calculated using the standard formula [20]. The 5ml of ORF was taken in a crucible and was placed in oven at 105°C till the constant weight was reached. The percentage of MC was calculated according to the formula below [21].

$$MC (\%) = W_1 - W_2 / W_1 \times 100$$

Where W_1 = Weight of ORF (before drying)
 W_2 = Weight of ORF (after drying)

Determination of Iodine value (IV) of ORF: IV of any oil sample measures the degree of unsaturation and it is measured as the number of gm of halogen (iodine) absorbed/100gm of the sample. 25ml of Wij's solution was added to 2gm of ORF sample in a stoppered bottle and was left undisturbed for 2 hours. After adding 10ml of KI solution in the flasks, titration was carried with standard 0.1N sodium thiosulphate solution till pale yellow colour appeared. 1ml of the starch solution as an indicator was added to the reaction mixture and titrated again till blue colour disappeared. The experiment was repeated with blank. The number of ml sodium thiosulphate was used for blank (B) and sample (S) was noted

Preparation of Wij's Solution:

200ml glacial acetic acid was added to 26gms of sublimated I_2 . The mixture was heated until iodine dissolved in acetic acid. It was cooled and later on used for further procedure i.e. titration with 1N sodium thiosulphate solution. By following the same procedure a blank titration without using ORF sample was carried out as the control.

$$IV = \frac{(V_B - V_S) \times N \times 12.69}{\text{Weight of sample(g)}}$$

V_B = Vol. of sodium thiosulphate used in blank titration

V_S = Vol. of sodium thiosulphate used in ORF sample titration

N = Normality of thiosulphate solution

Determination of Acid value (AV) of ORF: AV of oil and biodiesel measures the degree of FFAs in the sample, influencing the fuel quality [22]. AV was measured using ASTM D 664 standard method. 2g ORF was taken in a 250ml conical flask and 50ml of a mixture of equal volumes of ethanol and ether (1:1) was added to it. The reaction mixture was heated until completely dissolved, then it was cooled and titrated with potassium hydroxide (1N), using 1ml of phenolphthalein as indicator. The flask was shaken constantly till persistent pink colour is obtained. AV was calculated from the following formula.

$$\text{Acid Value} = \frac{(a) \times 56.1 \times \text{Normality of KOH}}{W}$$

Where a = ml of KOH used in titration

W = Weight of oil sample in grams

Determination of Peroxide value (PV) of ORF: PV measures content of oxygen (as peroxide) in a substance and which specifies the rancidity level of oil sample [23]. PV was calculated by ASTM Cd8b-90 standard method. 5gm of the sample was poured in 250 ml flask. 30 ml of acetic acid/chloroform (3:2) solution was added to the sample mixture and was swirled to dissolve the oil. By using Mohr pipette 0.5ml of saturated solution of KI was added to the reaction mixture. The flask was stoppered and swirled again for one minute (light brown colour was observed). After adding 20 ml distilled water the contents were shaken vigorously to liberate unreacted iodine. The reaction mixture in the flask was titrated using 0.1N sodium thiosulphate solution, until the pale yellow colour was observed. Then 1ml of starch solution (as an indicator) was added and titration was continued until blue colour disappeared. By following the same procedure a blank titration was also done. PV was calculated by using the formula:

$$\text{Peroxide value} = \frac{(S-B) \times N \times 1000}{W}$$

S = Vol. of sodium thiosulphate used in titration of ORF sample

B = Vol. of sodium thiosulphate used in blank titration

N = Normality of sodium thiosulphate solution

W = Weight of oil sample in grams

Spectroscopic Analysis of ORF to Detect Relevant Hydrocarbons

Fourier Transform Infrared Spectroscopy (FTIR): FTIR, Perkin Elmer model was used to evaluate functional groups present in our ORF sample under a range of 250cm^{-1} to 4000cm^{-1} and has the resolution of 1cm^{-1} . The readings from the spectrum of FTIR in the computer were recorded. When oil interacted with infrared light, the chemical bonds contracted, stretched and absorbed light at a specific wavelength (λ). On this basis, the functional groups present in the sample were identified [24].

Double beam UV-Visible Spectroscopy of ORF: The ORF obtained was analyzed by double beam UV-Visible spectrometer (Systronics 2200) having λ range from 190nm-3300nm. It refers to the absorption spectrum in the ultraviolet-visible spectral region. It has been used to determine the organic and inorganic compounds present in the sample. UV-Vis spectroscopy provides easier and faster results due to reduced analysis time.

Thin layer chromatography: Silica-gel TLC-plates (Polygram Sil G) were used for thin layer chromatography. The ORF sample and standard sample of fatty acids were loaded without any dilution at a distance of 1.5cm from each other and 2cm from the bottom. The spotting was done with help of capillary having diameter of 0.5cm. The solvent system used was

Hexane	:	Ethyl alcohol
90	:	10

After running in the aforementioned solvent system, the plates were air dried, and developed using 29% alcoholic H_2SO_4 .

Statistical analysis

In present study the oil rich fraction obtained from *C.pyrenoidosa* was tested for various physico-chemical parameters and results were expressed as mean \pm standard deviation (SD) from three independent parallel experiments.

III RESULTS AND DISCUSSION

Streak plate method was followed for isolation of the microalgal species from the sewage water. Inoculum of *C. pyrenoidosa* was taken from petri plate and was inoculated in Erlenmeyer culture flasks containing 250 ml of Fogg's medium using urea as nitrogen source. The culture flasks were incubated under white LEDs with 16:8 light/dark photoperiod with 1000 lux light intensity, temperature of $25 \pm 2^\circ\text{C}$ and 50-55% humidity. After 5-6 days of lag phase, algal growth increased exponentially and extensive

biomass i.e. $5.467 \pm 0.034 \text{ g/l}$ with lipid content of 27.2 ± 0.26 (% Dry Cell Weight) was obtained. The algal slurry was dried in an oven at 80°C and dark green powdered algal biomass was obtained. The lipid-rich fraction obtained using chloroform – methanol solvent system was further concentrated using rotary vacuum evaporator and dark brown, viscous and more concentrated oil rich fraction (ORF) was obtained at the end of extraction (Fig. 1)

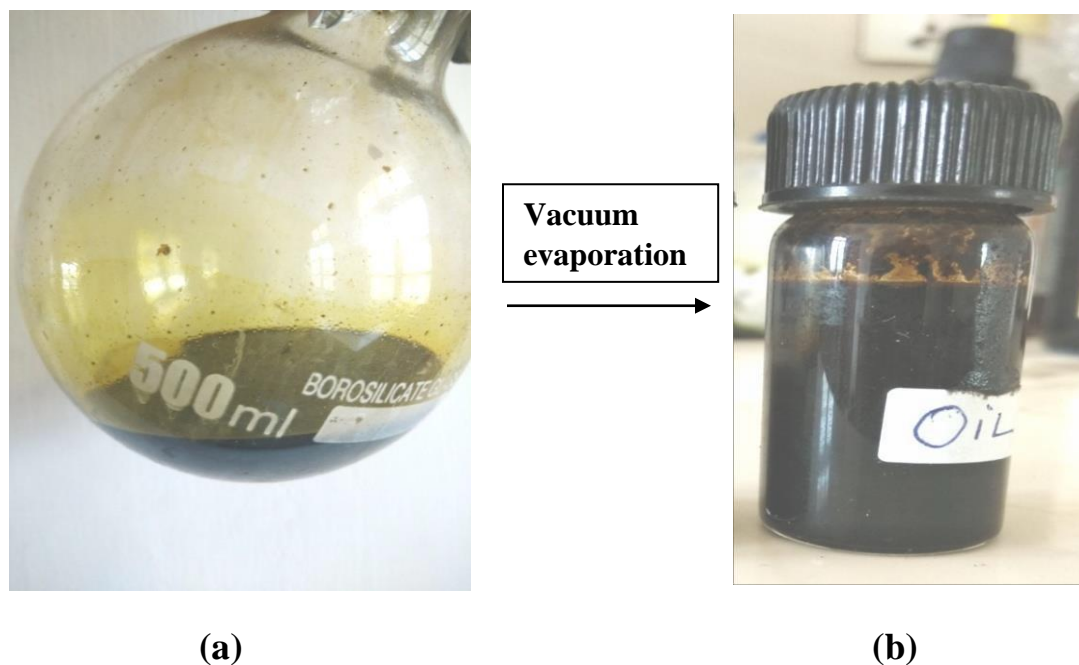


Figure 1(a) Lipid- rich fraction (b) Concentrated ORF

Analysis of physical parameters of ORF

The ORF of *C. pyrenoidosa* was studied for different parameters like moisture content (MC), iodine value (IV), peroxide value (PV) and acid value (AV). The results are presented in Table 1.

Table 1 Results of characterization of ORF of *C. pyrenoidosa*

S.No	Properties	Test method	Value of oil sample
1.	Appearance	Dark brown
2.	MC (%)	ASTMD2709	0.21%±0.089
3.	PV	ASTM Cd8b-90	1.1± 0.025
4.	IV	ASTM D554	102.45±0.12
5.	AV	ASTM D664	1.67±0.059

Our ORF sample has moisture content of 0.21% ±0.0089 and was compared with ASTM standard D6751-02 of *Jatropha*. Moisture content in oil obstructs the separation of biodiesel from glycerol. Oil should have moderate content of moisture otherwise; soap formation will be there, which will deteriorate the quality of biodiesel. High moisture content also creates hindrance in the trans-esterification process. High moisture content leads to microbial growth in the fuel so handling, storage and transportation of biodiesel become difficult. On the other hand, it can also cause corrosion in the internal combustion engine and reduces its shelf life [25].

Our sample of ORF has IV of 102.45± 0.129, when this value was compared with the standard ASTM value IV indicates the degree of unsaturation in an oil sample. It reflects the stability of an oil sample against oxidation [26]. Higher IV indicates greater unsaturation in a sample. While, the oil with low IV has tendency to get oxidise and polymerise easily leading to engine deposits [27]. It shows that our oil sample upholds the good qualities to use for making biodiesel.

ORF of *C.pyrenoidosa* has AV of 1.67±0.0059 which is almost equal to ASTM standards. AV is a useful parameter as it indicates the mineral as well as corrosive FFAs in the oil sample which can corrode automotive parts and decrease the efficiency of the engine. It is an important quality parameter, as low conc. of the FFAs gives better quality of oil [28].

PV of our ORF has lower value of 1.1± 0.025, proving the oxidative stability of oil as high PV indicates more free radicals, causing auto-oxidation of the oil sample which decreases the efficiency of fuel. Good quality oil has low PV which is an indicator of greater stability of oil. [29].

Spectroscopic studies of ORF to detect relevant hydrocarbons

FTIR (Fourier Transform Infrared Spectroscopy): FTIR, Perkin Elmer model was used to evaluate functional groups present in the sample. Perkin Elmer spectroscopy range was 4000cm^{-1} to 250cm^{-1} and has resolution of 1cm^{-1} and result is presented in Fig. 2 and Table 2. The bands in FTIR spectrogram were identified using reference standards as well as already published data [30, 31, 32]. The OH stretching at 3423.03cm^{-1} indicates presence of alcohols & phenols and C-H stretching at 2929.34cm^{-1} corresponds to aliphatic hydrocarbons. The symmetrical and asymmetrical $-\text{CH}_2-$ stretching at this range indicates strong lipids and little proteins also [32, 33]. At 1660.41cm^{-1} strong C=C stretching vibrations corresponds to olefin group i.e. alkenes. The peaks at $1750.49 - 1382.71\text{cm}^{-1}$ confirm the presence of aryl ketones and aldehyde groups. The bands at 1076.08cm^{-1} indicate the presence of phenols and halo hydrocarbons in our sample [34]. During the combustion process, breaking of C-H and C-C bond takes place to release ample of energy. At the same time alkenes and alkynes break completely into smaller fragments and produce energy.

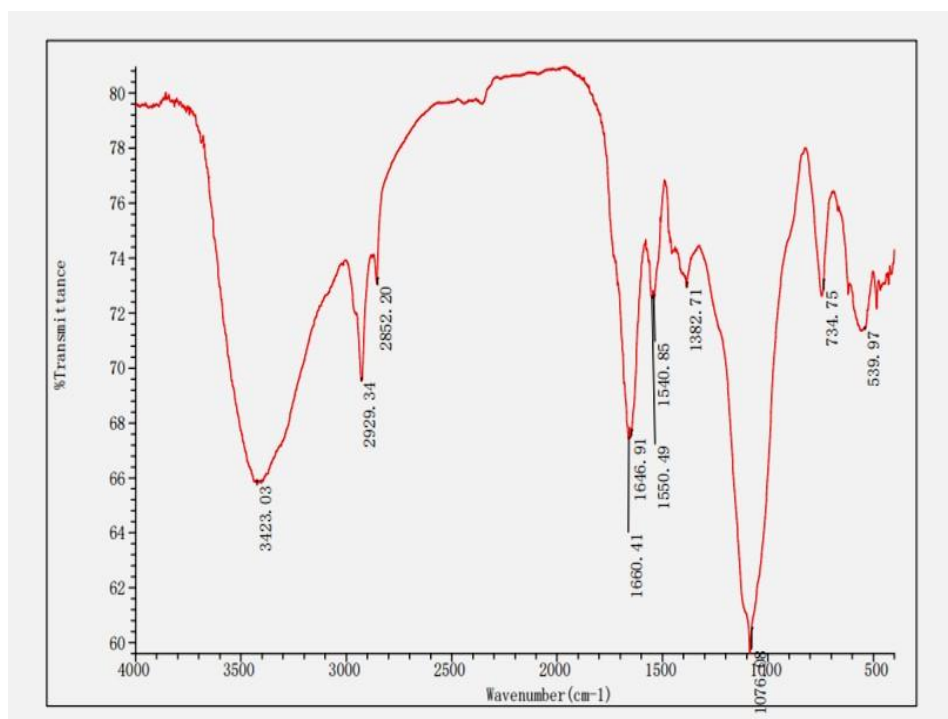


Figure 2 FTIR Spectrogram of ORF

Table 2 Interpretation of FTIR spectrogram of ORF [35]

S.No.	Stretching vibrations	Groups present	Position
1.	OH stretching	Alcohols and phenols	3423.03- 3010.2 cm^{-1}
2.	C-H stretching	Aliphatic hydrocarbon	2929.34-2852.20 cm^{-1}
3.	Symmetrical and asymmetrical -CH ₂ - stretching	Long chain FAs and lipids	2752.23-1739.14 cm^{-1}
4.	C=C stretching	Olefin group (alkene)	1660.41-1646.91 cm^{-1}
5.	C=O	Aryl ketones and aldehyde groups	1750.49 -1382.71 cm^{-1}
6.	C-X and C-OH	Phenols and halo hydrocarbons	1076.08-539.97 cm^{-1}

Double beam UV-Visible Spectroscopy of ORF: It refers to the absorption spectrum in the ultraviolet-visible spectral region. It has been used to determine the organic and inorganic compounds present in the sample [34, 35]. UV-Vis spectroscopy provides easier and faster results due to reduced analysis time. The ORF obtained was analyzed by UV-Vis spectrometer-lambda 750 from Perkin Elmer, having λ range from 190 nm-3300 nm (Fig. 3). UV-Vis spectroscopy technique was used to check the quality of oil sample. The absorption peaks were studied using reference standards. The oil showed maxima absorbance peaks in region of 200-250nm, 340-380nm and 450-520nm. The compounds showing peaks in the range of 200-250nm and 340-380nm belongs to hydrocarbons. As spectrogram of our oil sample has broad region absorption peaks at the range of 450-520nm; it confirms the high concentration of long chain fatty acids in our ORF sample which is required for a good quality biodiesel.

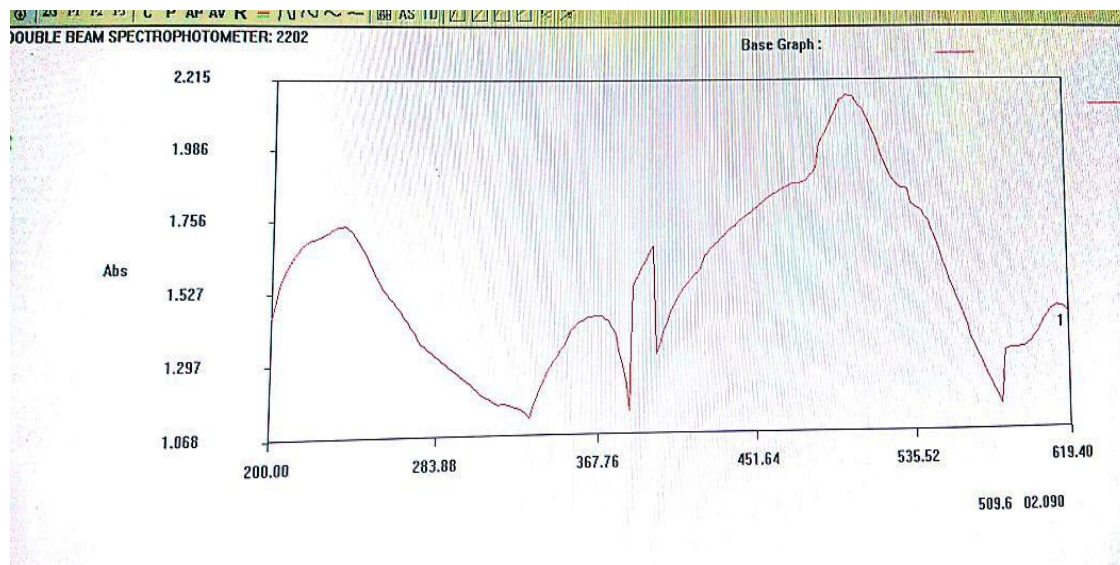


Figure 3 UV-Visible spectrogram of ORF

Chromatographic analysis of ORF by thin layer chromatography: ORF was investigated through thin layer chromatography (TLC) technique using standard samples of oleyl alcohol, oleic acid and stearic acid as reference with ORF of *C. pyrenoidosa*. TLC has confirmed the presence of oleyl alcohol, oleic acid and stearic acid in ORF and their retention factor (R_f) values are presented in Table 3. The presence of long chain fatty acids in ORF sample is a primary requisite for transesterification process to convert algal oil to biodiesel.

Table 3 Result of R_f values of hydrocarbons of ORF

S.No.	Fatty acid standards	Molecular formula	R_f value
1.	Oleic acid	$C_{18}H_{32}O_2$	0.43 ± 0.051
2.	Stearic acid	$C_{16}H_{36}O_2$	0.38 ± 0.034
3.	Oleyl alcohol	$C_{18}H_{36}O_2$	0.39 ± 0.043

CONCLUSIONS

A promising microalgal candidate for the production of biodiesel should have suitable hydrocarbons composition accompanied by high TAGs production. The results of FTIR, UV Visible Spectroscopy and TLC of ORF revealed that it has saturated & unsaturated alkenes, alkynes, ethers, alcohols and fatty acids. Low moisture content, high iodine value and low acid value indicated that our ORF sample has long chain of fatty acids with no free fatty acids. So *C.pyrenoidosa* is feasible to use as biofuel feedstock, for the trans-esterification process to make biodiesel. To optimize the usefulness of ORF extracted from *C.pyrenoidosa* related to its productivity and suitability to use as alternative fuel, requires further in-depth research.

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