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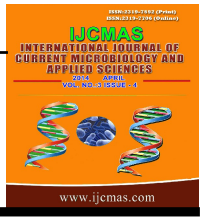

Original Research Article

Production of biofuel by using micro algae (*Botryococcus braunii)*

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A B S T R A C T

Microalgae are sunlight driven cell factories that convert carbondioxide to potential biofuels. The micro algae culture *Botryococcus braunii* was obtained from Algae Depot, USA and was maintained in Chu 13 medium. Further, the culture was mass produced in a specially designed photo bioreactor. The physical and environmental factors were established for the proper growth of the culture. Algal oil was extracted using Soxhlet apparatus through repeated washing or percolation with organic solvent such as hexane and petroleum ether for the 5weeks old culture. The final concentrated compound was separated using aqueous two phase extraction by mixing two incompatible polymers. Then it was purified and subjected to gas chromatography for the analysis. The growth of the culture in a fabricated photo bioreactor was found to be best at a temperature of 23^{o}C, a light intensity of 60 W/M^{2}, with a light period of 12 hours per day, and a salinity of 0.15 molar NaCl and a pH of 7.5. In the laboratory, *B.braunii* is commonly grown in cultures of Chu-13 media. The cell growth reached a maximum of 1.2g L-1 for *B.braunii* after 3 weeks in flasks. The growth was however higher in bubble column photobioreactor reaching a maximum of 4.1g L-1.

Keywords

Botryococcus braunii, microalgae, bubble column photo bioreactor, biofuels, *Saccharomyces cerevisiae*, Soxhlet apparatus.

Introduction

Microalgae are photosynthetic, heterotrophic organisms that have an extraordinary potential for cultivation as energy crops. Microalgae are potential source of biomass, which may have great biodiversity and consequent variability in their biochemical composition (Satyanarayana et al., 2011).

Under difficult agro-climatic conditions and are able to produce a wide range of commercially interesting byproducts such as fats, oils, sugars and functional bioactive compounds (Erika C Francisco *et al.*, 2010).

The Combustion of fossil fuels generates sulfuric, carbonic, and nitric acids, which fall to Earth as acid rain, impacting both natural areas and the built environment. Monuments and sculptures made from marble and limestone are particularly vulnerable, as the acids dissolve calcium carbonate. The algae oil is the best substitute for energy production and energy drink (K.Vairavan *et al*., 2010).

Botryococcus braunii The green colonial hydrocarbon rich unicellular microalgae *Botryococcus braunii* (Banerjee *et al.*, .2002; Metzger and Largeau, 2005) is widespread in freshwater, brackish lakes, reservoirs and ponds. It is also widely distributed in reservoirs at temperate, tropical and arctic latitudes (Tyson, 1995). It is recognized as one of the potent renewable resource for the production of liquid hydrocarbons.*B braunii* is classified into A, B and L races based on the type of hydrocarbons in the range of C21 to C33 odd numbered n-alkadiens,mono-, tri-, tetra-,and pentaenes and they are derived from fatty acids (Banerjee *et al.,* 2002; Metzger *et al*.,2005).The L race yields a single C40 isopropenoid hydrocarbon ,lycopa -14(E), 18(E)-diene (Metzger *et al*; 1990).The B race produces two types of triterpenes called *Botryococcus* of C30-c37 of general formula CnH2n-10 as mojor hydrocarbons and small amounts methyl branched squalene. Certain strains of B race also biosynthesize cyclobotryococcenes (David *et al*.,1988 ;Achitouv *et al*.,.2004). Also a feature common to all the three races is the presence of a highly aliphatic ,non-hydrolysable and insoluble bio macromolecule (algaenan) found in their outer cell walls (Achitouv *et al.*,2004). The highly resistant nature of the *B.braunii* algenn to degradation allows it to selectively preserves during fossilization, leading to fossil *B.braunii* remains, a major contributor to a number of high oil potential sediments (Simpson *et al.,* 2003). The algae *B.braunii* produces oil in the range 0f 29-75% (on dry weight basis). This variation in the content of hydrocarbon is due to the difference in the strains , the race it belongs and also depends on cultural and physiological conditions (Dayananda *et al.*,2006),these fuels were free from oxides of sulphur and nitrogen (SOX and NOX) after their combustion. Being a photosynthetic organism, it can reduce CO_2 emissions by 1.5 x 105 tons/yr and 8.4 x103 ha of microalgal cultivation area would be necessary (Sawayama *et al.*,1999).

Zhang *et al.*,(2010) have used reactors called raceway ponds systems and proposed closed type photobioreactors, for higher photosynthetic efficiency and temperature control of the culture medium. Yusuf Christi *et al.*,(2008) found that, the roles algal cultures can play economically with biofuel production and the long term benefits. According to Katsumi *et al.*, (1987) Algae efficiently use CO_2, and are responsible for more than 40% of the global carbon fixation. Thus this investigation was undertaken to Grow the cultures of *Botryococcus braunii* in photobioreactor and for the Biofuels Production.

Materials and Methods

Sample collection

The culture of *Botryococcus braunii* was obtained from Algae Depot, USA .It was cultivated in Chu-13 (1942) medium in small volumes in conical flasks for the stock culture and then it was inoculated to a shake flask after which it was inoculated to photobioreactor and was maintained at a constant pH 7.5 and a set temperature 23^{o}C.

Shake Flask Culture

The microalgae obtained cultured in 250ml shake flask culture method. It was maintained at room temperature (28$\pm$2) and, the light/dark hour cycle followed was 12:12 in rotary shaker at 100rpm for the adequate supply of oxygen. The

culture was harvested after 3 weeks of incubation and then transferred into photobioreactor.

Bubble column Photobioreactor culture

The 3 weeks old culture obtained from the shake flask was inoculated into bubble column photobioreactor specially designed for the present investigation. The bubble column photobioreactor consisted of two circular concentric glass columns of 30mm inside diameter and 65mm outside diameter. Height of 726 and 711mm/ the nominal working of 4.0L. The dispersion system for the reactor consisted of a 1.5cm diameter air diffuser located at the center of the column. The culture was exposed to the natural day and night photoperiod and red blue light of different wavelengths for the growth rate studies. The culture environment was maintained to a temperature of 23^{o}C, a light intensity of 60 W/M^{2} ,with a light period of 12 hours per day, and a salinity of 0.15 molar NaCl at pH of 7.5. The supplement of CO_2 to the culture would enhance the growth of the algae so, the photobioreactor was designed to supplement at a high rate of 1.5 litres/min air supply through an air sprayer/air stone to create a forced stress on the algae for exchange of gas.

Hydrodynamic Stress

The hydrodynamic stress is brought about by the use of an airlift PBR, which uses a sparger/air stone to propagate the algae from the dark zone to the dark zone to the light zone with high degree of turbulence offered by supplying air.

Biomass Estimation

The biomass yield of microalgae was calculated by using the following equation.

$$\text{Biomass yield content (mg ml}^{-1}\text{)} = \frac{\text{Final weight (mg)-initial weight (mg)}}{\text{Sample taken (ml)}}$$

Lipid Estimation Test

Extraction and fractionation of lipids

The lipid extraction was done by using Bligh and Dyer method 1.0g of the freeze-dried preparation was homogenized in 30ml of chloroform- methanol-water (1:2:0.8, v/v) with a Waring blender, and lipids were extracted after addition of 30 ml each of chloroform and water. The homogenate was filtered through Whatmann filter paper no. 2 on a Buchner funnel. It was repeated for three times and the chloroform layer was evaporated by keeping on a water bath to dryness and total lipids were measured gravimetrically. The silica gel column supplied by Amil was (Ixl5cm) packed with silica gel using 250ml of hexane, 150ml of chloroform, and 150ml of methanol to isolate hydrocarbons, nonpolar lipids except hydrocarbons and polar lipids, respectively to fractionation of total lipids. The isolated lipids in each eluate were measured gravimetrically after evaporation of the solvent.

Analysis of lipids

The composition of each fraction was analyzed by thin-layer chromatography on silica gel 60F254 (Merck, 0.25 mm thick) plates using the following solvents: hexane for hydrocarbons; ; petroleum ether diethyl ether diethyl ether-acetic acid (90 : 10 : 1, v/v) for nonpolar lipids; chloroform-methanol-acetic acid-water (100 : 20 : 2 : 5, v/v) for polar lipids. The spots were visualized by spraying with sulfuric acid-ethanol (1 : 9, v/v) followed by charring. Polar lipids were detected also by specific

spray reagents: Dragendorff and each spots were compared with the literature. For measurement of relative percentage of individual components, the lipids were chromatographed on silica gel-coated glass plates.

It was also calculated by using the following formula,

$$\text{Lipid content (mg ml}^{-1}\text{)} = \frac{\text{Final weight (mg)-initial weight (mg)}}{\text{Sample taken (ml)}}$$

Determination of Alcohols by Gas Chromatography

The algal sample was analyzed by gas-liquid chromatography the test was conducted at Azyme Biosciences Bangalore for the determination of alcohol by gas chromatography.

Production of Biofuels

The extraction thimble was closed with a fat free cotton swab. The thimble was inserted into the soxhlet extractor. The solvent such as hexane, petroleum ether, was filled into the solvent vessel. The oil extract was extracted at a temperature 110-130^{o}C for 10-20extraction cycles. The solvent was drained into a container by opening the spigot to the soxhlet extractor. The solvent vessel was heated until all the solvent evaporated and condensed in soxhlet extractor. The vessel containing the fat residue was placed in a drying oven at 103^{o}C and heated to constant weight.

Lipase catalyzed esterification

Fatty acids after the soxhlet exraction was esterified using immobilized lipase membrane in 50ml stoppered flasks without organic solvent.

Immobilization of lipase on fabric membrane

Lipase was immobilized by using an established immobilization procedure. Briefly, 0.1g of fabric (approximately 9cm^2) is presoaked for 1hour in 10ml of co immobilization solution consisting of 0.5g of gluten, 0.2g of lecithin, 0.2g of polyethylene glycol 6000 and 0.1g of magnesium chloride.

Fabric membranes were dried at room temperature and used as supports for the immobilization of lipase. Membranes were added into 10ml of enzyme solution (5,000 to 10,000 Uw/ml), stirred for 2 to 3 hours, lt was taken out and dried at room temperature under a vacuum. The activity of immobilized lipase was determined by using an olive oil emulsion method after grinding at 0°C, was 10,000 Uw/g membrane.

Production of Bioethanol
Extractive fermentation

The fermentor, input, output and sampling was sterilized by autoclaving. The lipid extracted algae debris after soxhlet extraction and other nutrients were sterilized separately and added aseptically to the reactor before inoculation of *Saccharomyces cerevisiae* (Anuradha Karunanidhi 2008).

After the sterilized medium cooled to the ambient temperature, it was inoculated with 6%v/v of inoculums growth with 1kg.m-3 biomass of algae. Working volume of the reactor was maintained 1liter.

The experiments were conducted in the batch mode. The temperature was maintained at 30^{o}C and the aeration at

0.5VVM. The initial pH was adjusted between 6.5-6.8 with 1M NaOH. The control pH was maintained at 5.0.The agitation was set at 160rpm.These conditions were maintained throughout the chemostat runs.

Results and Discussion

From the present investigation the following results were obtained. The microalgae sample Fig.1a had adapted to the Chu-13 culture medium with microscopic view of the culture Fig. 1b after 3 weeks of incaution in rotary shaker Fig.2, which was monitored in terms of its increase in biomass after which, it was transferred to the photobioreactor. The results were confirmed with the earlier studies made by David *et al.,* 1988. The culture was found to be yield well and the yield was measured by taking the total weight of the biomass and was found to be 1.2g/l from the shake flask studies.

Bubble column Photobioreactor culture

Botryococcus braunii inoculated to the photobioreactor showed much increase in the growth rate and the lipid content compared to that of shake flask culture. However, the apparatus designed and fabricated for the study was found to be suitable because the light intensity, temperature, humidity and co_2 supply was found to be more appropriate for the yield of total biomass from the algal sample when compared to the shake flask studies Fig. 3a & b. The result found to be 4.1g/l.

Growth rate

The growth rate curves of *Botryococcus braunii* in shake flasks and bioreactor are depicted in Fig.4..the cell growth reached a maximum of 1.2g L-1 for *B.braunii* after 3 weeks in flasks. The growth was however higher in photobioreactor ,reaching a maximum of 4.1g L-1. The results are concurrent with the earlier reports made by Chisti, Y. 2008.

Lipid Estimation

The total lipid was measured gravimetrically by using following formula.and the data was obtained as shown in the Table.4.1.

From the Table-1 It was found that, increase in the lipid content as the days increases. Maximum lipid content was found to be 78% later on the percentage of lipid content was showing constant as the increasing age of culture. The graph plotted, when compared with the investigation done by Katsumi *et al.,* 1987 was found to be 71.6% whereas in our investigation it is observed to be 78% of lipid content from the *B. braunii* grown in a bubble column photobioreactor supplemented with Chu 13 medium after five weeks of culture.

Determination of Alcohols by Gas Chromatography

The five weeks old sample when subjected to Gas chromatography, chromatogram of the sample was found similar to that of methanol chromatogram (Fig.6 & Fig.7). The peak area of the sample was calculated by comparing to the peak area of methanol and the percentage of

Fig.1a. Algal sample obtained from US depot. b. Microscopic image of *Botryococcus braunii*

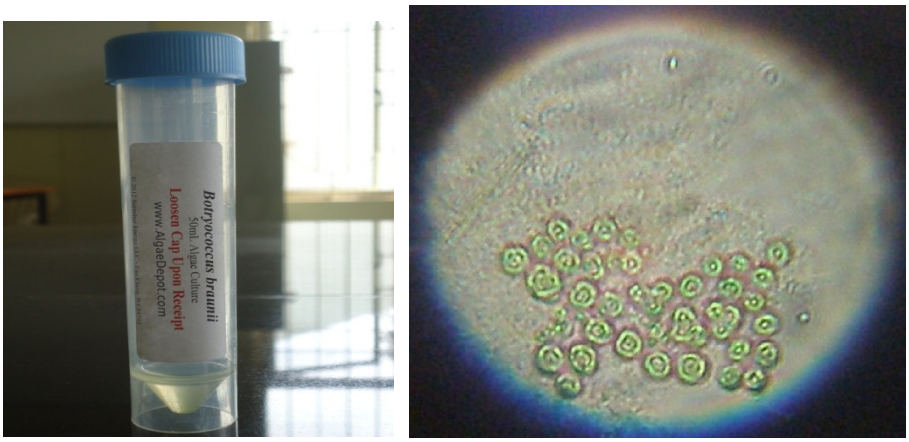


Fig.2 Weeks old Shake Flask Culture

Fig.3 Weeks old Bubble column Photobioreactor culture

Fig.4 Biomass growth rate curve of *Botryococcus braunii*

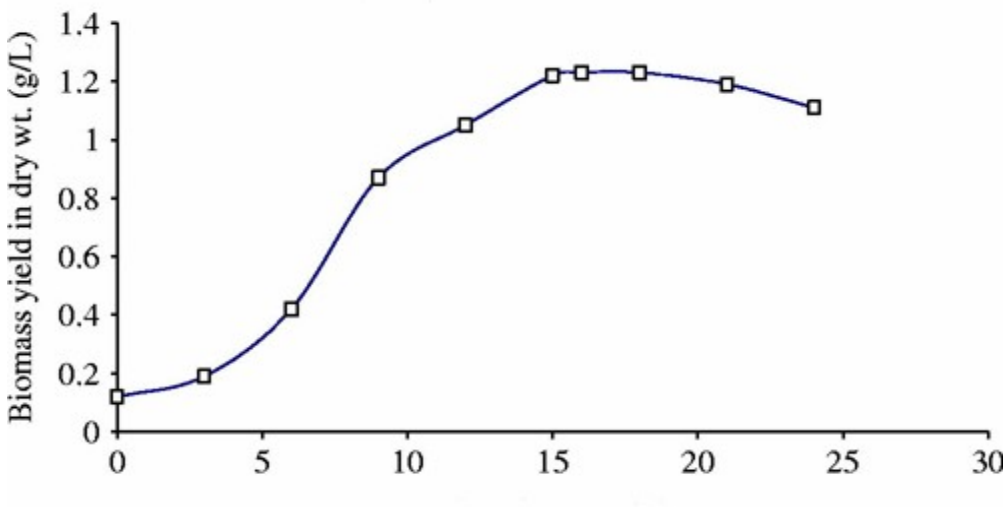


Table.1. Showing the percentage of oil in terms of grams

Age of culture (days)	Dry weight (grams)	Lipid extract weight (grams)	% Lipid content
0	5	0	0
7	5	0.45	9
14	5	0.91	18.2
21	5	1.8	36
28	5	2.89	57.8
35	5	3.9	78
42	5	3.8	78

Fig.5 Percentage v/s time in days, tested for lipid content

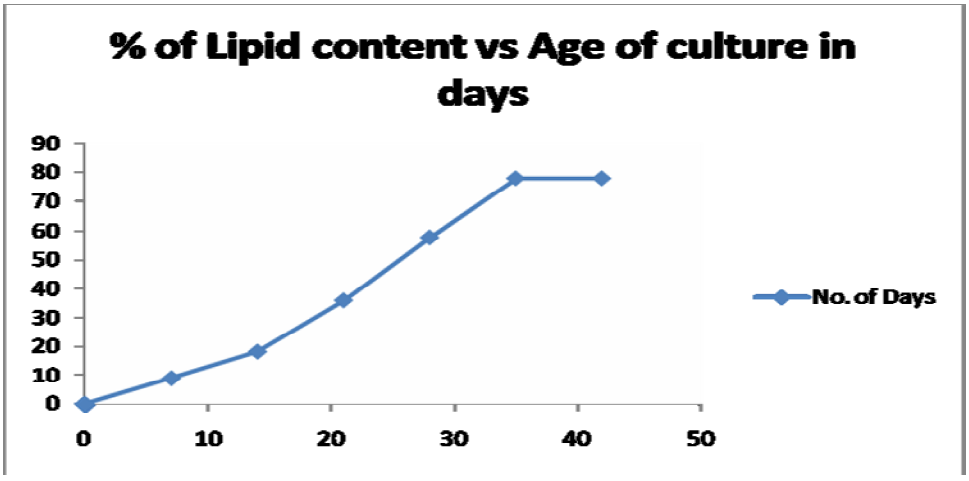


Fig.6 Chromatogram of Methanol

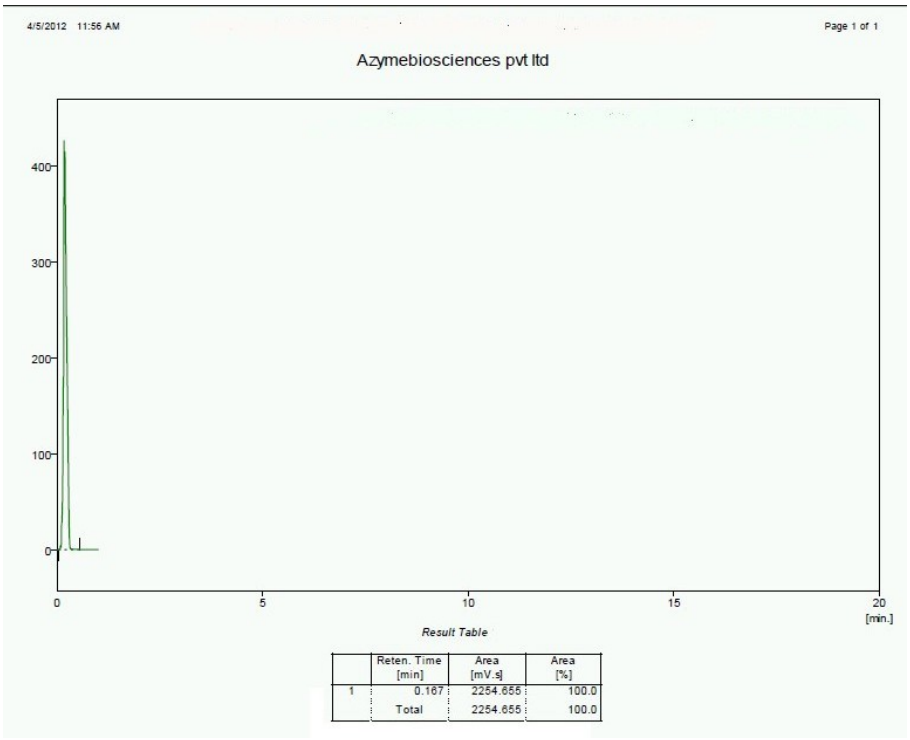
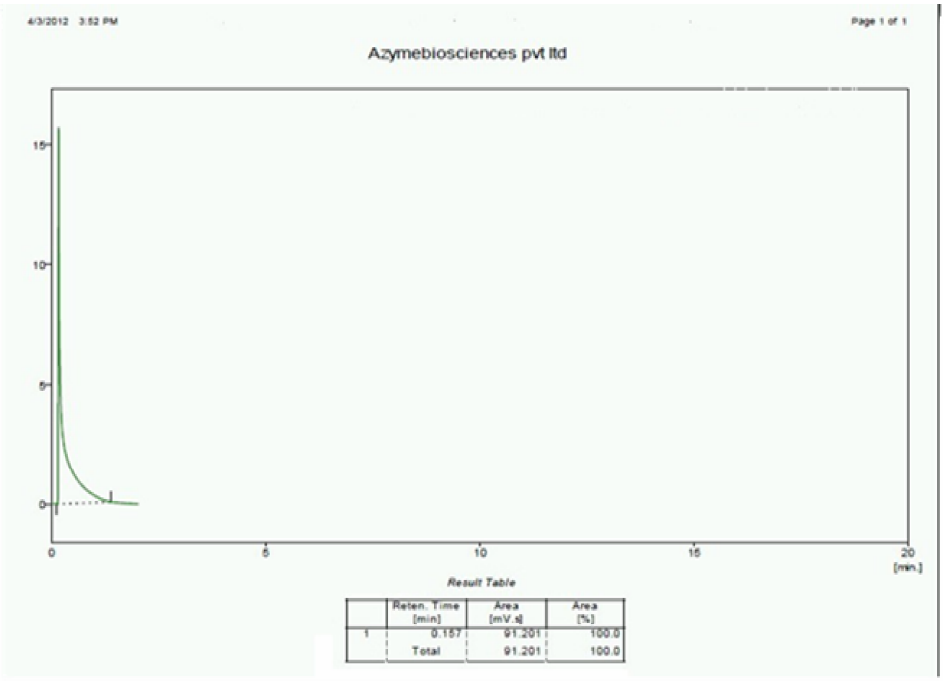


Fig.7 Chromatogram of Algae Sample



methanol in algae sample was found out by the formula.

Production of Biofuels

Algae oil extraction by Soxhlet extraction method

After the extraction, the solvent was drained into a suitable container by opening the spigot on the soxhlet extractor. The solvent vessel was heated until the solvent was evaporated and the algae oil was obtained.

Lipase catalyzed esterification

There was no separation of aqueous and organic phases when it was left undistributed after stirring slowly for 30minutes. The production of biodiesel by esterification was not possible for the *Botryococcus braunii* oil and it was later studied Aquatic Species Program (ASP) report that the *Botryococcus* oils are not fatty acid trigycerides but they are triterpenes and they lack the free oxygen atom needed for esterification process. Later the Botryococcus oil was used as a feedstock for hydrocracking.

In conclusion, the biomass yielded in a bubble column photobioreactor system. Subjected to extractive fermentation, for the lipid content and methanol production was found to be successful in production of biofuel from the microalgae *Botryococcus braunii*.

Acknowledgement

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