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# GROWTH RATE, PIGMENT COMPOSITION AND FATTY ACID PROFILE OF *Chlorella pyrenoidosa*

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**ABSTRACT**

*Chlorella pyrenoidosa* isolated from the water bodies of Madurai was maintained in Chu10 medium with a photoperiod of 12 hours light/ 12 hours dark, light intensity of 2000 lux at a temperature of 25$^0$C. Growth rate, pH, dry weight and pigments such as chlorophyll a, carotenoids were monitored at an interval of 10 days until the 50$^{th}$ day of growth. The amount of chlorophyll a and carotenoids was maximum on 40$^{th}$ day of growth. Nile red fluorescence indicated more number of lipid bodies on 40$^{th}$ day. At 460-480nm excitation light, the nile red fluorescence of lipid vesicles exhibited an emission maximum of 465nm and a quantum yield of 0.65. Lipid content (48.8% dry weight) was maximum on 40$^{th}$ day of growth. HPTLC analysis of lipids showed the presence of glycolipid, digalactosyl diglyceride, phosphatidylcholine, phosphatidyl ethanolamine, monogalactosyl diglyceride. Fatty acid analysis by GC indicated more amount of saturated fatty acids in *C. pyrenoidosa*. Hydrocarbon analysis by GC-MS was done using the hexane extract of the algal strain. Linear regression analysis showed a significant relationship between OD and dry weight, OD and pigments, dry weight and lipid weight.

**Keywords**: Fatty acid, GC-MS, Hydrocarbon, Lipids, Nile red.

## INTRODUCTION

More recently, algae have received wide attention and popularity as they have the potential of high yield of oil that is used as a feedstock for synthesis of biodiesel. Algae thus appear to be a significant source for biofuels. Their photosynthetic efficiency is much higher than that of terrestrial plants. Some microalgae species have the potential to accumulate lipids at more than 50% of their biomass. Lipid obtained from microalgae can be categorized in three parts: crude lipids, neutral lipids and total lipids. Crude lipids include neutral lipids and pigments. Neutral lipids are comprised of triglycerides, free fatty acids, hydrocarbons, sterols, wax and sterol esters and free alcohols. Total lipids comprises of pigments, phospholipids, glycolipids, and the neutral lipids (Yogesh Sharma *et al*., 2011). Lipids can be recovered from algal biomass by means of physical or chemical processes or both (Jae-Yon *et al*., 2010). Many algal species have been found to grow rapidly and produce substantial amounts of triacylglycerol or oil, and are thus referred to as oleaginous algae (Sheehan *et al*., 1998). Biomass productivity, total lipid content and lipid productivity are among the main parameters that determine the economic feasibility of using microalgae as a source of biofuels (Wijffels *et al*., 2010). Oil content in microalgae can exceed 80% by weight of dry biomass (Meeting, 1996; Spolaore *et al*., 2006). The presence of lipids, hydrocarbons and other complex oils is dependent on the algal species (Banerjee *et al*., 2002). The analysis of the overall fatty acid profiles as well as the occurrence of fatty acids in different lipid classes in microalgae is an emerging field which is expected to reveal the identification of novel

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fatty acids with a variety of new functional groups (Berge and Barnathan, 2005). The aim of this work was to isolate microalgae species with high biomass, high lipid content, to determine the fatty acid profile in algal strain.

## MATERIALS AND METHODS

### *Microorganism and culture conditions*

*Chlorella pyrenoidosa* isolated from the water bodies of Madurai was maintained in Chu10 medium with a photoperiod of 12 hours light/ 12 hours dark, light intensity of 2000 lux at a temperature of 25$^0$C. Growth rate, pH, dry weight and pigments such as chlorophyll a, carotenoids were monitored at an interval of 10 days until the 50$^{th}$ day of growth (Subramanian, 1993). Pigments were separated by TLC using the solvent petroleum ether : acetone (7:3).

### *Nile red assay*

### *Nile red staining for lipid determination*

Nile red (9-diethylamino-5H- benzo [α] phenoxa phenoxazine-5-one) staining was carried out to detect intracellular lipid droplets. Nile red solution (0.1mg/ml in acetone) was added to cell suspensions and incubated for 10min. Nile red stained cells were observed in a Fluorescence microscope (Lee *et al*., 1998). Excitation and emission fluorescence spectra were determined with a Fluoromax 4 spectrophotometer with a 450 watt xenon lamp. Stained cell fluorescence was measured after 30 minutes using the excitation wavelength as 460nm.

### *Extraction of crude lipids*

Crude lipids were extracted from the *C.pyrenoidosa* at different days interval (Bligh and Dyer, 1959). The content of crude lipids were determined gravimetrically after oven drying (80$^0$C) the extract for 20min. TLC screening for lipids was carried out by spotting a concentrated lipid extract on silica gel plates. TLC plates were developed in hexane: diethyl ether: acetic acid (18:2:1) mobile phase and air dried. The same solvent system was used for HPTLC analysis. The iodine vapour was used to visualize the separated lipid compounds. The presence of lipid compounds was detected by brown spots against a white background. Crude lipid extracts were further purified by column chromatography.

### *Fatty acid analysis by GC*

GC analysis was performed at different days intervals on Gas Chromatograph 2010 Plus (Shimadzu, Japan) using Flame Ionization Detector (FID). Injector and Detector temperature was set at 225$^0$C and 250$^0$C respectively. One microlitre was injected in split mode (35:1) at a flow rate of 184.9 ml/min with Nitrogen as the carrier gas onto a FAMEs-RTX-2330 column (length 105.0m, Film thickness 0.20 µm, total run time 40min). Peak areas were integrated using the GC solution software. The fatty acid methyl esters were identified by internal standards (Supleco, 37 FAMEs).

### *Hydrocarbon analysis by GC and GC-MS*

100ml of algal culture was taken and centrifuged at 10,000 rpm for 20 minutes. The pellet was taken and to it 5ml of hexane was added and mixed. The sample was centrifuged at 10,000 rpm for 15 minutes and the supernatant was recovered (Dayananda *et al*., 2005). The hydrocarbon samples were analyzed by GC and GC-MS. GC-MS analysis was performed using GC Clarus 500 Perkin Elmer mass detector with Elite-5MS column (30×0.25mm×0.25µm film thickness). Nitrogen was used as the carrier gas at a flow rate of 1ml/min. The injection port was maintained at 250$^0$C. Oven temperature programming was done from 110 to 280$^0$C at 100C/mins and it was kept at 280$^0$C for 5mins. Mass scanning range was from 45-450(m/z). Conditions maintained in the GC were 250$^0$C for injector port, 280$^0$C for detector temperature and 100$^0$C for capillary column.

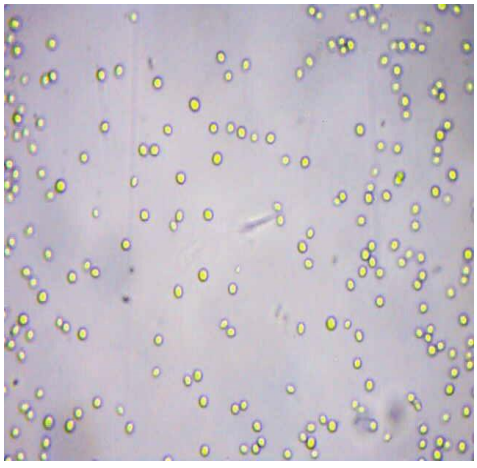
### *Statistical analysis*

All analysis was performed in triplicates. Data are presented as mean values ± S.D. Regression analysis was done using SPSS 11.5 software. Differences were considered significant at $p<0.05$.

## RESULTS

Light microscopic image of *Chlorella pyrenoidosa* is shown in Figure1. The average specific growth rate of *C.pyrenoidosa* was 0.72 at 665nm. Biomass productivity (0.009g dwt mL$^{-1}$) of this strain was maximum on 40$^{th}$ day. Pigments such as chlorophyll a, carotenoids content were monitored at 10 days interval for 50 days. The amount of chlorophyll a (2.1mg/ml) and carotenoids (3.5mg/ml) was maximum on 40$^{th}$ day of growth. TLC separation of pigments produced two fractions with an absorption maxima of 458nm and 660nm.

**Figure 1. Light microscopic observation of *C.pyrenoidosa***



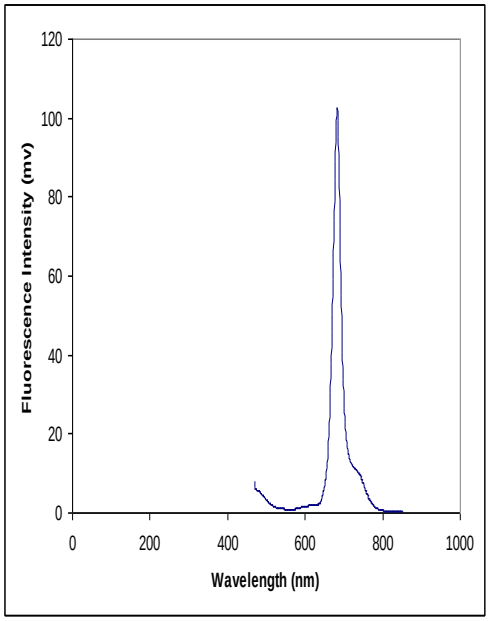
***Nile red assay***

Intracellular lipid droplets of microalgae were observed by nile red staining in a fluorescence microscope. Both yellow and red fluorescence cells were observed. *C. pyrenoidosa* showed a large number of lipids inside the cell and within the gelatinous matrix, as indicated by the yellow colour under blue light (Figure 2).

**Figure 2. Nile red stain of *C.pyrenoidosa*: chlorophyll emits red fluorescnec, lipid granules appeared as yellow**

The emission and excitation maxima of nile red in lipid suspensions depend upon the concentration of the dye. *C. pyrenoidosa* showed better fluorescence on 40$^{th}$ day and quantum yield was 0.65 (Figure 3).

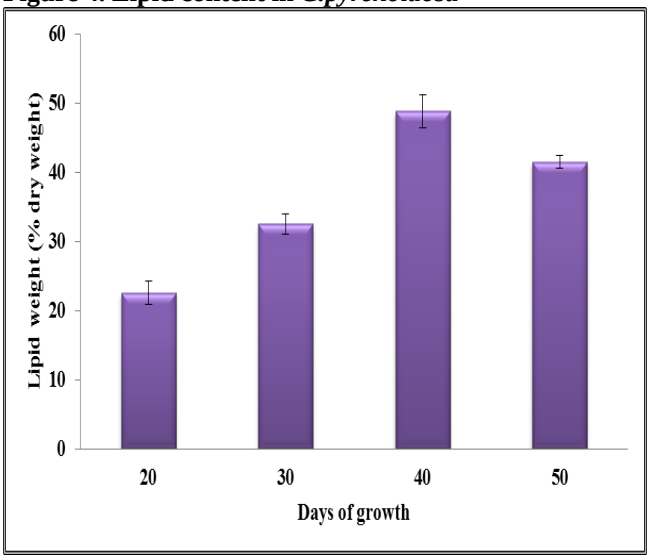
**Figure 3. Emission spectrum of *C.pyrenoidosa* with Nile red**




**Extraction and analysis of lipids**

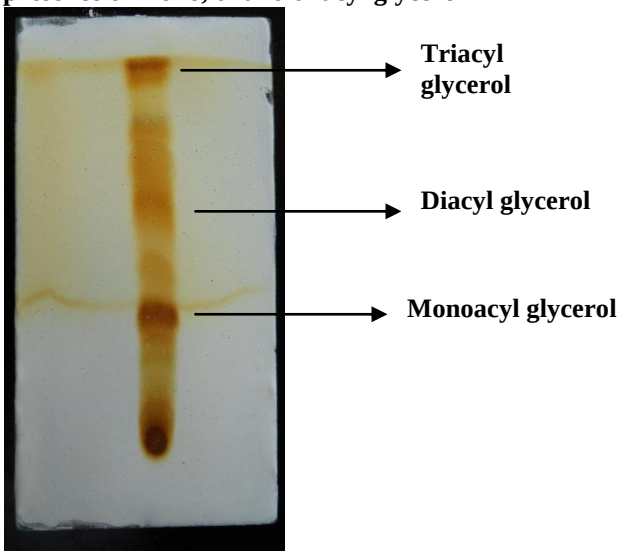
Lipid content was maximum (48.8% dry weight) on 40$^{th}$ day (Figure 4).

**Figure 4. Lipid content in *C.pyrenoidosa***




The lipids were fractionated by one-dimensional TLC on silica gel using the solvent system hexane/ diethyl ether/ acetic acid (70:30:1). Black spots were observed after continuous exposure to iodine vapour (Figure 5).

**Figure 5. TLC separation of lipids indicated the presence of mono, di and triacyl glycerol**




HPTLC analysis of lipids showed the presence of glycolipid, digalactosyl diglyceride, phosphatidylcholine, phosphatidyl ethanolamine, monogalactosyl diglyceride (Figure 6). The extracted lipids were further analyzed by column chromatography. The fractions were eluted initially in chloroform and then in acetone: methanol (9:1)

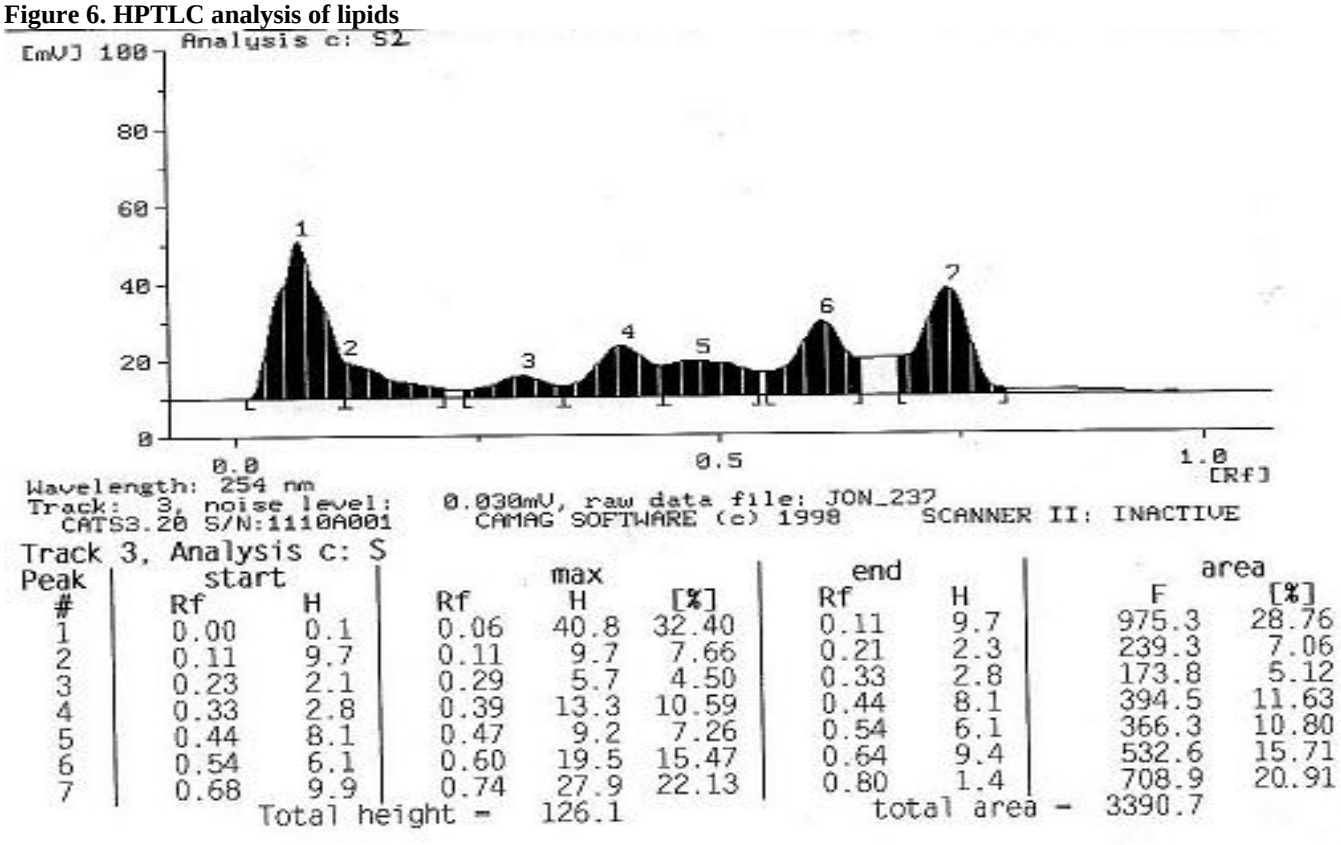
and finally eluted using methanol. The eluted fractions were subjected to GC analysis. *C. pyrenoidosa* was found to contain more amount of neutral and glyco lipids and a fewer amount of phospholipids.

***Fatty acid analysis by GC***

The fatty acid content of the algal biomass was compared with harvesting time. Based on the chromatogram, the composition of fatty acid samples was evaluated by comparing the retention time of each peak and its area with the standard. Fatty acid distribution from 20$^{th}$ to 50$^{th}$ days is represented in Figure 7.

The predominant fatty acids in *C. pyrenoidosa* were butyric acid (C4:0), caprylic acid (C8:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0) and lignoceric acid (C24:0). The percentage of saturated fatty acids increased with days of growth. The total saturated fatty acid content ranged from 49 to 73%.

**Figure 6. HPTLC analysis of lipids**




| Peak # | start Rf | start H | max Rf | max H | max [%] | end Rf | end H | area F | area [%] |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.00 | 0.1 | 0.06 | 40.8 | 32.40 | 0.11 | 9.7 | 975.3 | 28.76 |
| 2 | 0.11 | 9.7 | 0.11 | 9.7 | 7.66 | 0.21 | 2.3 | 239.3 | 7.06 |
| 3 | 0.23 | 2.1 | 0.29 | 5.7 | 4.50 | 0.33 | 2.8 | 173.8 | 5.12 |
| 4 | 0.33 | 2.8 | 0.39 | 13.3 | 10.59 | 0.44 | 8.1 | 394.5 | 11.63 |
| 5 | 0.44 | 8.1 | 0.47 | 9.2 | 7.26 | 0.54 | 6.1 | 366.3 | 10.80 |
| 6 | 0.54 | 6.1 | 0.60 | 19.5 | 15.47 | 0.64 | 9.4 | 532.6 | 15.71 |
| 7 | 0.68 | 9.9 | 0.74 | 27.9 | 22.13 | 0.80 | 1.4 | 708.9 | 20.91 |
| | | Total height = | | 126.1 | | | total area = | 3390.7 | |

**Figure 7. Fatty acid analysis by GC**

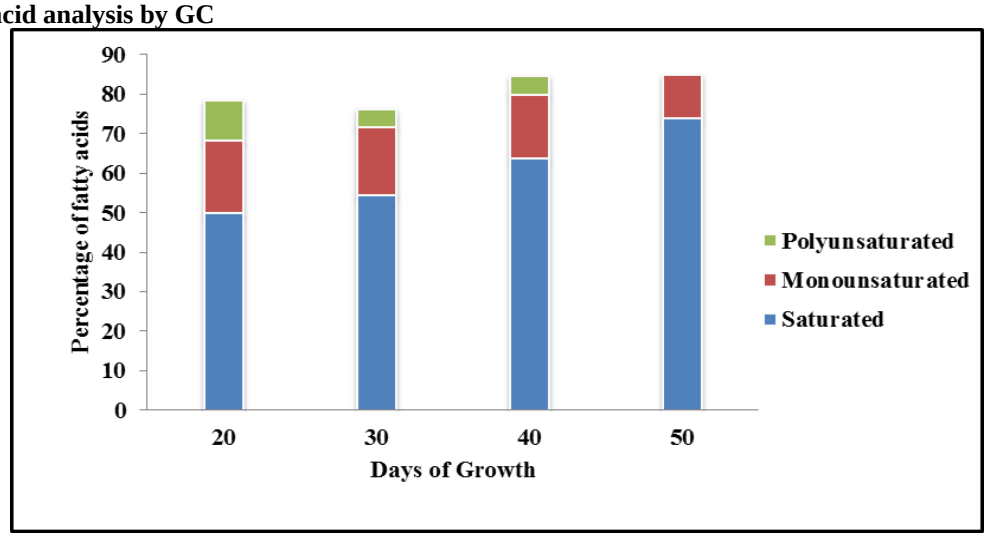


**Figure 8. Hydrocarbon analysis by GC-MS**

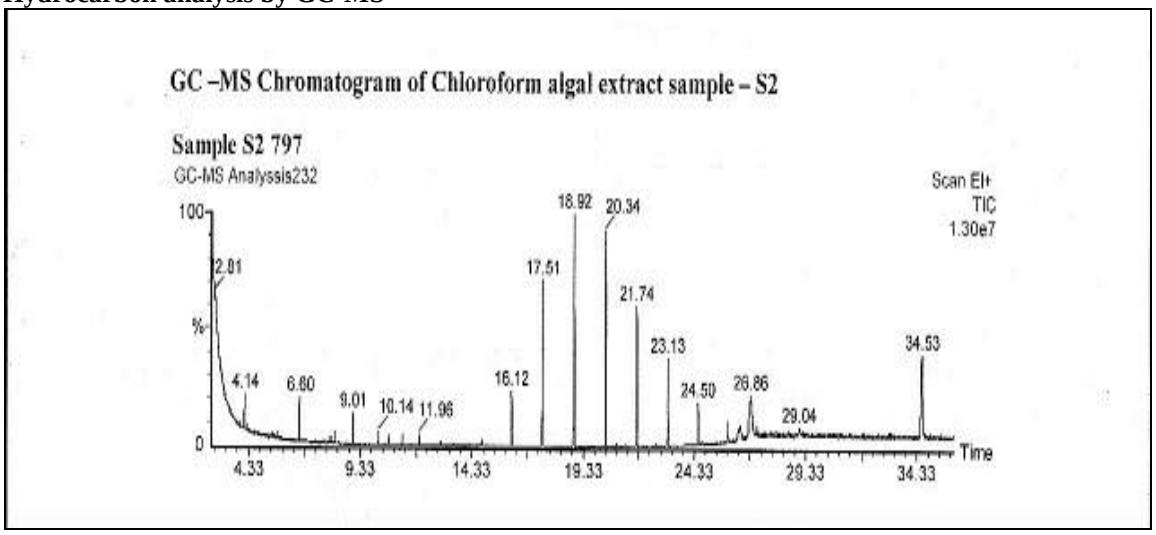



**Table 1. Identified hydrocarbons by GC-MS**

| RETENTION TIME | IDENTIFIED HYDROCARBONS | MOLECULAR FORMULA | MOLECULR WEIGHT | PEAK AREA % |
|---|---|---|---|---|
| 4.14 | Heptane, 2,2,3,3,5,6,6- heptamethyl | $C_{14}H_{30}$ | 198 | 2.68 |
| 9.01 | 1-Iodo Tetradecane | $C_{14}H_{29}I$ | 324 | 1.34 |
| 10.14 | 1-Iodo Nonane | $C_9H_{19}I$ | 254 | 0.45 |
| 10.60 | 1-Iodoundecane | $C_{11}H_{23}I$ | 282 | 0.45 |
| 11.22 | 5H-Tetrazol-5-amine | $CH_3N_5$ | 85 | 0.45 |
| 16.12 | Hexadecane | $C_{16}H_{34}$ | 226 | 3.57 |
| 17.51 | 2-Bromo dodecane | $C_{12}H_{25}Br$ | 248 | 12.05 |
| 18.92 | Heptacosane | $C_{27}H_{56}$ | 380 | 18.75 |
| 20.34 | 2-Ethyl-2-methyl Tridecanol, | $C_{16}H_{34}O$ | 242 | 17.86 |
| 21.74 | 3,8-dimethyl Undecane, | $C_{13}H_{28}$ | 184 | 12.05 |
| 23.13 | 1-Iodoundecane | $C_{11}H_{23}I$ | 282 | 7.14 |
| 24.50 | 1-Iodoundecane | $C_{11}H_{23}I$ | 282 | 3.57 |

***Hydrocarbon analysis by GC-MS***

The early stationary phase culture of *C. pyrenoidosa* was harvested and the hydrocarbons were extracted from the dry algal biomass with hexane. The GC-MS study of major peaks revealed the presence of straight chain and branched hydrocarbons ranging from C7-C27 (Figure 8). Hydrocarbons identified by GC-MS are summarized in Table 1.

**DISCUSSION**

Algal growth is directly affected by the availability of nutrients, temperature, light and pH (Xun Yang *et al*., 2012). Generation time was high in algal culture and proliferous rate was low (Huang *et al*., 2010). The duration of exponential phase in cultures depends upon the size of the inoculum, the growth rate and the capacity of the medium and culturing conditions to support algal growth (Fogg and Thake, 1987). Chlorophyll content decides the photosynthetic rate of a particular strain. Changes in chlorophyll level are probably controlled as algal density and climatic factors (light & temperature). Photosynthetic efficiency of microalgae is directly related to its growth and hence biofuel production.

Lipids including hydrocarbons and triglycerides were stained in yellow, while chlorophyll was stained in red (Guan Hua Huanga *et al*., 2009). Nile red permeates the algal cell walls and hence stained lipid droplets emit yellow fluorescence. There is linear correlation between nile red fluorescence and the total lipid content of microalgae, where an increase of lipids in aging algal cells was due primarily to neutral lipids rather than glyco or phospholipids (Cooksey *et al*., 1987). Nile red staining is a high throughput, rapid screening technique to determine the neutral lipid content of many algal strains (Lee *et al*., 1998). At 460-480nm excitation light, the nile red fluorescence of these vesicles exhibit an emission maximum of 465nm. The emission maxima at the lower concentration of the dye tend to be of a shorter wavelength than observed in the presence of higher concentrations. At low concentrations of nile red, both the core hydrophobic

and coat amphipathic components could be interacting with nile red, giving an emission maximum of approximately 570nm. With increasing concentrations of nile red, the hydrophobic core may become saturated and the excess nile red could then only interact with the coat phospholipids or hydrophobic proteins (Greenspan *et al*., 1985).

Many algal species exhibit rapid growth and high productivity and several microalgal species can be induced to accumulate substantial quantities of lipid, often greater than 60% of their dry biomass (Richmond, 2004). An increase in total lipid content in the stationary phase usually predominates due to an accumulation of neutral lipids in the form of triglycerides rather than polar lipids located in the cell membrane (Miao *et al*., 2004). *C.pyrenoidosa* contains short, medium and long chain fatty acids. Some microalgae present a larger fatty acid spectrum, when compared to oleaginous plants, containing a molecular structure with even more than 18 carbons (Belarbi *et al*., 2000). In a previous study (Lee *et al*., 2010), the most commonly synthesized fatty acids have chain lengths that range from C16 to C18, similar to those of higher plants, and palmitic, stearic, oleic and linolenic acids were recognized as the most common fatty acids contained in biodiesel. The function of fatty acids in algae is related to cell membrane, energy storage and metabolic processes (Orhan *et al*., 2003). The percentage of unsaturated fatty acids decreased with culture time. The dominant hydrocarbon is C27 and C16 in *C.pyrenoidosa*. Short chain hydrocarbons (<C20) were mostly present in this strain than compared to long chain hydrocarbons (>C19) (Matsumoto *et al*., 1996). GC-MS analysis showed that the hydrocarbons such as heptacosane and 2-ethyl-2methyl tridecanol were the major components among the hydrocarbons produced by *C. pyrenoidosa*.

## CONCLUSION

Nile red is a promising fluorescence dye for rapid quantification of cellular lipids in microalgae. TLC screening method indicated the presence of lipid compounds. Greater proportion of saturated fatty acids and the presence of monounsaturated fatty acids in *Chlorella pyrenoidosa* indicated its potential for production of oil. The results of this study indicate that the naturally isolated microalga *C.pyrenoidosa* is valuable for use in biofuel production.

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