



Fatty acids methyl ester analysis of potent micro algae *Scenedesmus dimorphus* (Turpin) Kützing and *Chlorococcum infusionum* (Schrank) Meneghini isolated from effluents of Neyveli thermal power station expansion 1

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ABSTRACT

The present study reports the production of fatty acids methyl esters by microalgae under the influence of light and dark condition. Microalgae are renewable resource containing rich lipids in their body and has the potential to refill the partial energy demands in an eco-friendly way. We isolated an indigenous green alga from Neyveli Thermal Power Station Expansion I (*Scenedesmus dimorphus* and *Chlorococcum infusionum*) as a potent source for biodiesel. To get a better yield of biodiesel, the growth of the microalgal isolate was optimized with the addition of nutrients and salts under light and dark conditions. The lipid fractions were extracted from the biomass through solvent extractions and the fractions were analyzed for biodiesel under GC-MS. The percentage of lipids synthesized from *Scenedesmus dimorphus* and *Chlorococcum infusionum* under light and dark conditions were analyzed and compared. The *Scenedesmus dimorphus* and *Chlorococcum infusionum* from dark samples shows rich in saturated fatty acids (Tridecanoate acids, Dodecanoate acids, Decanoate acids and Heptanoate acids), considerable amount of Poly Unsaturated Fatty Acids (PUFA) (Heptadecadienoate acids, Eicosadienoate acids, Octadecadienoate acids, Hexadecadienoate acids and Docosadienoate acids) and Mono Unsaturated Fatty Acids (MUFA) *Scenedesmus dimorphus* in light condition, *Chlorococcum infusionum* in dark condition (Tetradecenoate), when compared to algae was grown under light condition. Hence, the algae grown in dark condition is an excellent source for high yield of saturated fatty acids.

Key words: Biofuel, biodiesel, *Scenedesmus dimorphus*, *Chlorococcum infusionum*, Nile red staining, FT-IR, GC-MS.

INTRODUCTION:

Biodiesel has become more attractive recently because of its environmental benefits and the fact that it is made from renewable resources. The cost of biodiesel, however, is the main hurdle to commercialization of the product. The used cooking oil and algae are used as raw material, adaption of continuous transesterification process and recovery of high quality glycerol from biodiesel by-product (glycerol) are primary options to be considered to lower the cost of biodiesel (Fangrui *et al.*, 1999). The transesterification of natural triglycerides (eg:- oils and fats) is employed to obtain fatty acid methyl esters (FAME) which are key reagents in the chemical industry (Loupy *et al.*, 1993, Ahn *et al.*, 1982). The FAME are the raw materials for the production of long chain carboxylic acids, detergents, alternative fuels for diesel engines (Bio Diesel) and mono and triglycerides (Sonntag 1982). Lipid qualification and quantification can be carried out by several means including Nile Red fluorescence microscopy, Nile Red spectrofluorometry, Fourier transform infrared micro-spectroscopy (FTIR), Thin-layer chromatography (TLC) and Gas Chromatography (GC) with Mass Spectrometry (MS) (Medina *et al.*, 1998). The present study aims the production of fatty acids by microalgae

under the influence of light and dark condition. Microalgal samples were collected from various environmental conditions from Neyveli Thermal Power Station Expansion 1. The algal microspore accumulates larger volume of lipid droplets due to light stress that is a good source for biodiesel production. The algae present in the samples were identified and the amount of biodiesel produced in light and dark conditions were analyzed and compared by IR, GC-MS. In future, molecular level research will be carried out for revealing high algal oil production from these microalgae.

MATERIAL AND METHODS:

COLLECTION SITE

In the present study, sample collected from various environmental conditions from Neyveli Thermal Power plant expansion I like the 1.Intake water 2.Cooling Tower Water Outlet 3.Stagnant Water 4.Cooling Tower Outlet Water 5. Pump Outlet Water 6.Outfall Water. More than 24 algal strains were identified from 6 sites. The temperature, pH, specific conductance, and water depth were recorded at each collection site by using YSI Multi parameter. Most of the water samples had the temperatures ranged from 18°C to 45°C, pH ranged from 6.1 to 10.2.

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ISOLATION OF MICROALGAE

Compare with the other microalgae, collected from 6 different sites, *Scenedesmus dimorphus* & *Chlorococcum infusionum* were dominant in the present study. So, further work were carried out in these two dominant species. *Scenedesmus dimorphus* & *Chlorococcum infusionum* present from the algal mixtures of samples were isolated using BBM medium. Different types of dilutions viz., 1/10, 1/100 was prepared using the half strength BBM medium. Then 0.1 ml of each dilution spread on BBM agar (2%) medium and kept under the laboratory condition. Upside down, in order facilitate diffuse light to the wall less organisms. Discrete green colonies appeared, after 15 days were isolated and transferred to BBM medium and kept under laboratory conditions. Then, they were subcultured and maintained.

IDENTIFICATION

Scenedesmus dimorphus & *Chlorococcum infusionum* isolated successfully from the algal mixtures were identified based on their morphological Characteristics viz., cell shape, size, flagella length, presence or absence of vacuoles, presence or absence of granules and presence or absence of pyrenoid, lipid composition, they were identified.

GROWTH KINETICS

The growth rate of the experiments on growth of *Scenedesmus dimorphus* and *Chlorococcum infusionum* at optimum temperature 28°C, 0.06g urea and different light: dark illustration, like light condition 12:12 and dark condition 6:18. Determination of growth rate was done Spectrophotometer Hitachi U 2900. Optical wavelength of 680nm was determined for the cultures and the results range from 677nm to 684nm. The wavelength of 680nm was confirmed by literature (Lee 2011). Growth rate (K) and generation time (G) was calculated by the equations 1 and 2 (Qin 2005).

Equation 1: Calculation of growth (K) rate for microalgae using optical densities

$$K = \log (\text{ODf}-\log\text{ODi}) / T \times 3.322 \text{ ----- (1)}$$

ODf: final optical density, Odi: initial optical density, T: time in days

Equation 2: Calculation of generation time (G) in days

$$G = (0.301)/K \text{ ----- (2)}$$

SELECTION OF GROWTH MEDIA

Both *Scenedesmus dimorphus* and *Chlorococcum infusionum* were grown in 2 different media to establish the best media for growth of the cultures. BG11 medium is used as a general medium for freshwater cultures but is preferred for growth of microalgae with high nutrient requirements as the concentrations of nitrate and phosphate are exceptionally high. BBM is also a general culture medium but differs in that it contains high levels of trace

elements. Cultures were grown in 5L glass jars with a working volume of 2.5L under ambient conditions and exposure to 150µmol/m²s with a light: dark cycle of light condition 12:12 and dark condition 6:18 for a period of 21 days. Culture density measurements were performed daily. Growth rates were calculated using equations 1 and 2.

PREPARATION OF NILE RED SOLUTION

The 0.5g of Nile Red powder was suspended in 1ml of acetone, used as stock solution. From this 0.05ml was mixed with 50ml of glycerol mixture (75:25, Glycerol and water). This solution was directly used for staining the lipid bodies of algal cells (Elumalai *et al.*, 2011).

NILE RED STAINING

Nile red [9-(Diethyl amino) -5H benzo [a] phenoxazin- 5-one] staining was conducted to detect intracellular lipid droplets. 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 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 2009).

DIFFERENT EXTRACTION METHODS

In the present study, the different solvent extraction methods used, like Tom Lewis *et al.*, method (2000), Tony R. Larson & Ian A. Graham method (2001), Lepage & Roy method (1984), Folch, Lees & Sloane Stanley (1957) and Sharif Hossain & Salleh method (2008). GAS CHROMATOGRAPHY AND MASS SPECTROSCOPIC STUDIES (GC AND MS)

The collected biodiesel from sample was processed with GC and MS (JEOL GC mate). Lipid fraction was resuspended 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 or Fossil fuel diesel and gasoline oils.

The methyl esters of fatty acids were quantified by a gas chromatograph (Agilant-JEOL GC AND MS). The column (HP5) was fused silica 50m x 0.25 mm I.D. Analysis conditions were 20 minutes at 100°C the 3°/ 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

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identified by gas chromatography coupled with mass spectrometry (Tadashi 2009).

FOURIER TRANSFORM INFRA-RED SPECTROMETRY (FT-IR)

A Perkin Elmer model spectrum-I PC was used. FTIR spectra (Resolution: 4 cm-1, Scan Number: 3) were performed after evaporation of the Lipid fraction on the Thallium bromide tablets (Elumalai *et al.*, 2011).

RESULTS AND DISCUSSION:

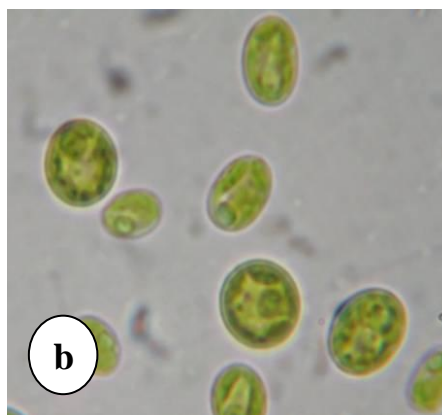
MICROSCOPIC OBSERVATION OF PURE STAIN

Scenedesmus dimorphus: Colonies 4 – 8 celled with the cells arranged in a linear or subalternating series (eight –

celled colonies always in subalternating series). Differ from *Scenedesmus obliquus* in the outer cells of the colony being more or less lunate and the apices of the cells being attenuated. The cells range is 2 – 8 μ broad, and 14 – 35 μ long (Fig. 1a).

Chlorococcum infusionum: Cells usually spherical, rarely ovoid or elongated and of variable dimensions, solitary or in flat irregular colonies. The Chloroplast is a hollow sphere with a notch on one side and with a single pyrenoid. The Cells range is 10-109 μ, rarely up to 135μ in diameter (Fig. 1b).

Fig. 1 The Light microscopic observation of algae samples, (a) *Scenedesmus dimorphus* (b) *Chlorococcum infusionum*.



GROWTH RATE AND GENERATION TIME OF SCENEDESMUS DIMORPHUS AND CHLOROCOCCUM INFUSIONUM

Dark Condition exhibited a consistently low culture density throughout the period of cultivation reaching a maximum OD of 2.651. Growth rate periods of

2 to 30 days, and first 8 days growth rate similar after 8 to 30 days. Light Condition having high growth rate, reached a maximum OD of 3.989. Generation times for growth in *Scenedesmus dimorphus* Light and Dark Condition were 70.32 hours and 107.5 hours respectively (Table 3).

Table 3: Growth rates and generation time of *Scenedesmus dimorphus* grown in Light and Dark Condition for a period of 30 days.

Condition	Initial OD (680nm)	Final OD (680nm)	Growth Rate (K)	Generation Time (G) (hrs)
Light (12:12)	0.120	3.989	0.428	70.32
Dark (6:18)	0.120	2.651	0.280	107.5

Dark Condition exhibited a consistently low culture density throughout the period of cultivation reaching a maximum OD of 1.990. Growth rate for the period of 2 to 30 days, first 8 days growth rate similar after 8 to 30 days and the light condition

having high growth rate, reached a maximum OD of 3.909. Generation times for growth in *Scenedesmus dimorphus* Light and Dark Condition were 71.83 hours and 145.62 hours respectively (Table 4).

Table 4: Growth rates and generation time of *Chlorococcum infusionum* grown in Light and Dark Condition for a period of 30 days.

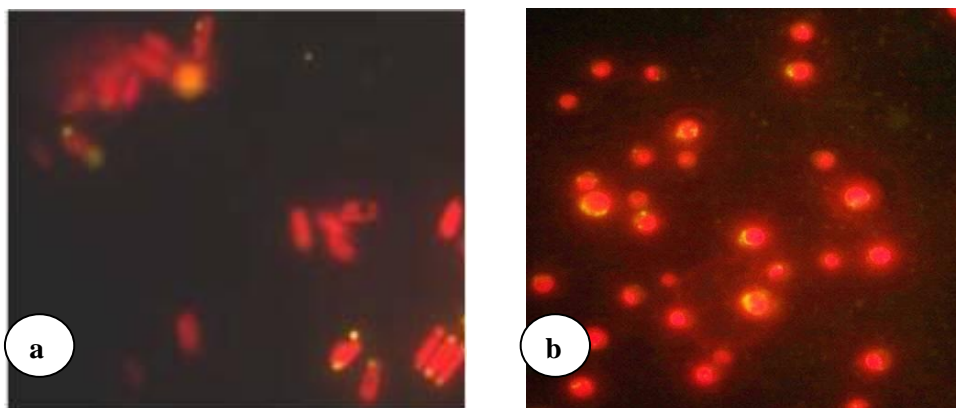
Condition	Initial OD (680nm)	Final OD (680nm)	Growth Rate (K)	Generation Time (G) (hrs)
Light (12:12)	0.123	3.909	0.419	71.83
Dark (6:18)	0.123	1.990	0.2067	145.62

NILE RED STAINING OBSERVATIONS

Intercellular lipid droplets of microalgae observed by Nile red staining under fluorescence

microscopy, neutral lipids including hydrocarbons and triglycerides were stained in red (Fig. 2 a & b).

Fig. 2. a – Nile red staining of *Scenedesmus dimorphus* b – Nile red staining of *Chlorococcum infusionum*



LIPID EXTRACTION FROM MICROALGAE

By comparing this five extraction methods Sharif Hossain & Salleh method is the best method. So, the main Sharif Hossain & Salleh method (Sharif 2008) adopted to the present study. Several important factors during the lipid extraction such as effect of solvent, drying temperature and incubation period was investigated to see which method give the best result and high yield in view of total lipid productivity.

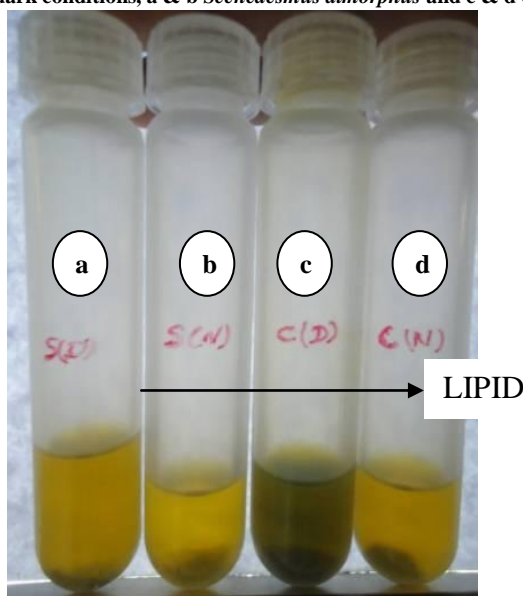
GAS CHROMATOGRAPHY AND MASS SPECTROSCOPY

By growing microalgae such as *Scenedesmus dimorphus* and *Chlorococcum infusionum* under light and dark condition in separately yields 0.5 g/l cells which can be collected as a paste through cooling centrifuge then eventually dried. After extraction the oil content growing under light shows yellowish liquid but the algae from dark condition give brownish colour (Fig. 3). In this study, petroleum ether solvent system was used for esterification of high lipids from microalgae biomass. Three types of fatty acids such as saturates, monoenes, polyenes found in extracts (Table 5).

Scenedesmus dimorphus under light condition in the percentage of Saturated, MUFAs and PUFAs fatty acids are 31.89, 17.50 and 50.59. *Scenedesmus dimorphus* under dark condition in the percentage of Saturated and PUFAs fatty acids are 51.27 and 48.72. *Chlorococcum infusionum* under light condition in the percentage of Saturated and PUFAs are 28.92 and 71.07. *Chlorococcum infusionum* under dark condition in the percentage of Saturated, MUFAs and PUFAs are 48.60, 27.05 and 24.33.

The high content of saturated fatty acids are presenting in both *Scenedesmus dimorphus* and *Chlorococcum infusionum* under dark condition. But the PUFAs are highly present in *Scenedesmus dimorphus* and *Chlorococcum infusionum* under light condition. But MUFA's totally different from light and dark condition. The presence of MUFA's was recorded in *Scenedesmus dimorphus* only at light condition where as *Chlorococcum infusionum* in dark condition. The biosynthesis of these molecules does not occur mainly during the exponential phase of growth.

Fig. 3. Shows the lipid layer of light and dark conditions, a & b *Scenedesmus dimorphus* and c & d *Chlorococcum infusionum*.



Where the carbon source gives rise to lipids that are essential for the development of the membranes 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 light and dark condition *Scenedesmus dimorphus* and *Chlorococcum infusionum* are compared (Table 5). By GC-MS, we were able to get different fractions such as saturated fatty acid, MUFAs and PUFAs are particularly interesting and it was compared in both light and dark grown algae. The GC and MS spectrum of light and dark sample shows the peak variations of fatty acids. High peak areas were obtained in dark condition sample (Fig. 5) and (Fig. 7) while compared to day time sample (Fig. 4) and (Fig. 6). The percentage of different fatty acids present in *Scenedesmus dimorphus* and *Chlorococcum infusionum*

was analyzed with light and dark grown samples which contain saturated fatty acids, MUFAs and PUFAs in various ratios (Table 6) and Molecular weight and retention time of saturated fatty acid (Table 7). The *Scenedesmus dimorphus* and *Chlorococcum infusionum* from dark sample shows rich in saturated fatty acids (Tridecanoate acid, Dodecanoate acid, Decanoate acid and Heptanoate acid), Considerable amount of PUFAs (Heptadecadienoate acid, Eicosadienoate acid, Octadecadienoate, Hexadecadienoate and Docosadienoate acid) when compare to *Scenedesmus dimorphus* and *Chlorococcum infusionum* grown under light condition and MUFAs (Tetradecenoate) were recorded in *Scenedesmus dimorphus* only at light condition where as *Chlorococcum infusionum* in dark condition. So the both *Scenedesmus dimorphus* and *Chlorococcum infusionum* grown in dark condition is an excellent source for high yield of saturated fatty acids.

Table 5. Composition (%) of group of fatty acids from *Scenedesmus dimorphus* and *Chlorococcum infusionum* under day and night conditions.

Fatty acids	Double bonds	<i>Scenedesmus dimorphus</i> under light (%)	<i>Scenedesmus dimorphus</i> under dark (%)	<i>Chlorococcum infusionum</i> under light (%)	<i>Chlorococcum infusionum</i> under dark (%)
Saturates	0	31.89	51.27	28.92	48.60
Monoenes	1	17.50	-	-	27.05
Polyenes	≥ 2	50.59	48.72	71.07	24.33

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Table 6. Relative composition of saturates, MUFAs and PUFAs from oils of *Scenedesmus dimorphus* and *Chlorococcum infusionum* day and Night.

Fatty acids	Percentage (%)			
	<i>Scenedesmus dimorphus</i> in day	<i>Scenedesmus dimorphus</i> in Night	<i>Chlorococcum infusionum</i> in day	<i>Chlorococcum infusionum</i> in Night
Saturated fatty acids				
Tridecanoate acids	-	16.68	-	-
Dodecanoate acids	-	20.33	28.77	-
Decanoate acids	18.6	15.7	-	24.61
Heptanoate	14.15	-	-	25.77
Mono Unsaturated Fatty Acids (MUFA)				
Tetradecenoate acids	17.98	-	-	28.05
Poly Unsaturated Fatty Acids (PUFA)				
Heptadecadienoate acids	34.94	17.02	24.99	-
Eicosadienoate acids	-	33.06	-	-
Octadecadienoate acids	17.02	-	-	-
Hexa decadienoate acids	-	-	24.36	25.23
Docosadienoate acids	-	-	21.33	-

Table 7. Molecular weight and retention time of saturated, MUFAs and PUFAs fatty acids obtain from GC-MS.

Fatty acids	Retention Time				Molecular weight (g/mol)
	<i>Scenedesmus dimorphus</i> in day	<i>Scenedesmus dimorphus</i> in Night	<i>Chlorococcum infusionum</i> in day	<i>Chlorococcum infusionum</i> in Night	
Saturated fatty acids					
Tridecanoate acids	-	21.25	-	-	222
Dodecanoate acids	-	21.56	21.02	-	206
Decanoate acids	19.24	22.91	-	18.37	186
Heptanoate acids	19.56	-	-	17.32	144
Mono Unsaturated Fatty Acids (MUFA)					
Tetradecenoate acids	18.23	-	-	18.64	240
Poly Unsaturated Fatty Acids (PUFA)					
Heptadecadienoate acids	17.45	19.93	19.86	-	280
Eicosadienoate acids	-	23.74	-	-	322
Octadecadienoate acids	16.91	-	-	-	294
Hexa decadienoate acids	-	-	18.64	19.96	266
Docosadienoate acids	-	-	17.31	-	350

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Fig. 4. GC of *Scenedesmus dimorphus* in dark sample

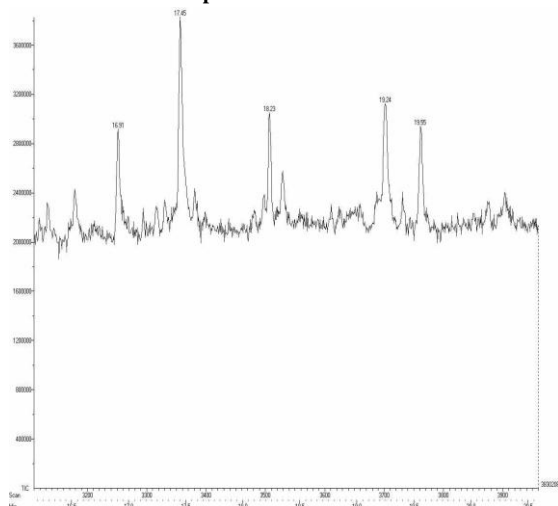


Fig. 6. GC of *Chlorococcum infusionum* in dark sample

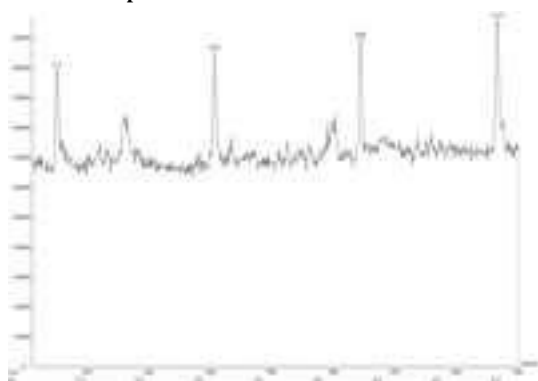


Fig. 5. GC of *Scenedesmus dimorphus* in light sample

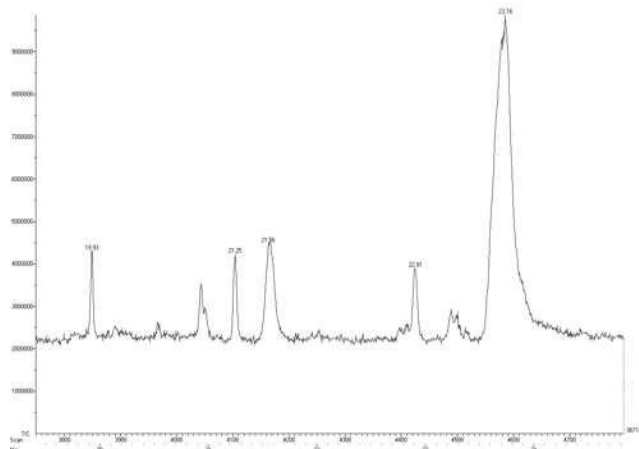
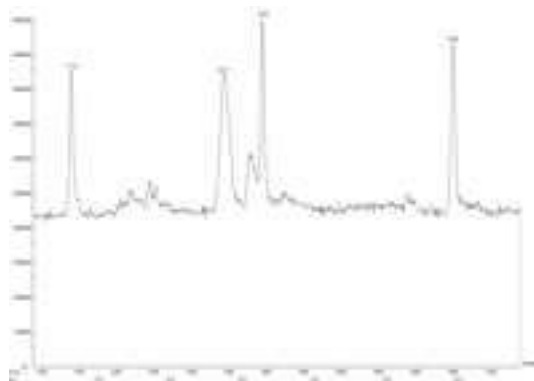


Fig. 7. GC of *Chlorococcum infusionum* in light sample



FT-IR 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. 8) so all are cis-isomer, as expected from alga lipid because trans isomers produce a strong band at 970 cm^{-1} and a weak band at 3012 cm^{-1} while cis-isomers gave medium nearby 720 and 3012 cm^{-1} bands. An analysis of the IR spectrum showed (Fig. 8, 9, 10 and 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.

- CO₂: they produce strong bands in between 2800–2000 cm^{-1} as well as in 700 cm^{-1} region.

Fig. 8. FTIR of night time *Scenedesmus Dimorphus* sample

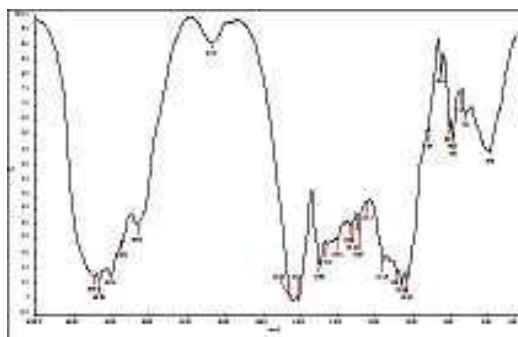


Fig. 9. FTIR of day time *Scenedesmus dimorphus* sample

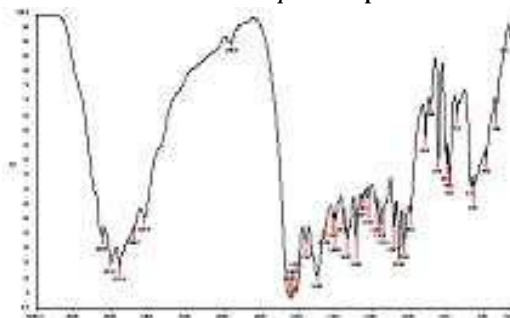


Fig. 10. FTIR of night time *Chlorococcum infusioenum* sample

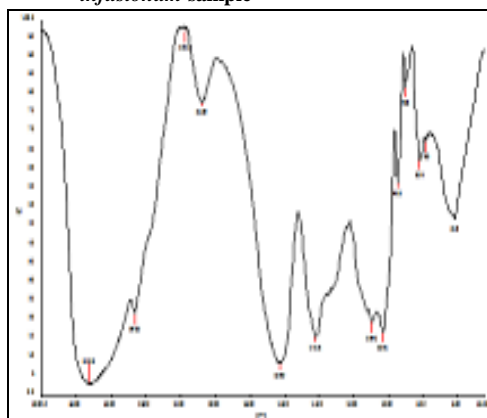
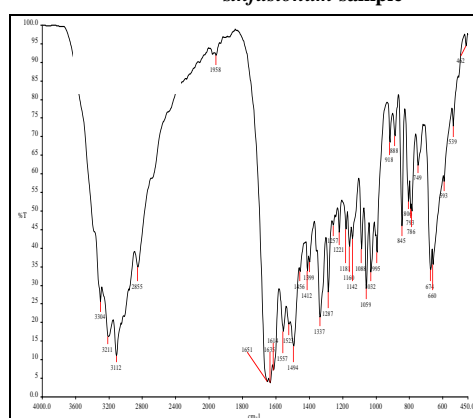


Fig. 11. FTIR of day time *Chlorococcum sinfusioenum* sample



• H₂O: the adsorption bands of water can be observed in the range of 1800-1200 cm⁻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 bio-fuel and other biomaterials (Benemann 1982). The potential advantages of algae as feed stocks for bio-fuel and biomaterials include their ability to:

- 1) Synthesize and accumulate large quantities of neutral lipids/oil (20–50% DCW).
- 2) Grow at high rates (e.g. 1–3 doublings/d).
- 3) Thrive in saline / brackish water / coastal sea water for which there are few competing demands.
- 4) Tolerate marginal lands (e.g. desert, arid – and semi – arid lands) that are not suitable for conventional agriculture.
- 5) Utilize growth nutrients such as nitrogen and phosphorus from a variety of waste water source providing the additional benefit of wastewater bio-remediation.
- 6) Reduce emissions of a major green house gas.

7) Produce value-added co-products or byproducts (e.g. bio polymers, proteins, polysaccharides, pigments, animal feed, fertilizer & H₂).

8) Grow in suitable culture vessels (photobioreactors) throughout the year with annual biomass productivity, on an area basis, exceeding that of terrestrial plants by approximately ten fold.

There is an increase in total lipids stationary phase of 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 neutral lipids. 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).

CONCLUSION:

The present work concluded that light stress conditions in the natural habitats of *S. dimorphus* and *C. infusioenum* did control the lipids in the two algae. Our results evidence that *S. dimorphus* and *C. infusioenum* is

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capable of regulating lipid metabolism depending on the light and dark conditions. *S. dimorphus* and *C. infusionum* is able to change the lipid content and the composition of fatty acids both in total lipid pool and individual lipids. In dark grown algae the concentration of saturated fatty acids increased while in *S. dimorphus* and *C. infusionum* grown at light this lipid was difference in lipid composition of *S. dimorphus* and *C. infusionum* grown under light and dark condition may be considered as one of the adaptive responses of the algal cells to the varying growth conditions. Therefore, the survival of algae under changed environmental conditions might be attributed to the functioning of the membranes. Since lipids are the structural components of the chloroplast membranes where photosynthetic apparatus is formed, and it is possible to assume that changes in the lipid content and their ratio are necessary for readjustment of the structure of chloroplast membranes of *S. dimorphus* and *C. infusionum* to provide the efficient lipid synthesis under the dark conditions.

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