



RESEARCH ARTICLE

Studies on Growth Characteristics of Hydrocarbon Producing *Botryococcus braunii*

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ABSTRACT

Botryococcus braunii is notable for its ability to produce high amounts of hydrocarbons, especially oils in the form of triterpenes. Samples of green colonial unicellular microalga *B. braunii* were collected from Kuruchi lake at Coimbatore. The culture was initially screened and purified. Based on the macroscopic and microscopic studies, the purified culture was identified as *B. brauni*. Macroscopic characters like clumpy, green colonies were produced by *B. braunii* on BG-11 medium. The colony characters like green, pyramid-shaped colloidal microalgae were observed. Studies were conducted to determine the effect of pH, sodium-bi-carbonate, dark and light, and to estimate the chlorophyll, lipid, sugar and protein content by the isolate. The culture attains log phase at third week. The growth of *Botryococcus* is observed to be maximum in pH 7 and a high yield of biomass produced at this pH. When 2.5% sodium-bi-carbonate is added along with the medium, the growth curve attains a log phase on the 6th day itself. The *Botryococcus* exposed to 24 hour light conditions showed an improved growth rate compared to *Botryococcus* exposed to 16 hours light. The amount of chlorophyll is 0.039% occurs in 100gm of *Botryococcus*. The maximum amount of protein was observed in hexane extract is 50%, and the mutagenesis for 30 minutes results in 55.6%. The maximum amount of lipid was observed in hexane extract is 16.5%, and the mutagenesis for 30 minutes results in 38.9%. The maximum amount of sugar was observed in ethanol extract is 78.15%, and the mutagenesis for 40 minutes results in 58.3% while extracting with hexane.

Keywords: Biofuel; *Botryococcus*; optimization; lipid yield

INTRODUCTION

Algae are a large and diverse group of simple plant-like organisms, ranging from unicellular to multicellular forms. The largest and most complex marine forms are called seaweeds. They are considered Plant-like because of their photosynthetic ability and simple because they lack the distinct organs of higher plants such as leaves and vascular tissue (Graham,1983). Though the prokaryotic cyanobacteria (Commonly referred to as BGA) were traditionally included as algae in older textbooks, many modern sources regard this as outdated and restrict the term algae to eukaryotic organisms. All true algae, therefore, have a nucleus enclosed within a membrane and chloroplasts bound in one or more membranes (Tanabe *et al.*, 2015).

Algae constitute a paraphyletic and polyphyletic group; they do not represent a single evolutionary direction or line, but a level or grade of the organization that may have developed several

times in the early history of life on earth. Algae lack leaves, roots, and other organs that characterize higher plants. They are distinguished from protozoa in that they are photosynthetic. Many are photoautotrophic, although some groups contain members that are mixotrophic, deriving energy both from photosynthesis and uptake of organic carbon either by osmotrophy, mixotrophy or phagotroph (Gouveia *et al.*, 2019).

Algae are most prominent in bodies of water but are also common in terrestrial environments. However, terrestrial algae are usually rather inconspicuous and far some common in moist, tropical regions and other adaptations to living on land. Algae are also found in other situations, such as on snow and exposed rocks in symbiosis with a fungus as lichen (Beneman *et al.*,1977).

Botryococcus braunii is a green, pyramid-shaped colloidal microalga of the order Chlorococcales (class Chlorophyceae) that is of potentially great

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importance in the field of biotechnology. Colonies held together by a lipid biofilm matrix can be found in temperate or tropical oligotrophic lakes and estuaries and will bloom when in the presence of elevated levels of dissolved inorganic phosphorus. The species is notable for its ability to produce high amounts of hydrocarbons, especially oils in the form of Triterpenes that are typically around 30-40 percent of their dry weight. Compared to other green algae species, it has a relatively thick cell wall that is accumulated from previous cellular divisions, making extraction of cytoplasmic components rather difficult. Fortunately, much of the useful hydrocarbon oil is outside of the cell (Tenauel *et al.*,1989)

Botryococcenes are the major oil constituents of the green algae *Botryococcus braunii*. The hydrocarbons this species produces can be chemically converted into fuels. Transesterification cannot be used to make biodiesel from botryococcenes, the major oil of *Botryococcus braunii*. This is because Botryococcene is not a regular vegetable oil (which is a fatty acid triglyceride) but is instead a triterpene and lacks the free oxygen for transesterification. It can be used as feedstock for hydrocracking in an oil refinery to produce octane (gasoline, petrol), kerosene, and diesel. Up to 86% of its dry weight can be long-chain hydrocarbons. Several challenges must be met in order to produce the desired alkanes, such as gasoline, economically. This will only be briefly covered in this article at this time, as it has only just begun (Gouveia *et al.*,2017).

First is that a suitable strain of *Botryococcus braunii* must be found. Several strains are available from algae specimen banks, but there is no guarantee that these are high-producing strains. Some plant patent applications have been filed and patents granted for high producing strains. It is within reason for even a small scale producer of botryococcenes to breed successively higher producing strains of *Bb*, due to the reasonably fast generation rate. However, in selecting *Bb* strains for high Botryococcene production, it is likely that other beneficial attributes may be bred out. For instance, resistance to disease, competitive advantages against other organisms, and survivability in less than ideal climates. In this case, a photobioreactor may be needed.

The practice of farming algae is known as algaculture. However, there are properties of *Botryococcus braunii* that make its harvest a bit different than the harvest of other algae. Compared to other green algae species, it has a relatively thick cell wall that is accumulated from previous cellular divisions, making extraction of cytoplasmic components rather difficult. Fortunately, much of the useful hydrocarbon oil is outside of the cell. This gives rise to the hope that the algae will not have

to be killed in order to extract its useful oil. Indeed, this is the case. Several methods are available to extract the botryococcene. One that shows the most promise is the use of hexane as a solvent. If used at the proper molarity, it does not kill the majority of the *Bb*, while extracting the botryococcene. However, like all organisms, *Bb* is less productive in old age. It is said they become senescent and produce less botryococcene (Hillen, *et al.*,1982).

MATERIALS AND METHODS

Collection and Isolation of Sample

The sample was obtained from kuruchi lake at Coimbatore. The samples were collected and cultured in a modified BG-11 medium (Bold,1949). The algae were subjected to purification by serial dilution followed by plating. The individual colonies were isolated and inoculated into a liquid medium (BG-11 medium) incubated at $25 \pm 1^\circ\text{C}$ under light intensity. The purity of the culture was ensured by repeated plating and by regular observation under a microscope.

Growth Curve (Dubey)

The BG-11 medium was prepared in 500mL and sterilized the autoclave at 121°C after cool it. The isolated colonies were inoculated into the medium and incubate in the light condition. The culture flasks were incubated for 3 weeks at $25 \pm 1^\circ\text{C}$ and take the optical density value through spectrophotometer interval between 24 hours and plotted graph.

Effect of pH, Sodium-Bi-Carbonate (NaHCO_3) and Dark and Light

The effect of pH on the growth of the alga and hydrocarbon yields was studied using BG11 media in the pH range of 6, 7 and 9. The experiment was carried out in the conical flask (500ml) containing 100ml of BG-11 medium and the pH of the medium was adjusted before autoclaving. All the flasks were inoculated uniformly at 25% (v/v) inoculum of 2 weeks old *Botryococcus braunii* culture. The culture flasks were incubated for 3 weeks at $25 \pm 1^\circ\text{C}$ and take the optical density value through spectrophotometer interval between 24 hours and plotted graph. The cultures were harvested and the cells were washed with distilled water after centrifugation at 5000 rpm. Then the pellet was collected and freeze-dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight.

A two-tier Erlenmeyer flask was used for photoautotrophic growth experiments. The medium in upper chamber was inoculated with 25% (v/v) of two weeks old *Botryococcus braunii* culture. The mouth of the upper and lower compartments was sealed tightly with cotton plug. The mixture of

sodium-bi-carbonate (3M) solutions were added in different concentrations (2.5% and 5%). The culture flasks were incubated for 3 weeks under 24 hours light and take optical density value through spectrophotometer interval between 24 hours and plotted a graph.

The effect of dark and light on growth of the algae was studied using BG-11 medium. The experiment was carried out the conical flask (500 ml) containing 100mL of BG-11 medium. All the conical flasks were inoculated uniformly at 25% (v/v) inoculum of 2 weeks old *B. braunii* culture. The culture flasks were incubated for 3 weeks at $25 \pm 1^\circ\text{C}$ and 16:8 hrs light, dark cycle and take optical density value using spectrophotometer and plotted a graph. The cultures were harvested and the cells were washed with distilled water after centrifugation at 6000 rpm. Then the pellet was freeze-dried. The dried culture was used in further processes.

Chlorophyll Estimation (Gron D.I. 19426)

Weight 250 mg of finely cut and well mixed representative samples of leaf or fruit tissue into a clean mortar. Grind the tissue to a fine pulp with the addition of 20mL of 80% acetone. Centrifuge and transfer the supernatant into a 100mL volumetric flask. Grind the residue with 20mL more of 80% acetone, centrifuge and transfer the supernatant to the same volumetric flask. Repeat this procedure until the residue is colorless, wash the mortar and pestle thoroughly with 80% acetone and collect the washing in the volumetric flask. Make up the volume to 100mL with 80% acetone. Read the absorbance of the made-up solution at 645 and 663nm against solvent (80% acetone) blank. The absorption of acetone extract may be measured from 400 to 700nm to get the absorption spectrum of the pigments. Calculate the amount of chlorophyll present in the extract in mg chlorophyll per g of tissue according to the following equations:

$$\text{Chlorophyll a mg per g tissue} = 12.7 (D_{663}) - 2.69 (D_{645}) \times$$

$$\text{Chlorophyll b mg per g tissue} = 22.9 (D_{645}) - 4.68 (D_{663}) \times$$

$$\text{Total chlorophyll mg/g tissue} = 20.2 (D_{645}) - 8.02 (D_{663}) \times$$

Where,

D = absorbance at specific wavelengths,

V = final volume of 80% acetone - chlorophyll extract and

W = fresh weight of tissue extracted.

Preparation of Extract

The preparation of extract was using the three solvents such as 80% ethanol 100% chloroform, and hexane. Three conical flasks (100mL) was taken and clean dry. All the flasks taken with the 0.5 gm dried culture, add three solvents respectively and close tightly incubate the 24 hrs at room temperature.

After incubation, the empty Petri plates are sterilized, weighed and marked 3 solvents separately. The solvents are filtered the Petri plate and evaporate it after the weight the Petri plate and calculate the weight extraction.

Protein Estimation (Lowry's, 1951)

Various concentrations of working standard solution 1 to 5 mL were taken in a different test tube as S_1 to S_5 . The volume was made up to 5mL with distilled water. 0.1mL of the sample in a test. To all the above tubes, added 4.5mL of Lowry's reagent and allow it to stand for 10 minutes. Then add 0.5 mL of Folin's phenol reagent. The color development was read at 620nm after 10 minutes using a red filter. A standard graph was drawn by taking the concentration of the protein on the x-axis and the optical density on the y-axis. The amount of protein present in the sample was calculated from the graph.

Lipid Estimation

Place 100 μL of the lipid extract in a 1x -1mL test tube. Evaporate solvent in speed Vacuum concentrator at about 45°C . Add 400 μL of concentrated sulphuric acid and vortex. Close tube tightly, or if not pressure-resistant, cover it with a pear-shaped glass bulb (or marble) and heat for 10 minutes to 100°C in water or dry bath. Cool for 5minutes in a water bath at 20°C . Add 3mL of phosphoric acid vanillin reagent and vortex. Measure absorbance after 60-65 minutes at 528 nm. Run cholesterol standards in the same way. Calculate lipid content as cholesterol equivalents from absorbance reading of sample and the standard curve.

Sugar Estimation

The working standard solution was taken in the range of 0.1, 0.2, 0.3, 0.4, and 0.5 mL in different test tubes labeled as s_1 , s_2 , s_3 , s_4 , and s_5 respectively, A blank was taken with 1mL of distilled water. To 0.1mL of the sample was added 2mL of trichloroacetic acid in order to precipitate protein. Allow to centrifugation for 5minutes 1mL of protein-free filtrate was taken in the test tube and marked as T. The above tubes were added with 3mL of orthotoluidine and kept all the tubes in a boiling water bath for 15 minutes. Cooled and the bluish-green color was read at 630 nm. A standard graph was drawn by taking concentration on the x-axis and optical density on the y-axis. The amount of sugar was calculated from the graph.

RESULT AND DISCUSSION

Algae are a large and diverse group of simple plant-like organisms and by producing an oil, *Botryococenes* are the major oil constituents of the green algae *Botryococcus braunii*. This

species produce the hydrocarbons this species produces can be chemically converted into fuels. Macroscopic characters like clumpy, green colonies were produced by *B.braunii* on BG -11 Medium.

Microscopic characters like green, pyramid-shaped colloidal microalgae was observed. The *Botryococcus braunii* reaches the log phase on the fifth day remains in the log phase till the third week (Figure 1 and 2).

Botryococcus braunii



Figure 1. Growth curve of Botryococcus in BG11 medium

Effect of pH, sodium –bicarbonate and dark and light

The effect of pH on the growth of *Botryococcus* was carried out under varying pH conditions such as pH6. pH 7 and pH 9, the growth of *Botryococcus* is observed to be maximum in pH 7. In pH6 the growth is less when compared with pH 7. In pH 9 the growth is retarded.

Table 1. Growth of Botryococcus at different pH of the media

Day	Optical density at 680 nm		
	pH 6	pH 7	pH 8
1	0.008	0.019	0.046
2	0.010	0.037	0.0295
3	0.008	0.071	0.036
4	0.059	0.105	0.0735
5	0.097	0.1625	0.088
7	0.236	0.271	0.219
9	0.431	0.367	0.294
11	0.488	0.610	0.365
12	0.737	0.719	0.4185
14	0.981	0.990	0.5015
15	0.996	1.171	0.526
16	1.200	1.341	0.6195
17	1.164	1.419	0.597
18	1.188	1.431	0.6055
19	1.210	1.505	0.6625
25	1.2455	1.753	0.805
26	1.414	1.751	0.917

Hence we can conclude that in alkaline conditions the *Botryococcus* does not grow well (Table 1 and Figure 3). The effect of sodium-bi carbonate on the growth of *Botryococcus* was carried out under various concentrations such as 2.5% and 5%; when 2.5% NaHCO3 is added along with the medium, the

growth curve attains a log phase on the 6th day itself (Table 2).

The effect of dark and light on the growth of *Botryococcus* was carried out under light intensity, such as 16 hours light and 8 hours dark compared to the 24 hours light. *Botryococcus* when exposed to 24 hr. light conditions, the growth rate is higher compared to *Botryococcus* exposed to 16 hr. light (Figure 4).

Table 2. Growth of Botryococcus at different concentrations of NaHCO3

Day	Optical density at 680 nm		
	2.5% (NaHCO3)	5% (NaHCO3)	Control
1	0.01	0.01	0.02
2	0.04	0.03	0.04
3	0.13	0.10	0.07
5	0.45	0.35	0.10
6	0.55	0.45	0.16
7	0.65	0.49	0.27
8	0.63	0.47	0.37
9	0.75	0.52	0.61
10	1.23	0.58	0.72
16	1.28	0.64	1.341
17	1.46	0.76	1.419

Chlorophyll, Protein, Lipid, and Sugar estimation

The amount of chlorophyll present is 100 gm of the *Botryococcus* is 0.039%. The amount of protein present in the 0.01g hexane extract of *Botryococcus* is 50% In chloroform and ethanol extract of *Botryococcus* is less compared to the hexane extract of *Botryococcus* in protein estimation. The amount of lipid present in the 0.01g hexane extract of *Botryococcus* is 16.5%. In chloroform and ethanol extract of *Botryococcus* is less compared to the hexane extract of *Botryococcus* in lipid estimation. The amount of sugar present in the 0.08g ethanol extract of *Botryococcus* is 78.15%. In chloroform and

hexane extract of *Botryococcus* is less compared to the ethanol extract of *Botryococcus* in sugar estimation (Figure 5).

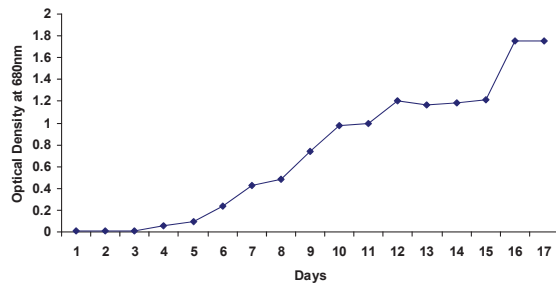


Figure 2. Growth curve of different pH

The algae require different levels of carbon-dioxide for their photoautotrophic adaptability, and 2% of carbon-di-oxide supplementation was found to be better for growth and hydrocarbon production (Tripathi et al., 2001).

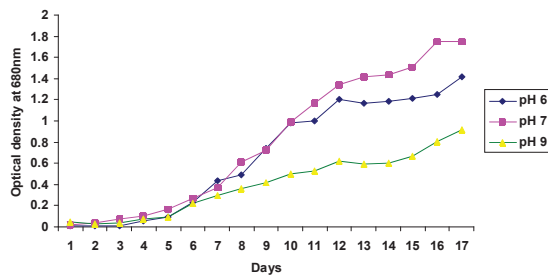


Figure 3. Growth curve using different concentration of NaHCO₃

In this study, the effect of sodium bicarbonate (2.5%) was highly efficient for the growth of *B. braunii*. The fat content of the organism was found to be 22%(w/w) while palmitic and oleic acids as the major fatty acids constituting 40.6% and 22.3% respectively (Fang et al., 2004).

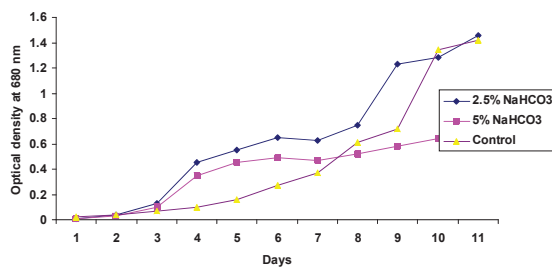


Figure 4. Growth curve in different light conditions

In this study, the amount of lipid in hexane extract of *Botryococcus* is 16.5% and the mutagenesis for 30minutes in 38.9%, respectively. The effect of pH on the biomass yield and pH 7.5 the algae showed maximum growth and production of hydrocarbons (Dayananda et al., 2006). In this study, the growth of *Botryococcus* is observed to be maximum in pH 7 and the production of hydrocarbons.

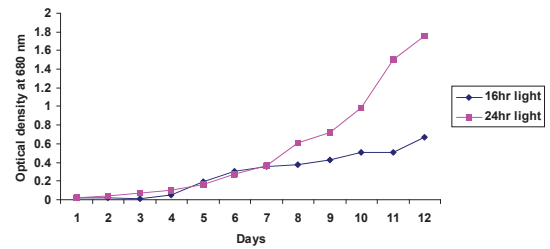
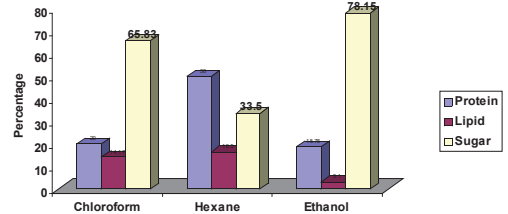


Figure 5. Extraction of Botryococcus



CONCLUSION

Botryococcus braunii is notable for its ability to produce high amounts of hydrocarbons, especially oils in the form of Triterpenes. Studies were conducted to find out the effect of pH, sodium-bi-carbon-ate, dark and light and to estimate the chlorophyll, lipid, sugar and protein, mutagenesis and to analyse the hydrocarbons and the results were summarized here. Samples of green colonial unicellular microalga *B. braunii* were collected from kuruchi lake at Coimbatore. The culture was initially screened and purified. Based on the macroscopic and microscopic studied the purified culture was identified as *B. brauni*. In this species reaches the log phase till the third week. The growth of *Botryococcus* is observed to be maximum in pH 7 and high yield of biomass. When 2.5% sodium-bi-carbonate is added along with the medium the growth curve attains a log phase in the 6th day itself. The *Botryococcus* exposed to 24hour light a condition the growth rate is higher when compared to *Botryococcus* exposed to 16hours light. The amount of chlorophyll is 0.039% occurs in 100gm of *Botryococcus*. The maximum amount of protein was observed in hexane extract is 50% and the mutagenesis for 30minutes in 55.6%. The maximum amount of lipid was observed in hexane extract is 16.5% and the mutagenesis for 30minutes in 38.9%. The maximum amount of sugar was observed in ethanol extract is 78.15% and the mutagenesis for 40minutes in hexane extract is 58.3%. The doubling time of *Botryococcus* through mutagenesis is highly efficient and the time is 432 hours.

REFERENCES

- Benemann, J.R. 1997. CO₂ mitigation with microalgae systems. *Energy Convers*, **38**: 475-479.
- Bold HC., 1949. The morphology of *Chlamydomonas chlamydogama*, sp. *Bot Club*, **76**: 101-108.
- Dayananda, Ravi sarada, Vinod Kumar and Ravi shankar. 2006. Isolation and characterization of hydrocarbon

- producing green alga *Botryococcus braunii* from Indian freshwater bodies. *Pytochemistry*, 10-11.
- Dubey, R.C and Chan,S. Textbook of *Biotechnology*, 375.
- Fang, Jain Y, Chiu, Hsien C, Jiunn and Yin R. 2002. Fatty acids in *B.braunii* accelerate topical delivery of flubiprofen into and across skin. *Pharmaceutics*, **276 (1-2)**: 163-173.
- Gouveia D, Jie Lian, Georg Steinert, Hauke Smidt, Detmer Sipkema, Rene H. Wijffels and Maria J. Barbosa. 2019. Associated bacteria of *Botryococcus braunii* (Chlorophyta). *J Biotechnol*, **27**.
- Gouveia J, Ruiz J, van den Broek L, Hesselink T, Peters S, Kleinegris DM, Smith AG, van der Veen D, Barbosa M, Wijffels R. 2017. *J Biotechnol*, **248**: 77-86.
- Graham L.E and Wilcox, L.W. 1983. The occurrence and phylogenetic significance of putative placental transfer cells in the green algae *coleochaete*. *American Journal of Botany*, **70**: 113-120.
- Gron D.I. 1946. *Plant Physiology*, **24**: 1-15.
- Hillen, L.W. 1982. Hydrocracking of the oils of *B. braunii* to transport fuels. *Biotechnology and Bioengineering*, **24**: 193-205.
- Lowry, O.H., Rosenbrough, N.J., Farr AL and Randall, R.J. 1951. Protein measurement with Folin phenol reagent *Biochemistry*, **193**: 268-275.
- Manuel A.S. Graca, Felix Barlocher and Mark, O. General Methods to study littler decomposition. *A practical Guide*, 134-139.
- Tanabe Y, Okazaki Y, Yoshida M, Matsuura H, Kai A, Shiratori T, Ishida K, Nakano S, Watanabe MM. 2015. *Sci Rep*. **1(5)**:10467.
- Tenauel, M., Ohmori, M and Miyachi, S. 1989. In organic carbon and acetate assimilation in *B.braunii* (chlorophyta). *Journal of phycology*, **2**: 662-667.
- Tripathi, Usha, Sarada, R and Ravishankar, G. 2001. A culture method for microalgal forms using two-tier vessel providing carbon-dioxide environment: studies on growth and carotenoids production. *Microbiology and Biotechnology*,**17(4)**: 325-329.