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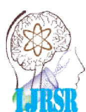

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Research Article

GROWTH RATE, PIGMENT COMPOSITION AND FATTY ACID PROFILE OF *Micractinium* sp and *Chlamydomonas* sp

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ABSTRACT

Microalgae have emerged as one of the most promising feedstock for biodiesel production. *Micractinium* sp and *Chlamydomonas* sp were isolated from the water bodies of Madurai. These microalgae were maintained in Modified BG11 medium with a photoperiod of 12 hours light /12 hours dark, light intensity of 2000lux at a temperature of 25°C. Growth rate, proliferation rate, dry weight and pigments such as chlorophyll *a*, carotenoids were monitored at an interval of 5 days until the 30th day of growth. The amount of chlorophyll *a* and carotenoids were maximum on 30th day of growth. Nile red indicated more number of lipid bodies during the declining phase of growth. Lipid, dry weight content was maximum on 30th day of growth. TLC separation of lipids revealed the presence of monoacyl glycerols, diacyl glycerols and triacyl glycerols. The fatty acids profile of *Chlamydomonas* sp and *Micractinium* sp were quantified by gas chromatography and found to produce significantly high level of saturated fatty acids.

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INTRODUCTION

Biofuel in the form of biodiesel and bioethanol has been produced from various edible and non-edible sources, viz., animal fat, waste frying oil, soybean, corn, palm, rapeseed, jatropha and sugarcane, but their production is very less to satisfy the energy demand moreover, their large-scale production requires huge amount of arable land, which directly competes with the production of food crops [1]. Algal biofuels, so called third generation biofuels, appear to be promising in delivering sustainable and complementary energy platforms essential to formulate a major component of the renewable and sustainable energy mix for the future. Algal biomass can be converted into various portfolios of biofuel products, such as bio-hydrogen, biodiesel, bioethanol and biogas [2]. Microalgae are eukaryotic photosynthetic microorganisms that are masters at using sunlight, CO_2 and water to produce biomass [3]. Microalgae are considered as a promising feedstock for biodiesel production due to their ability to grow rapidly and to accumulate a high quantity of lipid (20–50 % of dry weight) especially triacylglycerol (TAG) and many valuable by products, which are excellent feeds or fertilizers[4] [5]. Unlike other higher plants, microalgae have high growth rates, they require very less land area and do not compete with other food crops. In addition, they can be grown in wastewater and can mitigate the problem of water pollution [6] [7]. Most of the biodiesel produced from microalgal lipid and fatty acid methyl ester (FAME) were found to be in accordance with the biodiesel standards [8] [9]. Commercialization of biodiesel has led to the formulation of specific biodiesel standards and guidelines with the properties of biodiesel directly determined by the fatty acid composition of the microalgal feedstock. The two most important factors are i) the length of the carbon chain with the long chain C16- C18 TAGs being the best substrate for biodiesel production and ii) the number of double bonds which affect other biodiesel properties such as cetane number (ignition quality), cold-flow properties, viscosity and oxidative stability. To meet the specific technical biodiesel standards and to achieve the best balance between these properties, the most desirable TAGs are those containing the monounsaturated fatty acids (MUFAs) C18:1 (oleic acid) and C16:1 (palmitoleic acid). High concentrations of saturated fatty acids (SFAs) negatively affect the cold-flow properties and the polyunsaturated fatty acid (PUFA) C18:3 (α-linolenic acid) content is required to be below 12% as higher concentrations result in poor oxidative stability. Thus the fatty acid composition is important in determining the quality of the microalgal feedstock for biodiesel production [10]. The main goal to this study was to determine the dry wt, lipid content and fatty acid profile of *Micractinium* sp and *Chlamydomonas* sp.

MATERIALS AND METHOD

Algal growth condition

Chlamydomonas sp *and Micractinium* sp isolated from the water bodies of Madurai was maintained in modified BG11

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medium with a photoperiod of 12 hours light / 12 hours dark, light intensity of 2000 lux at a temperature of 25°C. Growth rate, protein content, carbohydrate content, dry weight and pigments such as chlorophyll a, chlorophyll b and carotenoids were monitored at an interval of 10 days until the 30th day of growth [11]. 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 10 minutes. Nile red stained cells were observed in a Fluorescence microscope [12]. Excitation and emission fluorescence spectra were determined with a Fluoromax 4 spectrophotometer with a 450 watt xenon lamp.The fluorescence of cells were measured after 30 minutes using the excitation wavelength at 460 nm.

Extraction of crude lipid

Crude lipids were extracted from the *Micractinium* sp and *Chlamydomonas* sp at different days interval [13]. The content of crude lipids were determined gravimetrically after oven drying (80°C) the extract for 20 minutes. 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 plates were developed by exposing the vapors of iodine crystals to stain the plates for visualizing the lipids. 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°C and 250°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).

Statistical analysis

The data are calculated and expressed as Mean±SD. Regression analysis was done using Microsoft Excel 2007.

RESULTS

Light microscopic image of *Micractinium* sp and *Chlamydomonas* sp is shown in Figure 1. The prolifertaion rate and generation time of *Micractinium* sp and *Chlamydomonas* sp was (0.195) and (0.274) respectively at 660nm. Biomass poductivity of *Micractinium* sp was (0.653 mg/ml) and *Chlamydomonas* sp (0.645 mg/ml) was maximum on the 30th day of growth. Pigments such as chlorophyll *a*, chlorophyll *b* and carotenoids, were monitored at 10 days interval for 30 days. The amount of chlorophyll *a* in *Micractinium* sp and *Chlamydomonas* sp was 2.5mg/ml and 3.2mg/ml respectively. Chlorophyll *b* content was 0.82mg/ml and 0.53mg/ml and carotenoids was 0.06mg/ml and 0.47 mg/ml and it was maximum on the 30th day of growth.

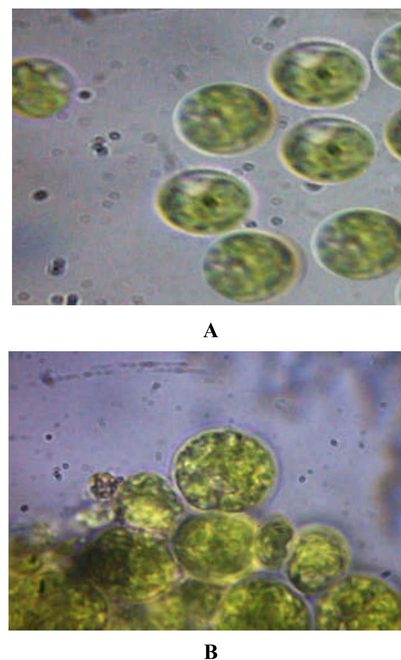


Figure 1 Light microscopic observation of (A) *Micractinium* sp and (B) *Chlamydomonas* sp

Nile red assay

After staining the cells with Nile Red 1. Intracellular lipid droplets of microalgae were observed in a fluorescence microscope. 2. Both yellow and red fluorescence cells were observed. Yellow fluorescence indicated the lipid droplets and red fluoresence was due to autofluorescence of pigment. *Chlamydomonas* sp and *Micractinium* sp 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).

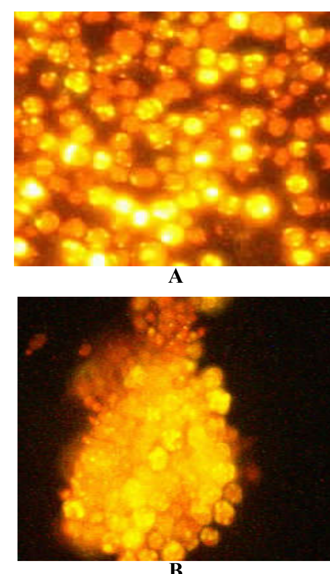


Figure 2 Nile red stain of (a) *Micractinium* sp and (b) *Chlamydomonas* sp . Lipid droplets appeared as yellow.

The emission and excitation maxima of Nile Red in lipid suspensions depend upon the concentration of the

dye. *Micractinium* sp and *Chlamydomonas* sp showed better fluorescence on the 30th day and quantum yield of *Micractinium* sp was 0.221 and *Chlamydomonas* sp was 0.258.

Extraction and analysis of lipids

Lipid content was maximum in *Micractinium* sp (23.0 % dry weight) and *Chlamydomonas* sp (30.4 % dry wt) on the 30th day of growth (Figure 3).

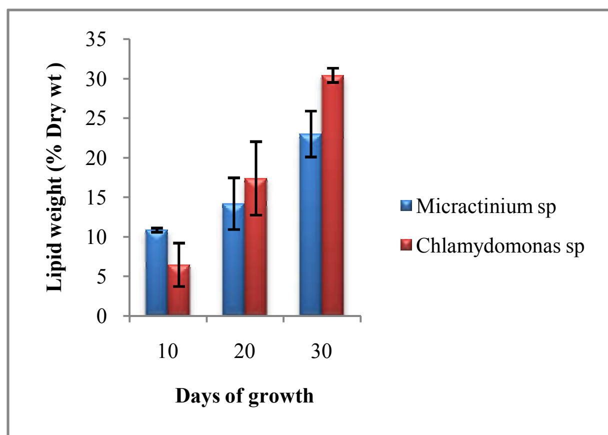


Figure 3 Lipid content in *Micartinium* sp and *Chlamydomonas* sp

The lipids were fractionated by uni- 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 4).

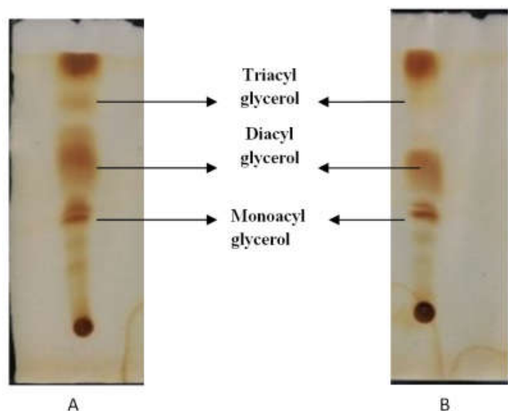


Figure 4 TLC separation of lipids indicated the presence of mono, di and triacyl glycerol in (A) *Micractinium* sp (B) *Chlamydomonas* sp

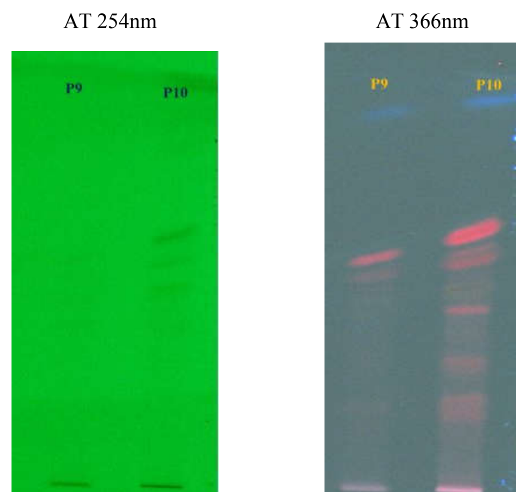
HPTLC analysis of lipids showed the presence of glycolipid, digalactosyl diglyceride, phosphatidylcholine, phosphatidyl ethanolamine, monogalactosyl diglyceride (Figure 5). The extracted lipids were further analyzed by column chromatography. The fractions were eluted initially in chloroform and then in acetone: methanol (9:1) and finally eluted using methanol. The eluted fractions were subjected to GC analysis. Was found to contain more amount of neutral and glyco lipids and a fewer amount of phospholipids.

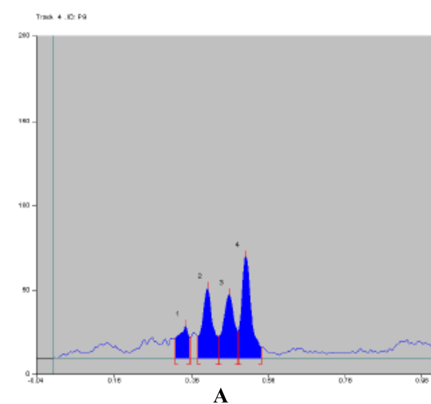
Fatty acid analysis by GC

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.

The predominant fatty acids in *Micractinium* sp and *Chlamydomonas sp* were butyric acid (C4:0), 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 and in *Chlamydomonas* sp 53 % of saturated fatty acid, 31 % unsaturated and 14% polyunsaturated fatty acid were observed on the 30th day of growth. In *Micractinium* sp unsaturated fatty acid 58% was maximum compared to saturated fatty acid 35% and polyunsaturated 5% fatty acids.

HPTLC fingerprinting profile of chloroform extract (based on lipids) Photo documentation under UV





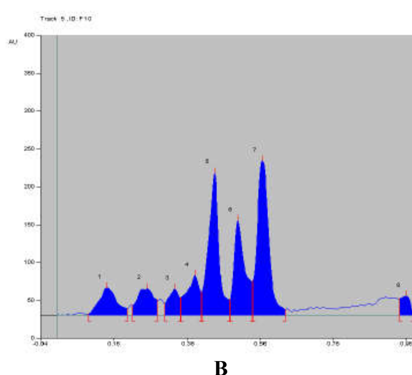


Figure 5 HPTLC analysis of lipids in (A) *Micractinium* sp and (B) *Chlamydomonas* sp

DISCUSSION

Research for the mass production of oil has recently given greater focus on microalgae. The preference towards

microalgae is due largely to its less complex structure, fast growth rate, and high oil content [14]. Microalgae prevailing in the local habitats directly influence higher proliferation rate and lipid accumulation capability that determine the suitability as the biodiesel feedstock [15]. In this perspective, results of present study showed that the proliferation rate and generation time increased from 10th day of growth and the cells attained the stationary phase on the 20th day of growth. Higher Chl-a content results in higher biomass production. Higher Chl-a content leads to the ability to capture more light energy and in consequence, the enhancement of photosynthesis and the acceleration of cell division process, which is followed by higher biomass production [16]. Chlorophyll a and carotenoid content were high in *Chlamydomonas* sp while chlorophyll *b* was high in *Micratinium* sp. The relationship between biomass and chlorophyll *a* were also reported in other green microalgae [17]. Nile red penetrated the cell wall and hence stained the lipid globules and emitted yellow colour. Nile red staining acts as a rapid diagnostic tool to measure the amount of biodiesel-convertible lipid that the cells accumulate. Nile red dye was able to interact with the intracellular lipid globules within the TS cells. The lipid globules emitted orange/yellow fluorescence upon interaction with the Nile red dye, while chlorophyll emitted a red auto fluorescence [18]. Variations in the optimal values of excitation and emission wavelengths are known to depend on the lipid content and composition of the investigated microalgal species. The emission wavelength of lipid fluorescence increases with lipid polarity. Thus, the emission of polar lipids generally peaks in the 590–635 nm, while neutral lipids have optimal emission fluorescence in the 560–590 nm [19]. Triacylglycerol (TAG) formation of oleaginous algae is often observed during the stationary phase [4]. The production of biodiesel, fatty acids with a chain length of 14–18 carbons are preferable. Saturated fatty acids C14, C16, and C18 and unsaturated fatty acids such as C16:1, C16:2, C18:1, and C18:2 are the most important for producing good biodiesel quality. This is because the other unsaturated fatty acids with 3 or 4 double bonds have reduced stability in storage [20]. The percentage of saturated fatty acids increased with days of growth and in *Chlamydomonas* sp 53 % of saturated fatty acid, 31 % of unsaturated and 14% polyunsaturated were observed on the 30th day of growth . In *Micractinium* sp unsaturated fatty 58% was maximum compared to saturated 35% and polyunsaturated 5% fatty acids. The degree of polyunsaturated fatty acid decreased with the age of culture [21] .The most common fatty acids of microalgae are Palmitic-(hexadecanoic-C16:0), Stearic-(octadecanoic-C18:0), Oleic (octadecenoic-C18:1), Linoleic-(octadecadienoic-C18:2) and Linolenic-(octadecatrienoic-C18:3) acids. Most algae have only small amounts of eicosapentaenoic acid (EPA) (C20:5) and docosahexaenoic acid (DHA) (C22:6), however, in some species of particular genera these PUFAs can accumulate in appreciable quantities depending on cultivation conditions [22]. *C. reinhardtii* was the most dominant in producing 16:0 palmitic acid [23].

CONCLUSION

Thin layer Chromatography and HPTLC techniques enable intial screeening of the presence of lipids (Monoacylgycerol, Diacylglycerol and Triacylglycerol). The Nile Red flurometric assay provided evidence of lipid content in *Chlamydomanas* sp and *Micractinium* sp. In order to identify the various classes of lipid present, gas chromatography was employed. Greater proportion of saturated fatty acids and monounsaturated fatty acids in both *Chlamydomonas* sp and *Micractinium* sp indicated its potential for production of oil. These characteristics indicated that both *Chlamydomonas* sp and *Micractinium* sp can be used for future exploitation as an alternaive renewable fuel source.

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