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Biodiesel production from local mixed algal culture of Rourkela, Odisha.

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Abstract

Algae biomass could be possible valuable resources towards the relief from upcoming petroleum indigence. The local algal diversity was exploited in order to find suitability for extraction of biofuel. In this study, locally available biomass was successfully cultured in the outdoor bioreactor and the effects of various common lipid extraction methods were quantified on the basis of gravimetric estimation. Finally, FAME was studied qualitatively by Gas chromatography-mass spectrometry, to check their suitability for biodiesel standards. Obtained results conform that among considered population predominant species were found to be *Chlorella*, *Anabaena*, *Euglena*, *Oocystis* and *Sphaerocystis*. Combination of dried algal biomass with the Folch's method yields >27% lipid which is comparatively higher than the traditional Soxhlet methodology i.e. 15%. More than 50 % of total fatty acids consists of carbon 18 acids i.e. linolenic acid (C18:3), linoleic acid (C18:2) and oleic acid (C18:1). The variety of comparatively short carbon chain fatty acids were also observed that was considered to give the best fuel properties. Therefore, this local algal mixed diversity was found to be suitable for biofuel as well as various other fatty acids production.

Keywords: *local algae biomass, biodiesel, lipid, extraction, fatty acids.*

Introduction

Fossil fuel reservoir beneath the earth surface is fixed and non-renewable. According to IEA (International Energy Agency) and BP (British Petroleum) reports techniques are in progress to discover new depositories but one day will not be sufficient to satisfy the demand of exponentially growing population (Radmer et. al. 1996, Clarens et. al. 2010). Increased consumption of conventional fuel majorly for industrialization and transportation is immersing our ecological balance day by day. Greenhouse gases accumulation, hole in ozone layer, acid rain, climate change, aquatic life trauma by oil spills are severe infrastructural damage to nature (Manuell et. al. 2007).

The present era demands new technologies to overcome these situations, and biofuels are the excellent alternative. These renewable energy feedstocks could be derived from the wide range of organic materials or even waste products. Leftovers could be efficiently recycled, could be exposed to open environment as these are biodegradable. Biodiesel exhaust has the less harmful impact on human health than petroleum diesel fuel as it contents comparatively lower nitric oxide, carbon and sulphur compounds (Hawkins et. al. 1999). Dependency from foreign petroleum outsourcing may decrease and will also provide employment within the country.

The current supply of biodiesel in comparison to other conventional fuel is very less as raw products usages fertile land, water, competitive with food or other consumables and seasonal based supply of raw material. One of the capable substitutes is algae that overcome these limitations (Clarens et. al. 2010). Selective algae stains have also benefited from higher growth rate, potential to produce higher triglycerides per area than terrestrial plants, can be cultivated in the useless land, polluted water, uptakes greenhouse gases, heavy metals from the environment and can be the source of various commercial products (Radmer et. al. 1996).

Although plenty of research confirmed that algal biomass is a promising source of biofuel, still large scale production is in infancy. Algae cultivation needs the selection of fast-growing, productive strains, optimised for the local climatic conditions for algal mass culture. Energy conversion steps also require innovations in steps of harvesting, oil extraction methodologies for economically feasible products such as biodiesel. Therefore, in the present study, we exploit this geographical environment for the selection of algal culture that could be successfully grown in the outdoor bioreactor. Finally, those strains were subjected to lipid extraction by different methodologies.

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Materials and Methods

Chemicals and reagents

All reagents were of analytical reagent grade were used. Double deionised water (Milli-Q Millipore) was used for all dilutions. Chemicals and reagents for Algae culture growth media and organic solvents (Petroleum ether, chloroform and methanol) (Merck, Mumbai, India) were all analytical grade.All the plastic and glassware were cleaned by soaking in dilute HNO_3 (1/9, v/v), autoclaved and were rinsed with distilled water prior to use.

Algae culture

Water samples were collected from local water deposits (22˚15'19.5"N 84˚54'14.5" E), and microalgae culture was purified by serial dilution, streak plating, the addition of antibiotics and antifungal agents at least to remove bacterial and fungal contamination. Isolated mixed cultures were preliminarily identified as per Prescott (Prescott. 1954). The morphological study was performed under inverted microscope (Olympus Corporation Tokyo, Japan).

Obtained strains were maintained in modified Bold Basal Media in incubator shaker with the constant shaking of 120 rpm, at 25 ± 2˚C under the light intensity of 50 ± 5 µmol m-2sec-1 and 18:6 (light: dark) duration photoperiod. For large scale culture, cultivation cells were transferred in thirty litres outdoor bioreactors and were aerated with compressed air. Temperature and illumination intensity were not controlled but was dependent on day-to-day weather conditions.

Growth Measurement

Optical density was used to monitor the algae growth. Ten milliliter samples were collected daily from the cultures. After centrifugation the culture was mixed with 2 ml acetone. Glass beads were used to break the cell and was vortexed for 10 min. Centrifugation at 5000 rpm for 2 min settles the cell debris. The supernatant was collected carefully and the entire procedure was conducted in dark. The same processes were repeated three times. Finally, OD of bulk cultures was measured at 680 nm (Perkin Elmer, Lambda 25).

Extraction pre-treatment

Algal cultures were left unaerated for few days for gravity settling. The concentrated cell cultures were dewatered further using a bench top centrifuge at 4500 g for 10 min. The resulting microalgal paste was rinsed with deionised water to remove residual salts. In experiments both the wet and dried microalgae was used. The microalgal paste was dried at 60 ˚C in an oven for overnight. A mortar and pestle were then used to grind the dried biomass into powder. The experiment using wet microalgal paste was obtained by centrifugation. Further cell disruption of wet paste was also done by putting the micro vial in liquid nitrogen and disrupting the with glass beads (Sigma-Aldrich, Bangalore, India) in a vortex mixer (Remi CM-101 Plus Cyclo Mixer) for 7 min. The procedure could be repeated until the sample forms a fine powder. Microalgal samples were stored at 5 ˚C for no longer than 1.5 months before they were used for lipid extraction.

Lipid Extraction

Total lipids were extracted from dry algal biomass by using a modified Folch method. The basic procedure was improved by replacing water by 1M NaCl to increase the yield of lipids (Gigliotti et. al. 2011). After that the settled down biomass was collected from the test tube by centrifugation and the solvent was removed to a weighed vial. Extraction of biomass was repeated twice as described above. The organic extractions pooled into a weighed vial, were dried and weighed to establish the total lipid.

Petroleum ether extraction was also experimented using Soxhlet apparatus. Briefly, the microalgal powder was packed in a cellulose thimble inside the extraction chamber of the Soxhlet unit. Pure petroleum ether (300 mL) was used to extract the lipid for 7.5 h at the rate of 10 refluxes per hour. The extracted lipid was gravimetrically quantified and transferred into a sealed glass vial for storage in the dark at 5 ˚C for less than one month. Transesterification of lipids was performed using a molar ratio of oil : methanol : HCl :: 1 : 82 : 4 with a reaction temperature of 65 ˚C and reaction time of 6.4 h. The top organic phase, which contained FAMEs (Fatty acid methyl esters) was pipetted out for analysis using Gas Chromatography–Mass Spectroscopy (GC–MS).

Analytical methods

The extracted lipids were characterized by using GC/MS- QP 2010 SHIMADZU and with flame ionization and mass spectrometry detection. GC-MST analysed the fatty acid composition of the lipids with VF-NAX MSA capillary wax column (L=30m and D=0.25mm). Helium gas (99.99% purity) was used as carrier gas at the flow rate of 1 ml/min. The initial oven temperature was set to 90°C for 2 min and then increased to 220°C. Each component was identified by comparing retention time and fragmentation with standards using the NIST library. Biodiesel properties were also estimated by "Biodiesel Analyzer Ver. 1.1". (http://www.brteam.ir/biodieselanalyzer) (Talebi et. al. 2013, Verduzco et. al. 2012)

Results and Discussion

Growth and Identification of algae

The presence of species with different geographical, regional, ecological niche could be the probable region for distribution of the different biological organism. Therefore, in the present study, we collected samples from local water bodies within NIT Rourkela campus. Freshly collected samples were serially diluted and grown in bold basal media along with antibiotic treatment. Antibiotic and antifungal treatment to isolate axenic algal culture has been employed successfully over the past years. Most algae tolerate higher concentrations of antibiotics than bacteria but as in environment wide range of bacteria exists; therefore, it is necessary to use the antibiotics mixture. Similarly, algae also differ in their sensitivity, but closely related species sometimes have quite related tolerances (Jones et. al. 1973). In present work, gentamicin, vancomycin, penicillin and nystatin were used in combination as per prescribed dose in earlier literature (Jones et. al. 1973).

Growth index of mixed algae culture in thirty litres outdoor bioreactor was measured in order to monitor the cell condition on routine basis. The culture was maintained in bold basal media and was routinely provided as per requirement of cell condition. The exponentially growing culture in incubator was transferred in bioreactor but still it follows the general trend of lag phase followed by log phase (Fig. 1).

Morphological features of algae culture such as cell wall, chloroplasts, and the cell dimensions were carefully observed. Few predominant species in the mixed algae culture were found to be *Chlorella*, *Anabaena*, *Euglena*, *Oocystis* and *Sphaerocystis*. The microscopic image with and without lugol's reagent was shown in Fig 2. The morphological feature

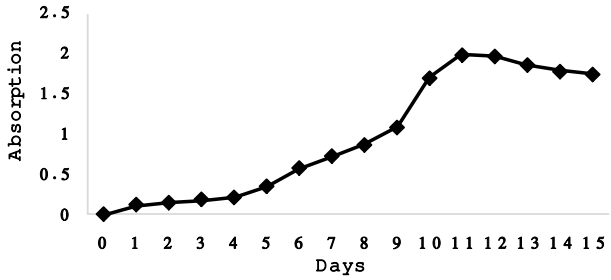

Figure 1. Growth curve of algae culture used in experiment.

of Chlorella includes single-cell green algae, spherical shaped, about 2-10 µm in diameter, without flagella, positive starch test (Bellinger et. al. 2015). *Anabaena* was identified as filamentous structure, presence of hetrocysts, surrounded by mucilage layer, non-motile and negative starch test with lugol's reagent. Optical microscopic observation of samples showed solitary, motile, green eukaryotic cell surrounded by a thin outer wall, centrally located nucleus, containing several green and red bodies like chloroplasts, negative starch test, typically about 30-60 µm long and 6-10 µm wide. These characteristics are very similar to those described for the *Euglenoid* sp. (Sittenfeld et. al. 2002). The observation reveals that the few species belongs to *Oocystis* genus as it is green, colonies within a thin mucilaginous envelope, oval or globular shape, autospores colonies released by rupture of the mother cell wall, positive starch test, 7-50 µM diameter during the different phase of cell cycle (Bellinger et. al. 2015). The study examined *Sphaerocystis* sp. as immotile, green algae, spherical colony, globose cells embedded in outer mucilage cover, sometimes colonies are even more than 1 mm diameter, chloroplasts are cup-shaped, mostly found in freshwater and positive starch test (Prescott et. al. 1954).

These species had a high growth rate, survived well in outdoor conditions and displayed wide temperature tolerances over the period of the study. Isolation, purification, and maintenance of monoculture is hectic and wastage of infrastructure and economy. Cell culture banks specially invest money and manpower to maintain those culture, but still few organisms are difficult to preserve alone, therefore, they are stored with symbionts (Amaral et. al., 2013). That problem could be automatically reduced if we directly utilize the mixed algal culture.

Selection of lipid extraction methodology

In this part of the experiment, the algal cells were subjected to lipid extraction from different viable techniques. Different methods of extraction were compared on the basis of extracted lipid percentage that could be useful to increase the reliability of results on lipid content. Fig. 3 shows that the most effective extraction method, among those taken into consideration, was the combination of dried algal biomass with the Folch method, which led to a yield (>27%) that by far exceeded those obtained by the traditional Soxhlet (15%). Moreover, solvent polarity matters a lot while extraction of lipid from the cell. As the algae contain various polar and non-polar lipid within cell that could be better extracted by combination of solvent mixtures. Chloroform and methanol binary mixtures were reported to extract higher oily fraction from different cell and tissues previously (Ramluckan et. al. 2014).

However, such a methodology did not show significant differences when applied to dried and wet biomass, the maximum lipid extraction yields being ~27 and ~25%, respectively. Lipids were also extracted from wet biomass by cell disruption. Lipid yield was higher in comparison to uncrushed algal biomass. It was clear from the result that, breaking down algal cell walls is essential to enhance extraction of intracellular compounds. Based on these results, it was decided to extract the lipid fraction using Folch method from dry biomass as this protocol could be applied to extract the highest oily fraction. The previous literature also states the similar finding (Converti et. al. 2009).

However, derivation of pure lipid from the mentioned protocol could not be claimed. As the liposoluble intracellular pigments are readily co-extracted into crude oil. Presence of chlorophyll and other pigments decrease the transesterification efficiency and combustion efficiency of biodiesel. However, little literature available regarding the purification of these pigments and even by using existing protocol is yet not satisfactorily accomplished. However according to literatures pigments are highly unstable in the presence of high light irradiance, acids, bases, and oxygen (Li et. al. 2016). Therefore during further transesterification reaction the pigment degradation may occur.

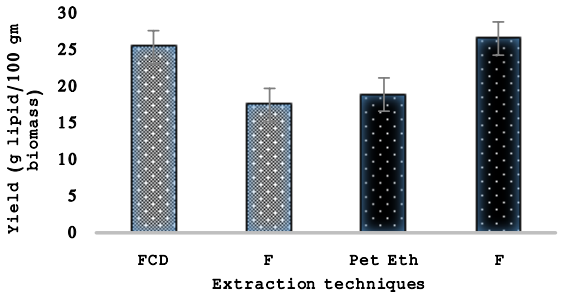

Figure 3. Lipid yield obtained by different techniques of extraction. Grey bar indicates wet biomass and black bar indicates dried biomass. F (Folch's method of lipid extraction), CD (Cell disruption), Pet Eth (lipid extraction using Petroleum ether as solvent)

FAME profile analysis

Further, analysis of fatty acid methyl esters produced by the reaction between fatty acids and methanol (transesterification), yielded very high values of myristic acid (14:0), which constituted about 8% (mol/mol) of the overall lipid fraction (Table1). A total of ~10 different fatty acid substrate from C12-C24 were identified by mass spectrometry. Out of these fatty acids, the majority are low carbon chain compounds which are very suitable for biodiesel production. According to retention time, Linolenic acid (C18:3), Linoleic acid (C18:2) and Oleic acid (C18:1) consists of more than 50% of total FAs. Moreover, the variety of fatty acids were present in total FAME fractions. These varieties could be due to the presence of the different variety of algae community (Lang et. al. 2011). Basic properties and application of these compound have also been described in Table 1 (Ramluckan et. al. 2013).

Determination of biodiesel properties are useful parameter to know the characteristics of the fuel (Table 2). The Biodiesel Analyzer Version 1.1 requires only FAME data input that reduces the task of obtaining heavy amount of oil for these important analysis (Devi et. al. 2012). Variation in fatty acids profile effects the overall biodiesel quality.

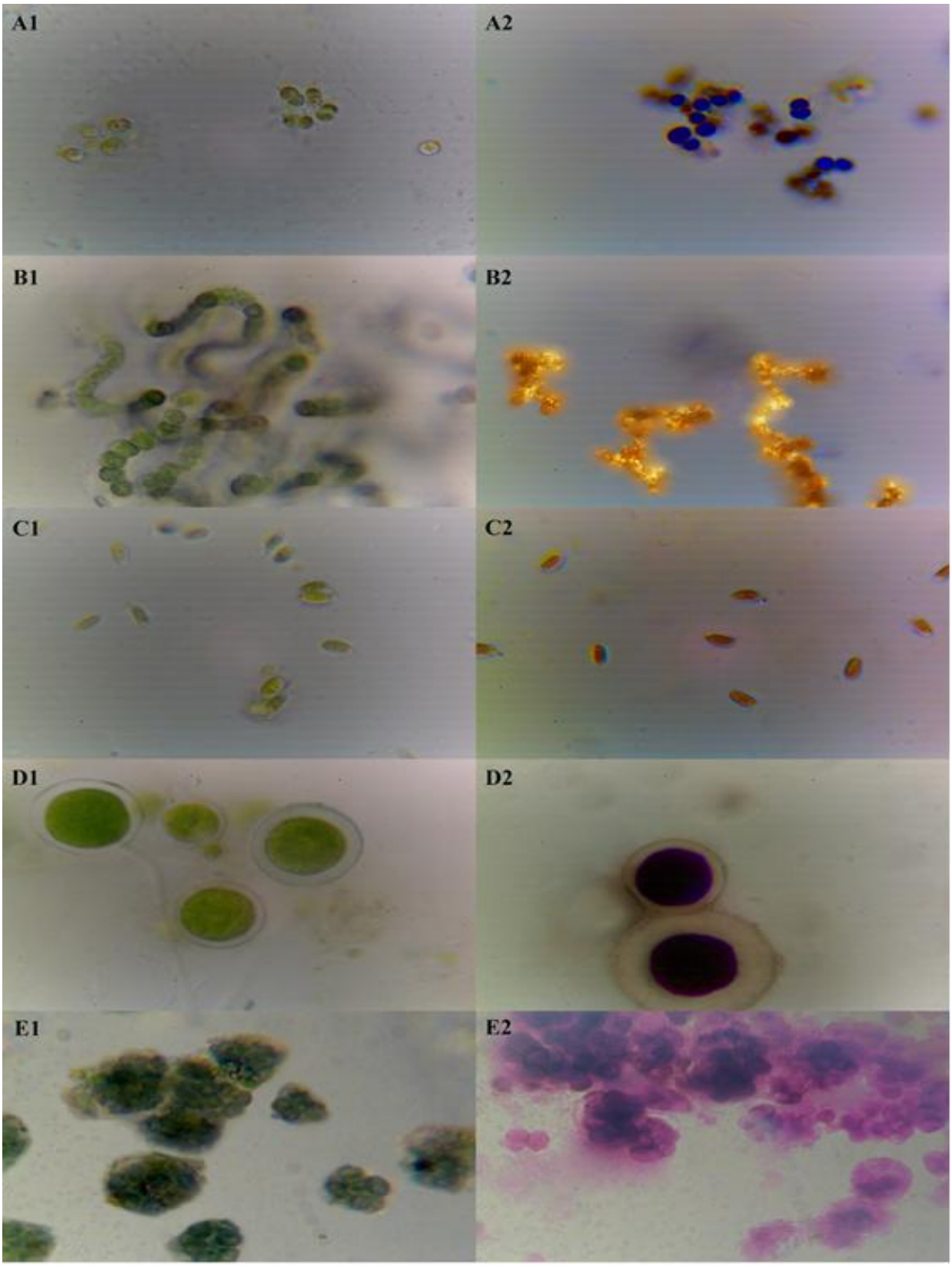

Figure 2. Microscopic view of predominant species present in the study. Panel 1 and Pannel 2 represents the image of before and after addition of lugol's reagent. A. Chlorella sp. B. Anabaena sp. C. Euglena sp. D. Oocystis sp. E. Sphaerocystis sp.

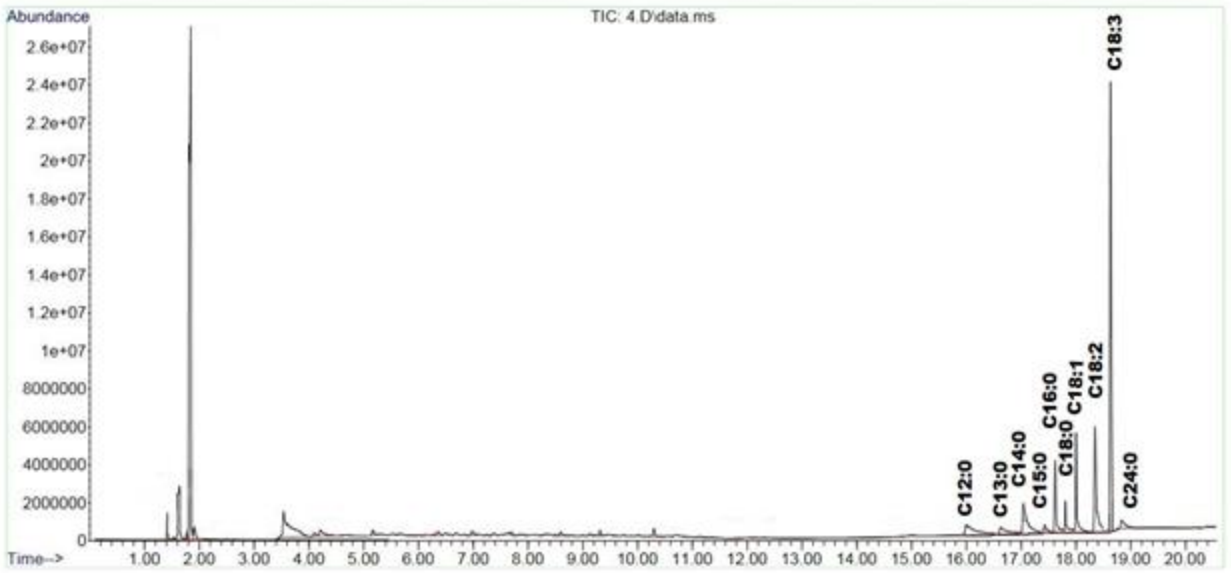

Figure 4. Chromatograms of FAMEs obtained during experiment.

Table 1. Fatty acid profile detected at various nutrient conditions.

Fatty acid	Lipid number	Relative content (%)	Application
Saturated			
Lauric acid	C12:0	4.56%	Antibacterial, antioxidant and antiviral inhibitor
Tridecyclic acid	C13:0	3.58%	Ingredient in methyl ester formation
Myristic acid	C14:0	8.46%	Manufacturing of Biofuels, cosmetics and tropical medicines
Pentadecanoic acid	C15:0	1.66%	Combustion, marker in butter fat composition
Palmitic acid	C16:0	6.32%	Biofuels and cosmetics preparation
Stearic acid	C18:0	3.44%	Biofuels and dietary supplements preparation
Lignoceric acid	C24:0	2.85%	Widely used in research.
Unsaturated			
Oleic acid	C18:1	9.12%	Used in food industry
Linoleic acid	C18:2	11.01%	Making of soaps, emulsifiers, beauty products, anti-inflammatory agent
Linolenic acid	C18:3	31.94%	Main component in drying of oils

Table 2. Analysis of Bio-diesel properties.

Bio-diesel Properties	Values
Saturated Fatty Acids (SFA)	30.87
Monounsaturated Fatty Acids (MUFA)	9.12
Polyunsaturated Fatty Acids (PUFA)	42.95
Degree of Unsaturation (DU)	95.02
Long-Chain Saturated Factor (LCSF)	8.05
Saponification Value (SV)	175.89
Iodine Value (IV)	115.56
Cetane Number (CN)	51.33
Cold Filter Plugin point (CFPP) (˚C)	8.81
Cloud Point (CP) (˚C)	-1.67
Oxidation Stability (OS) (hours)	5.34
Higher Heating Value (HHV)	32.41
Kinematic Viscocity (μ) (mm^2/s)	0.96
Density (ρ) (kg/m^3)	730

Normally polyunsaturated fatty acids are present in more quantity in algae that decreases stability of fuel but on the other hand it have low melting point and better cold weather properties in comparison to other bio-oil (Talebi et. al. 2014). In present study the values of estimated saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, degree of unsaturation and long-chain saturated factor are 30.87, 9.12, 42.95, 95.02 and 8.05 respectively. The estimated saponification value of bio-diesel from algae is 175.89 which is very near to Jatropa i.e. 198.85. Similarly, Iodine value of our sample is nearly equivalent to soyabaen i.e. 115.56 and 120.52 respectively (Gopinath et. al. 2009). According to the European specification minimum cetane number of 51 is required to meet the specification but like other bio-diesel algae bio-diesel is also just in boundary line. Cold Filter Plugin point and Cloud Point is very similar to palm bio-diesel.

Oxidative stability not only depends on FAME content but also varies from age of bio-diesel, storage condition and so on. Therefore, present result could be difficult to justify. But generally, oxidative stability of algal bio-diesel differs from vegetable oil profiles which is slightly astonishing. Higher Heating Value (HHV) is lower in biomass due to high oxygen content. Our result follows the same trend and have even less HHV than coconut bio-diesel. The kinematic viscosity by ASTM D6751 and EN 14214 standard is within range of 1.9–6.0 mm2/s and obtained value is 0.96. Whereas 860–900 kg/m3 is for density but biodiesel from algae do not satisfy the range hence extra effort is required in these particular case (Hoekman et. al. 2012).

Conclusion

Concluding, the study was about to culture local freshwater algal species so that it can thrive in outdoor conditions easily. The observation revealed that *Chlorella sp., Anabaena sp., Euglena sp., Oocystis sp.* and *Sphaerocystis sp.* were dominant species to adapt the environment throughout the large scale culture experiment. The best feasible lipid extraction methodology was developed, and Folch's method from dried algal sample was finally selected by repeating the experiment thrice. FAME conversion states that the final product was the mixture of lower carbon chain compounds which are very suitable for biofuel production. Bio-diesel properties were very similar to other plant derived oils. However, algae biomass pre-treatment, crude oil refining, modification in lipid extraction or direct transesterification technologies could improve the quality of bio-diesel and will be economically more feasible.

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