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Ultra Structural and Analytical Studies of Biodiesel Producing Microalgae (*Chlorella vulgaris* and *Senedesmis* sp.) Collected from Tamil Nadu, India

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Abstract

Microalgae are an economical choice for biofuel production, because of its availability and low cost. Our studies prove that biodiesel can be produced from Freshwater (Temple tanks, Forest lagoons, Inland lakes, Rock ponds) microalgae (*Chlorella vulgaris, Senedesmis* sp). The Nile Red fluorescence method described in our present study provides a rapid, easily manipulated and reliable method for *in vivo* quantification of neutral lipids in various microalgal taxa, particularly those belonging to the *Chlorophyceae*, that were previously found difficult to stain with Nile Red method. The optimum drying period of microalgae biomass is determined to be 120 min. The percentage of lipid content during n-Hexane soaking period (*Chlorella vulgaris*) is 49% during 210min. Extraction of lipid fragments through solvent extraction method from biomass and the fractions were analysed for biodiesel under FT-IR and GC-MS.

Key Words: *Chlorella vulgaris*, *Senedesmis* sp. Biodiesel, Nile red staining, FT-IR, GC-MS.

Introduction

Energy is the next burning issue in the upcoming year when every country requires a huge amount to sustain their economical progress. As the world is rapidly motorized by engines it requires a huge amount of oil to pump it and the oil price is continuously increasing for the last few years. Biodiesel is produced currently from plant and animal oils, but not from microalgae. This is likely to change as several companies are attempting to commercialize microalgae biodiesel. Microalgal, which are one of the most abundant organisms in the world, got final attention because of its highest capacity to produce bio-fuel in per acre as compared to other power crops. These tiny organisms does not require big lands or farm, money, they can be grow in open ponds, plastic box, glass vessels or bioreactors. The oil extracted by these microalgal proven to be utilizes, to ignite the engines of road vesicles and jets. There are thousands of species belongs to microalgae out of 300 have exported to produce enough quantity of hydrocarbons contents for forming the fuel. Conventional methods of lipid determination have many complicated steps, i.e., extraction, purification, concentration, and determination, which are time - consuming. A spectrophotometric method using Sudan black B (Thakur, *et al.,* 1989) and flurescence spectrometric methods using Luminor 490PT (Pomoshchnikova, *et al.,* 1981) and Nile red (Cooksey, *et al.,* 1987; Lee, *et al.,* 1997; Col, *et al.,* 1990) were reported to determine the lipid content of yeasts, algae, and ciliates, all of which were dispersed cells without forming large aggregations.

Materials and Methods

Isolation of microalgae

In the present study, a survey was conducted to study the population and examined the Nile Red and Bodypy method with two microalgal genus (*Chlorella vulgaris, Senedesmis* sp). Various physical and chemical treatments were applied to the existing Nile Red method to improve the effectiveness and efficiency. Species and Genus of microalgae were obtained from the cultures isolated from various environmental conditions from Tamil Nadu like Temple tanks, Rock ponds, Forest lagoons and inland lakes.

Culture identification

The algae culture was identified through the manual, "Microalgae identification for Aquaculture" by Barry H. Rosen (1990).

Nile red staining

Nile red (9-(Diethylamino) -5H benzo [a] phenoxazin- 5-one) staining were carried out to detect intracellular lipid droplets (Greenspan, *et al.,* 1985). Microalgal cells (0.5 ml) were collected by centrifugation at 1,500 rpm (Rotation per minute) for 10 min and washed with physiological saline solution (0.5 ml) several times. After the collected cells were re-suspended in the same solution (0.5 ml), the Nile red solution (0.1 mg/ml in acetone) was added to cell suspensions (1:100 v/v) and incubated for 10 min. After washing once, stained microalgal cells were observed by fluorescent microscopy (Tadashi Matsunaga, *et al.,* 2009).

The expression process, the amount of lipids from the algal biomass is shown in (Fig 1). For example, at a drying period of 30min, the lipid cotent in the expressed liquid was determined to be 38%. For a drying period of 90 and 120min, the lipid content was 42% and 46% respectively. However, a drying period of 240min resulted in a lipid content of 32%. The reason for low lipid content at 30min was the presence of excess water in the expressed algal liquid. At drying periods of 90 and 120min, the lipid content in the expressed algal liquid

showed the maximum amount or value. Further increasing the drying period up to 240 min resulted in a lower yield in the lipid content, because the algae biomass was overdried. Therefore the optimum drying period was determined to be 120min. Hence, further experiments were performed by air – drying the sample for 120min.

Lipid Extration

Freeze-dried cells were weighted in 10mL screw-top tubes, to which a fresh solution of the transesterification reaction mix (methanol/hydrochloric acid, chloroform, 10:1:1 v/v/v) was added. Cells were suspended in this solution by vortex mixing and immediately placed at 90ºC for 60min for transesterification. Transesterification reaction tubes were removed from the heater and cooled to room temperature. One milliliter of water was then added to each tube and heater and cooled to room temperature. One milliliter of water was then added to each tube and the fatty acids methyl esters extracted (hexane/chloroform, 4:1 v/v and 3×2mL). Samples were diluted with chloroform containing a known concentration of 19:0 (nonadecanoic acid) as the internal injection standard and injected into the chromatograph. (Tom Lewis*, et al.,* 2000).

Gas chromatography and mass spectroscopic studies

The collected biodiesel from sample was processed with GC and MS (JEOL GC mate). Lipid fraction was re suspended in n-hexane and applied to silica gel column chromatography. Aliphatic hydrocarbon fraction passes through the column fatty acid & carotenoid fractions were trapped. Passing through fraction was defined as hydrocarbon fraction, lipid components in hydrocarbon fraction were identified by GC/MS. The sample (1µl) was evaporated in a split less injector at 300°C. The results were compared with the petro based diesel and gasoline oils (Tadashi Matsunaga, *et al.,* 2009).

The methyl esters of fatty acids were quantified by a gas chromatograph (Agilant-JEOL GC & MS). The column (HP5) was fused silica 50m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C the 3°C / min to 235°C for column temperature, 240°C for injector temperature, helium was the carrier gas. The weight percentages of fatty acids were approximated by the area of the detector response. The fatty acid methyl esters were identified by gas chromatography coupled with mass spectrometry.

Fourier Transform Infra-Red Spectrometry:

A Perkin Elmer model spectrum-I PC was used and FTIR spectra (Resolution: 4 cm^{-1}, Scan Number: 3) were performed after evaporation of the Lipid fraction on the Thalium bromide tablets.

Results & Disscussion

Microscopic Observations

Algae from Temple tanks, Rock ponds, Forest lagoons and inland lakes shows vegetative cells are in unicellular, spherical about 5-10 µm in size and surrounded by a cell wall which consists of two regions (Fig. 3. a, b), an outer thin layer and an inner bulky microfibrillar layer. No trilaminar outer component of sporopollenin (Atkinson, *et al.,* 1972) was observed at any stage of the cell cycle, the cells were identified as *Chlorella vulgaris* and *senedesmis* sp.. Whole cells and cell wall fragments showed an intense light blue fluorescence when stained with Calcofluor white stain and were positive. (Takashi Yamada, *et al.,* 1982).

Nile red staining

Intracellular lipid droplets of microalgae observed by Nile Red staining under fluorescence microscopy, Fish-Fluorescence *in-situ* hybridization, neutral lipids including hydrocarbons and triglycerides were stained in yellow, while polar lipids were stained in red (Fig. 4).

The air dried microalgal biomass, as stated in the drying process, was subjected to soaking in the n – Hexane for various time periods, viz. 30 – 300 min. The results showed that n – Hexane soaking followed by expression helped to increase the extraction of lipids from algal biomass as shown in (Fig. 2). The algal biomass *Chlorella vulgaris,* which was subjected to soaking in n –Hexane for a period of 30 min, was subjected to soaking in n – Hexane for a period of 30, 60, 120, 150, 180,210, 240, 270, 300 min, resulted in a lipid content of 40%, 42%, 48%, 48%, 48%, 49%, 48%, 48% respectively. In the case of an algal drying period of 240 min followed by soaking in n – Hexane for the above mentioned time period, resulted in a lipid content of 37%, 37%, 37%, 36%, 37%, 38%, 42%, 44% and 47% respectively. A gradual increase in lipid content of expressed liquid content of the expressed liquid was observed as the soaking period in n – Hexane was increased, which could be due to softening of the dried algal biomass by the solvent action.

Gas Chromatography & Mass Spectroscopy

The high content of saturated fatty acids algae harvested from microalgae *Chlorella vulgaris* were compared with *Senedesmis* sp to be noted. But the PUFAs are highly present in algae from *Senedesmis* sp. The biosynthesis of these molecules does not occur mainly during the exponential phase of growth where the carbon source gives rise to lipids essential for the development of the membranes and also ATP for built up all the molecules necessary for the construction of the cell. As in the case for the biosynthesis of secondary metabolites, the fatty acids appear after the end of the exponential phase of growth. The Retention time and molecular weight of Monoethylenic fatty acids and PUFAs of *Chlorella vulgaris* and *Senedesmis* sp. algae are compared (Table. 2).

By GC-MS, we were able to get different fractions such as saturated fatty acid, MUFAs and PUFAs, in these the Saturated fatty acid and PUFAs are particularly interesting and it was compared in both *Chlorella vulgaris* and *Senedesmis* sp. samples.

The GC & MS spectrum of *Chlorella vulgaris* and *Senedesmis* sp. sample shows the peak variations of fatty acids. High peak area were obtained in *Chlorella vulgaris* sample (Fig. 6 & 7) while compared to *Senedesmis* sp. sample (Fig. 8 & 9).

The percentage of different fatty acids present in microalgae were analysed with *Chlorella vulgaris* and *Senedesmis* sp. samples which contain Saturated fatty acids and PUFAs in various ratios (Table. 3). The algae from *Chlorella vulgaris* sample shows rich in saturated fatty acid (capric acid, lauric acid, myristic acid) and comparable amount of PUFA (hexadecatrienoic acid, stearidonic acid Eicosapenaenoic acid, Docosahexaenoic acid) to algae grown

in normal light. So the *Senedesmis.* sp. grown in is an excellent source for high yield of saturated fatty acids.

Table 1: Composition (%) of group of fatty acids from *Chlorella vulgaris* and *Senedesmis* sp.

Fatty acids	Double Bonds	*Chlorella vulgaris* (%)	*Senedesmis* sp. (%)
Saturates	0	38	55
Monoenes	1	21	15
Polyenes	≥2	40	29

Table 2: Molecular Weight and Retention time of Saturated fatty acid obtain from GC-MS.

Fatty acids	Retention Time		Molecular Weight (g/mol)
	Chlorella vulgaris	*Senedesmis* sp.	
Saturated fatty acids			
Propionic acid	3.23	3.03	74.08
Butyric acid	-	4.7	88.11
Caprylic acid	8.05	7.27	144.21
Capric acid	10.33	9.5	176.26
Lauric acid	12.36	12.68	200.31
Myristic acid	14.15	13.06	228.37
Poly Unsaturated Fatty Acids			
Hexadecatrienoic acid	15.74	15.94	264
Stearidonic acid	17.21	17.6	290
Eicosapentaenoic acid	19.77	19.16	316
Docosahexaenoic acid	21.99	22.61	342

Table 3: Relative composition of Saturates, and PUFAs from oils of *Chlorella vulgaris* and *Senedesmis* sp. samples.

Fatty Acids	Percentage (%)	
	Chlorella vulgaris	*Senedesmis* sp.
Saturated fatty acids		
Propionic acid	13.07	17.03
Butyric acid	-	28.88
Caprylic acid	05.53	10.37
Capric acid	16.82	27.99
Lauric acid	43.16	14.87
Myristic acid	21.83	0.82
PUFAs		
Hexadecatrienoic acid	17.02	18.57
Stearidonic acid	21.73	18.02
Eicosapentaenoic acid	25.54	17.89
Docosahexaenoic acid	35.80	45.49

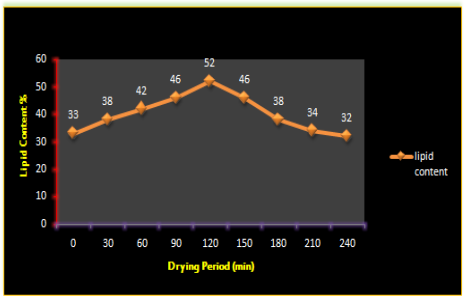


Fig 1. Percentage of Lipid content in Chlorella vulgaris during drying period

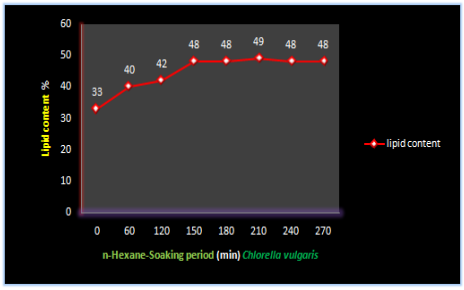


Fig 2. Percentage of Lipid content during n – Hexane soaking period (Chlorella vulgaris)

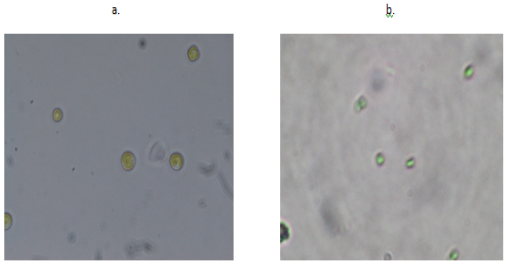


Fig 3. Light microscopic observation of algae samples (a) *Chlorella vulgaris* (b) *Senedesmis* sp.

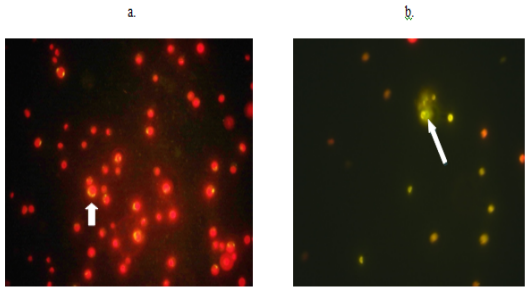


Fig 4. Fluroscence Microscopic images of *Chlorella vulgaris* stained by Nile Red. The lipid drops are yellow in colour (a and b).

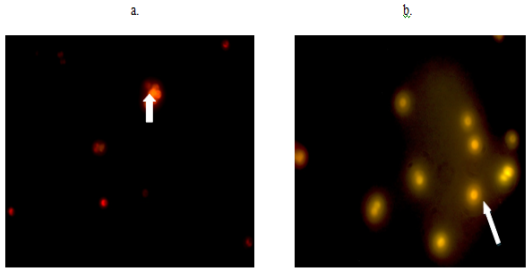


Fig 5. Fluroscence Microscopic images of *Senedesmis* sp. stained by Nile Red. The lipid drops are yellow in colour (a and b).
Fig 6. GC of *Chlorella vulgaris* Sample

Gas Chromatographic Spectrums

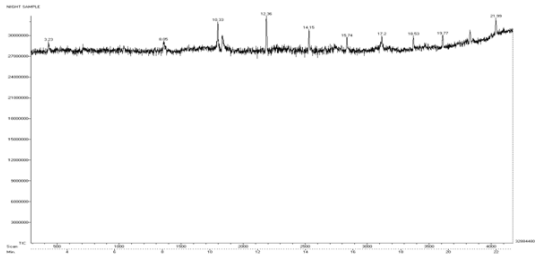


Fig 6. GC of *Chlorella vulgaris* Sample

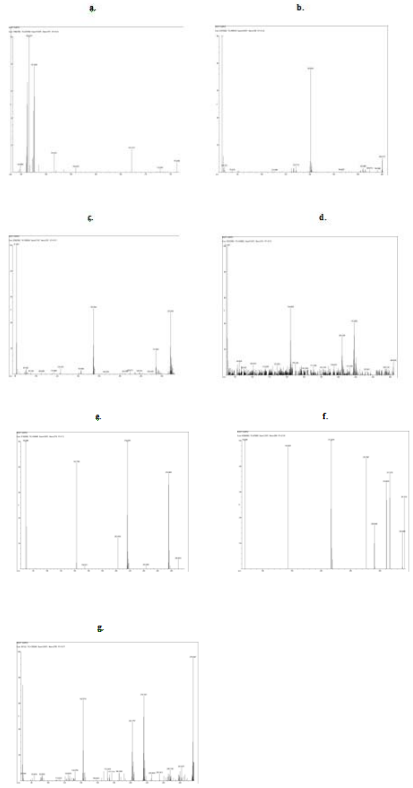
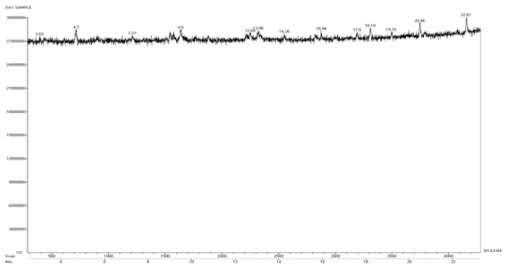


Fig 7. MS spectrum of Capric acid (a), Lauric acid (b), Myristic acid (c), Hexadecatrienoic acid (d), Stearidonic ancid (e), Eicosapentaenoic acid (f), Docosahexaenoic acid (g) from *Chlorella vulgaris* sample



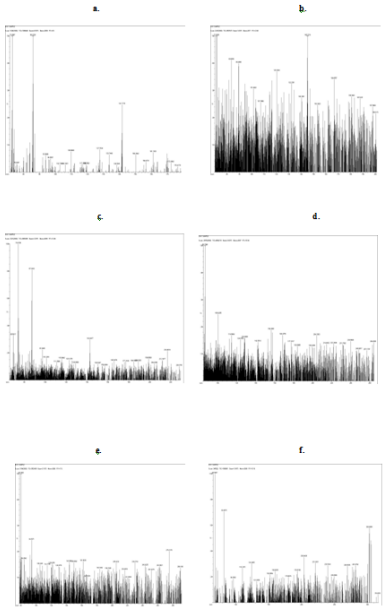


Fig. 9 MS spectrum of Caprica acid (a), Lauric acid (b), Myristic acid (c), Hexadecatrienoic acid (d), Stearidonic ancid (e), Eicosapentaenoic acid (f) from *Senedesmis* sp. sample

Infra Red Spectroscopic Spectrums:

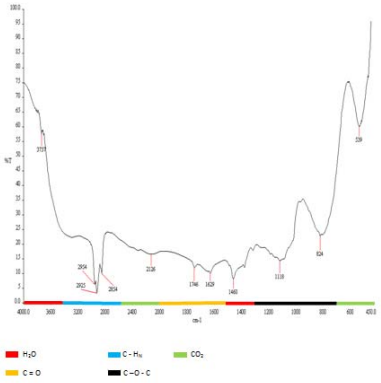


Fig 10. FT-IR of *Chlorella vulgaris* Sample

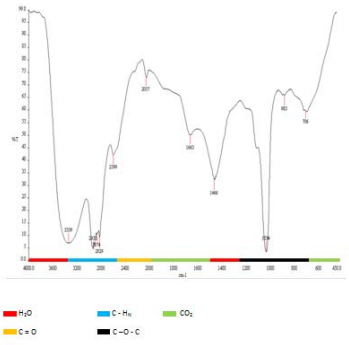


Fig 11. FTIR of *Senedesmis* sp. Sample

Fourier Transform Infra-Red Spectroscopy

Five maxima of the generation of volatile products can be found in the spectrum. All collected extractions give bands at 706 and 3339 cm^{-1} (Fig 10) so all are *cis* isomer, as expected from algae lipid because *trans* isomers have a strong nearby 970 cm^{-1} band and a weak nearby 3012 cm^{-1} while *cis* isomers gave medium nearby 720 and 3012 cm^{-1}.bands

An analysis of the IR spectrum showed (Fig. 10 & 11) the main composition stage, reveals the existence of the absorption bands characteristic of these five different bonds:

C═O: The main characteristic of the IR spectra of carbonylic compounds (aldehydes, acids, etc.) is the strong C═O stretching absorption band in the region of 1870–1540 cm^{-1}. In the case of esters, this band appears in the 1750–1735 cm^{-1}.

C—O—C: corresponding to ethers. These stretching vibrations produce a strong band in the 1200–900 cm^{-1} region.

C—H: absorption bands characteristic of the vibrations of C—H bonds, as an example, 2960 and 2875 cm^{-1} correspond to the asymmetric and symmetric vibrational modes of methyl groups, respectively, and 2929 and 2850 cm^{-1} correspond to the asymmetric and symmetric vibrational modes of methylene groups, respectively.

CO_2: they produce strong bands in between 2800-2000 cm^{-1} as well as in 700 cm^{-1} region.

H_2O: the adsorption bands of water can be observed in the range of 1800-1200cm^{-1}

As many algal species have been found to grow rapidly and produce substantial amounts of TAG or oil, and are thus referred to as oleaginous algae, it has long been postulated that algae could be employed as a cell factories to produce oils and other lipids for biofuel and other biomaterials (Benemann, *et al.,* 1982). The potential advantages of algae as feed stocks for biofuel and biomaterials include their ability to:

I. synthesize and accumulate large quantities of neutral lipids / oil (20 – 50% DCW),
II. grow at high rates (e.g.1–3 doublings per day),
III. thrive in saline / brackish water / coastal sea water for which there are few competing demands,
IV. tolerate marginal lands (e.g. desert, arid – and semi – arid lands) that are not suitable for conventional agriculture,
V. utilize growth nutrients such as nitrogen and phosphorus from a variety of waste water source providing the additional benefit of wastewater bio-remediation,
VI. reduce emissions of a major green house gas,
VII. produce value-added co-products or by-products (*e.g.* bio polymers, proteins, polysaccharides, pigments, animal feed, fertilizer and H2),
VIII. Grow in suitable culture vessels (photo-bioreactors) throughout the year with an annual biomass productivity, on an area basis, exceeding that of terrestrial plants by approximately ten fold.
IX. To create more employment opportunities in rural India.

The increase in total lipids in aging algal cells or cells maintained under various stress conditions consisted primarily of neutral lipids, mainly TAGs. This was due to the shift in lipid metabolism from membrane lipid synthesis to the storage of neutrallipids. *De novo* biosynthesis and conversion of certain existing membrane polar lipids into triacylglycerols may contribute to the overall increase in TAG. As a result, TAGs may account for as much as 80% of the total lipid content in the cell (Klyachko Gurvich, 1974).

As the increase in enzymatic activity can be blocked by the addition of protein synthesis inhibitors, it was suggested that the enhanced Acetyl CoA carboxylase (ACCase) activity could also be the result of an increase in the rate of enzyme synthesis (Roessler et al. 1994). The gene that encodes ACCase in *Cyclotella cryptica* has been isolated and cloned. The gene was shown to encode a polypeptide composed of 2089 amino acids, with a molecular mass of 230 kDa. The deduced amino acid sequence exhibited strong similarity to the sequences of animal and yeast ACCases in the biotin carboxylase and carboxyl transferase domains. Less sequence similarity was observed in the biotin carboxyl carrier protein domain, although the highly conserved Met-Lys-Met sequence of the biotin binding site was present. The N-terminus of the predicted ACCase sequence has characteristics of a signal sequence, indicating that the enzyme may be imported into chloroplasts via the endoplasmic reticulum.

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