

PHYSIOLOGICAL ATTRIBUTES AND LIPID PROFILING OF ALGAL STRAIN *BOTRYOCOCCUS*

Ashu*, Devendra Kumar¹, Y.Vimala², Neha Dhillon³ and Priyanka Nehra⁴

*Research Scholar, Department of Microbiology, Chaudhary Charan Singh University, Meerut, India

¹Assistant Professor, Department of Microbiology, Chaudhary Charan Singh University, Meerut, India

²H.O.D. Department of Microbiology, Chaudhary Charan Singh University, Meerut, India,

³ Research Scholar, Department of Microbiology, Chaudhary Charan Singh University, Meerut, India,

⁴Research Scholar, Department of Microbiology, Kurukshetra University Kurukshetra, Haryana, India.

ABSTRACT

The sustainability of algal biofuel production systems is achievable. Fossil fuel energy resources are diminishing rapidly and most importantly the liquid fossil fuel will be diminished by the year about 2050. There is regular research on biodiesel fuel into finding more and more appropriate source to enhance oil yield in algal cells. Additionally, fossil fuels are directly related to air pollution, land and water degradation. In order to replace all fossil fuel usage entirely, a large amount of biomass is required to manufacture sufficient bio-oil. Research on biofuel presently focuses on high amount of oil yield. In these circumstances, biofuel from renewable sources like microalgae can be a substitute to reduce our dependency on fossil fuels and assist to maintain the healthy global environment and economic sustainability. In the present study lipid profiling of *Botryococcus* was done by GC-MS and the maximum value of lipid content observed.

Key words: Botryococcus, lipid profile and Physiological parameters and GCMS.

INTRODUCTION

The world has been confronted with an energy crisis due to depletion of finite resources of fossil fuels. Continued use of petroleum-based fuels is now widely recognized as unsustainable because of depleting supplies, accelerating price, emerging concerns about global warming that is associated with burning fossil fuels and contribution of these fuels to environmental pollution (Gavrilescu and Chisti 2005). The idea of using microalgae as a source of fuel is not new but it is now being taken seriously. Microalgae are microscopic photosynthetic cell factories having high surface area-to-volume ratios. These have been reported to be the primary synthesizers of organic matter in aquatic environments and are sunlight driven factories that convert CO₂ to potential biofuel, food, feed and high value bioactive compounds (Meting and Pyne 1986). These have high growth rates and their yield of biomass per ha is three to five folds greater than the yield from typical crop plants (Nenan et al. 1986). These organisms can provide several different types of renewable biofuels which include methane produced by anaerobic digestion of the algal biomass (Spolare et al. 2006), biodiesel derived from microalgae oil and photo-biologically produced biohydrogen (Akkerman et al. 2002; Ghiradi et al. 2000).

Microalgae have been reported to be rich source of lipids namely fats, oil and fatty acid (Chisti 2007) which are present as membrane components and metabolites. Algal oil possesses characteristics similar to those of fish and vegetable oils and therefore, is considered to be potential substitutes for products of fossil oil. Oil content in microalgae can exceed 80% by weight of dry biomass and oil levels of 20-50% are quite common (Metting 1996). India's transportation fuel requirements are unique in the world and consume almost five times more diesel fuel than gasoline. The fuel demand is also being used in large quantities in industrial and agricultural sectors. Due to higher demand, it is expected that crude oil production will start declining from the beginning of 2012. Fatty acids are the major component of lipids, and the physical, chemical, and physiological properties of a lipid class depend primarily on its fatty acid composition. Lipids and fatty acids are the primary metabolites of microalgae to produce biodiesel. *Chlorella vulgaris* (Gouveia et al., 2009), *Scenedesmus obliquus* (Da-Silva et al., 2009; Gouveia et al., 2009; Sahu et al., 2013), have been extensively studied. In order to define a lipid class, it is must to define its fatty acids also. Fatty acids are compounds which are synthesized naturally through condensation of malonyl coenzyme A units by a fatty acid synthase complex. They usually contain even numbers of carbon atoms in straight chains (generally C14 to C24), either saturated or unsaturated. Synthesis of neutral lipids in the form of (triacylglycerides) TAG can be induced in many species under stress conditions. These lipids are suitable initiators for biodiesel production (Miao and Wu, 2006; Hu et al., 2008). Trans-esterification of these algal lipids convert crude lipids into biodiesel on reaction with alcohol (generally methanol) and sodium hydroxide act as catalyst and glycerol is produced as byproduct.

MATERIALS AND METHODS

Test organism

Green algal strain Botryococcus was procured from algal biotechnology laboratory, Department of Microbiology, Chaudhary Charan Singh University, Meerut. (Fig.1)

Growth and Maintenance

Organisms were grown and maintained in BG-11 medium (Stanier et al., 1971) at $28 \pm 2^\circ\text{C}$ under a light intensity of $52\text{--}55 \mu\text{mol photon m}^{-2}\text{s}^{-1}$ and L:D cycles of 16:8 hr. The pH of the medium was maintained in the range of 7.1–7.3 for optimal growth of cultures. (Fig.2)

Physiological parameters

Estimation of Chlorophyll (McKinney, 1941)

A known volume (10 mL) of homogenized cyanobacterial suspension was taken and subjected to centrifugation (4000g, 10 min). The chlorophyll was extracted from pellet with equal volume of methanol (95%) in a water bath (60°C , 30 min). The suspension was centrifuged and the absorbance of the supernatant was measured at 650 and 665 nm against 95% methanol as blank.

Estimation of Total soluble proteins (Lowry et al. 1951; Herbert et al. 1971)

(i) Reagents:

(a) 1N sodium hydroxide solution

(b) (i) 5% sodium carbonate

(ii) 0.5% copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) solution in 1% sodium potassium tartarate

2 mL of reagent B (ii) was mixed with 50 mL of freshly prepared reagent B (i)

(c) 1N Folin-ciocalteu reagent

A known volume (0.5 mL) of homogenized cyanobacterial suspension was taken in test tubes. To this, 0.5 mL of reagent (a) was added. The tubes were then heated in a boiling water bath for 10 mins. and cooled in running tap water. Subsequently, 2.5 mL of reagent (b) was added in each and the tubes were incubated at room temperature for 10 mins. After this, 0.5 mL of reagent (c) was added and the tubes were kept at room temperature for 15 mins. The intensity of blue colour was read as absorbance at 650 nm against appropriate blank. The protein content was estimated using a standard calibration curve prepared from bovine serum albumin and expressed in terms of mg/mL.

Total lipids ($\mu\text{g}/\text{mL}$) or % lipid on dry wt. basis

Reagents :

1. 0.2N Perchloric acid (HClO_4)
2. (2:1 v/v) Chloroform-Methanol
3. Potassium Dichromate + Sulphuric acid (H_2SO_4)

Procedure:

30 mL of homogenized microalgae suspension was taken in 30 mL plastic centrifuge tube and centrifuged at 3000 rpm for 15 minutes. Tubes containing pellets placed in ice bath and 10 mL of ice cold 0.2N HClO_4 added. Contents thoroughly vortexed and kept at 4°C for 15 minutes. Contents were then centrifuged at 7000 rpm for 15 minutes in refrigerated centrifuge tube. 25 mL of (2:1 v/v) Chloroform-Methanol mixture was added in the pellets, vortexed and allowed to stand for 5 minutes at room temperature. Sample centrifuged at 7000 rpm for 15 minutes and retained the supernatant. 0.2 volume of water added to the supernatant, shaken well for 5 minutes and centrifuged at 4000 rpm for 10 minutes. Lower organic phase was collected and rest was discarded. Organic phase evaporated to a volume of 2 mL under a stream of nitrogen. 0.5 mL of aliquot was taken and evaporated the content to dryness under vacuum. 2 mL of Potassium dichromate solution was added in to tubes and simultaneously standard was prepared with standard palmitic acid. Placed the tubes in boiling water bath for 45 minutes. After cooling absorbance measured at 350 nm against potassium dichromate as blank. Lipid was expressed as $\mu\text{g}/\text{mL}$ or % lipid on dry wt. basis (Bligh and Dyer 1959).

Stress studies

Botryococcus culture in standard medium were subjected to different kind of stress conditions such as temperature, light intensity and pH during an incubation period of 28 days. Samples were taken for the estimation lipid content.

Temperature stress

The influence of temperature stress was examined on biochemical parameter (Lipid content). Three different temperatures viz. $25 \pm 2^\circ$, $30 \pm 2^\circ$, $35 \pm 2^\circ$ were employed and all other growth conditions were kept as described earlier.

Light stress

Different light intensities of 3, 4 and 5 Klux were employed. These were maintained at $28 \pm 2^\circ\text{C}$ temperature and 16:8 hours light/dark cycles.

pH stress

To examine the effect of pH, cultures were grown at 6.5, 7.0 and 7.5, all other growth conditions were kept same as described earlier.

Fatty acid profile

Sample was prepared by method given by (Bligh and Dyer 1959) described above in procedure (total lipids 3.5.7) final volume of 2 mL of lipid extract evaporated under vacuum then 25 mL Methanol added with 5 drops of concentrated Sulphuric acid this mixture were kept for heating on heating mantle at 60°C for 4 hrs. 2 g of Sodium Hydrogen Carbonate dissolved in 50 mL water

and added into sample portioning were carried out by Diethyl Ether. Upper layer was collected charcoal was added and filtered the sample through funnel by keeping cotton and Sodium Sulphate. vacuum dried the sample and rinse with 1-2 ml of Hexane. Clear sample were injected into gas chromatography. (Table-1)

Results

Botryococcus shows maximum chlorophyll concentration (.903µg/ml) and protein concentration (.578mg/ml) at 14th days of incubation.

Total Lipids

Botryococcus shows around 92% maximum concentration of total lipids. (Table -1) (Fig.6)

4.5 Stress Condition

Botryococcus culture in standard medium was subjected to different kind of stress conditions such as temperature, light intensity and pH during an incubation period of 28 days. Samples was taken for the estimation lipid content.

4.5.1 Temperature stress

Three different temperatures viz. $25\pm 2^\circ$, $30\pm 2^\circ$, $35\pm 2^\circ$ were employed and all other growth conditions were kept as described earlier. *Botryococcus* shows maximum lipid content at 30°C . (Fig.3)

4.5.2 Light stress

Different light intensities of 3,4 and 5 Klux were employed. *Botryococcus* shows maximum lipid content at 4KLux. (Fig.4)

4.5.3 pH stress

To examine the effect of pH, cultures were grown at 6.5, 7.0 and 7.5, all other growth conditions were kept same as described earlier. *Botryococcus* shows maximum lipid content at pH 7.5. (Fig.5)

Discussion

Botryococcus was selected for studies on the optimization of cultural conditions for enhanced lipid production. These were subjected to variable environmental and cultural conditions for a period of 28 days and the differential variables used were temperature (25 ± 2 , 30 ± 2 and $35\pm 2^\circ\text{C}$), light intensity (3, 4 and 5 Klux), pH (6.5, 7 and 7.5). Total lipids were determined on dry weight basis using homogenized microalgal suspension and palmitic acid was used as standard (Bligh and Dyer 1959). The fatty acid profile was done by using gas chromatography with triple axis detector. A variety of environmental factors including nutrient availability can induce lipid accumulation in microalgae as a reserve of energy after the stress is over (Guschina and Harwood 2006).

The lipids were highest at 4 Klux as compared to 3Klux and 5 Klux on dry weight basis. Overall mean lipid content was highest in *Botryococcus* at pH 7.5.

Chemical profiling of *Botryococcus* by GC-MS yielded 27 major and minor constituents containing saturated /unsaturated hydrocarbons, fatty acids and others. GC-MS analysis of the led to the identification of eleven constituents which include hydrocarbons (24.28%) and fatty acid methyl esters (61.44%). Interestingly, oil was rich in hydrocarbons (37.55%) followed by fatty acids (28.95%).

FAME profile of *Botryococcus* consisted of C-16:0, C-18:0, cis, and C-18:1 trans, which are considered suitable for biodiesel production (Knothe 2008; Tan and Johns 1991) found that fatty acid composition varied according to the cultural conditions with linolenic acid (C18:2) predominating. FAME profile was found to be suitable for biodiesel and can be considered as a promising microalgae to be used as a source of lipids for biodiesel production.

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Conclusion

The present study reveals that, my best strain in context to high lipid producer is *Botryococcus*

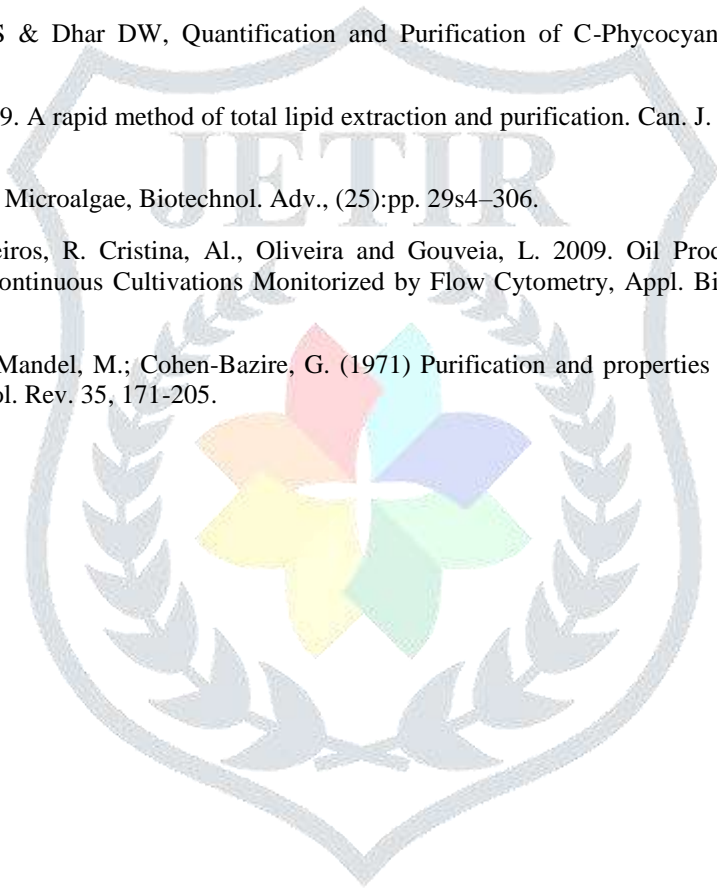
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S. No.	Rention time (min.)	Chemical name	Area (%)
1.	3.471	n-Decane	3.71
2.	4.793	n-Undecane	6.16
3.	6.212	n-Dodecane	5.50
4.	7.637	n-Tridecane	5.51
5.	9.010	n-Tetradecane	3.40
6.	15.276	Methyl hexadecanoate (Palmitate)	30.20
7.	16.832	Methyl 5, 9, 12-octadecatrienonate (Gamma-linolenate)	2.07
8.	16.987	Methyl 9, 12-octadecadienoate (Linolenate)	7.51
9.	17.050	Methyl 9, 12, 15-octadecatrienoate (Linolenate)	11.03
10.	17.095	Methyl 9 – Octadecenoate (Elaidate)	8.74
11.	17.284	Methyl octadecanoate (Stearate)	1.89

Table-1: Fatty acid profile of the lipids extracted from microalga *Botryococcus*

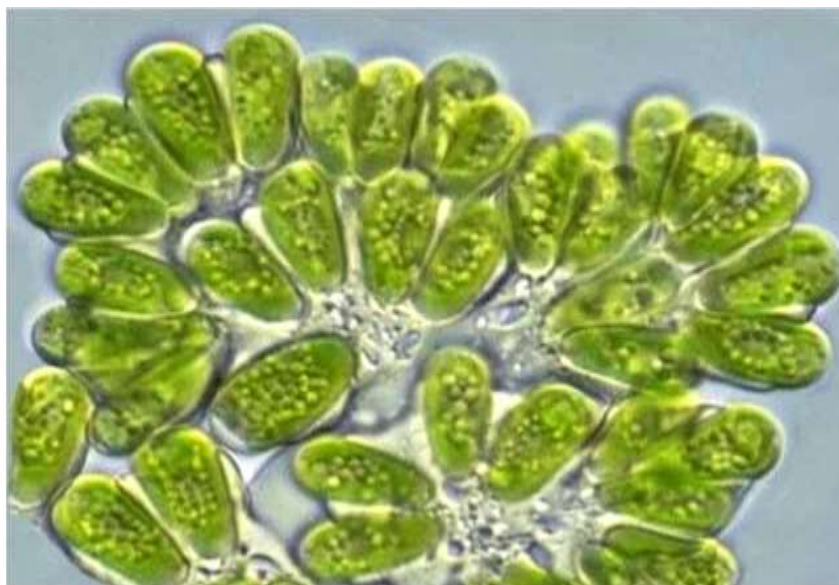


Fig.1: Photomicrograph of *Botryococcus*



Fig.2: Growth and Maintenance

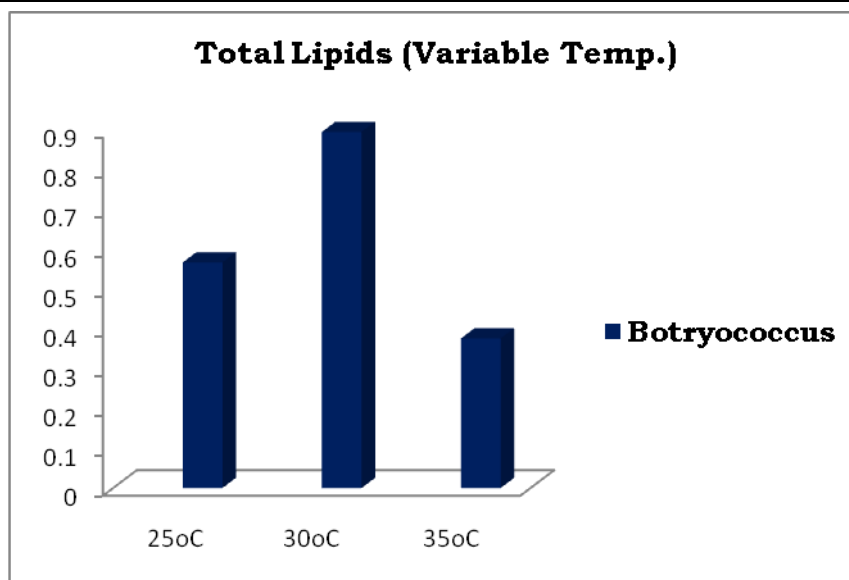


Fig.3

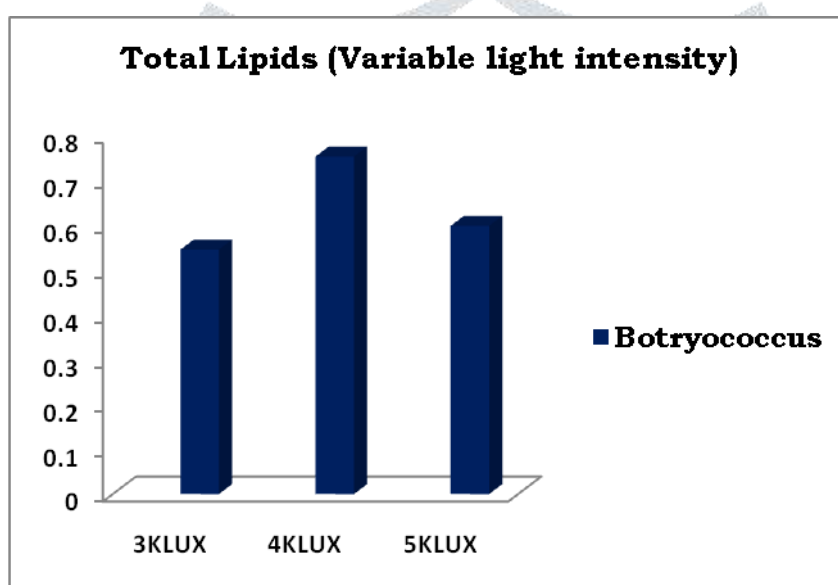


Fig.4

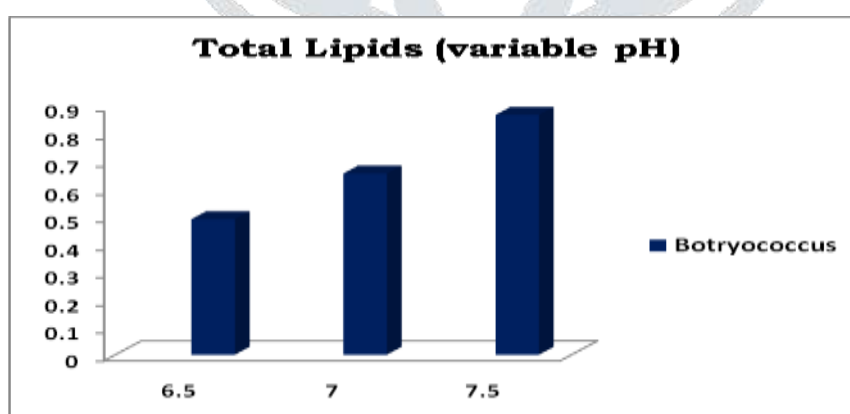


Fig.5

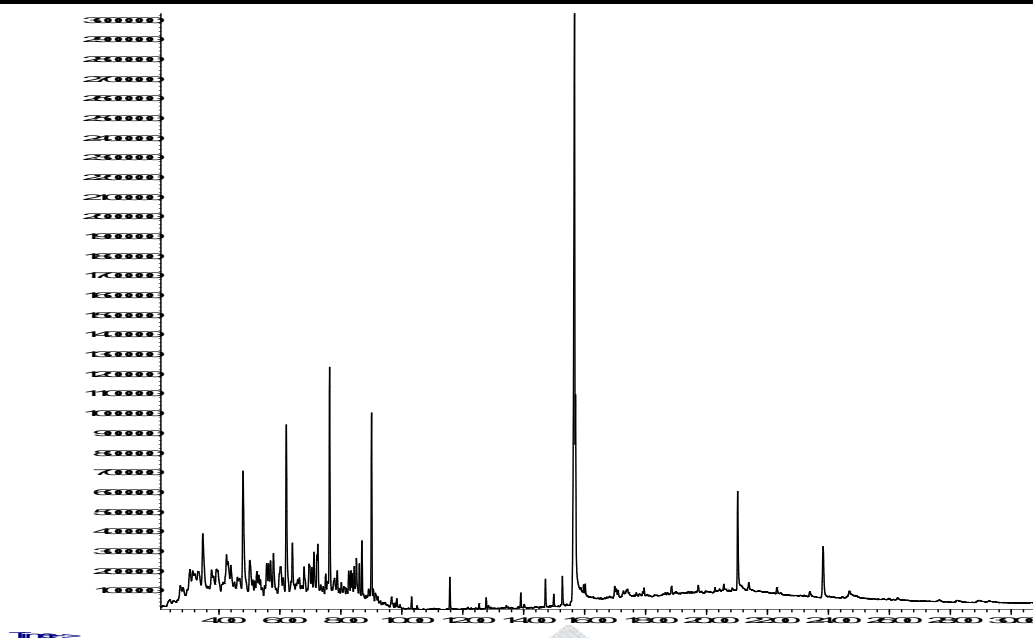


Fig. 6: Fatty acid profile of the lipids extracted from microalga *Botryococcus*

