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### RESEARCH ARTICLE

#### STUDIES OF BIOENERGY COMPOUNDS AND MOLECULAR CHARACTERIZATION OF BOTRYOCOCCENE SYNTHASE GENE FROM *BOTRYOCOCCUS BRAUNII* KUTZING.

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#### Abstract

Advanced generation biofuel production from microalgae *Botryococcus braunii* was investigated. The micro alga *Botryococcus* was first discovered and described by Kutzing in 1849 which has drawn more attention in the recent decades due to high accumulation of different types of hydrocarbons. The microalga *Botryococcus braunii* showed the growth kinetic and biochemical parameters include dry biomass, carbohydrate, protein and lipids. The extraction of fatty acid methyl esters (FAME) from the biomass of the microalga *B. braunii* were analyzed for biofuel compounds using gas chromatography mass spectrometry (GC-MS) and Fourier transform infra-red spectroscopy (FT-IR) the percentage of FAME were resulted. The presence of esters, alkanes and alkenes confirms the presence of hydrocarbons from the FAME. The phylogenetic relationship of the microalga *Botryococcus braunii* was analyzed by ClustalW and Neighbor joining method in MEGA5 program using the botryococcene gene. The dendrogram clearly shows that the isolated microalgal strain belongs to *Botryococcus braunii*.

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#### Introduction:-

The population explosion along with economic growth in the developing countries like India and China has major focus on transportation which led to rapid increase in demand for energy all over the world. The biofuel has the efficiency to become a sustainable and renewable energy because of the carbon released into air by combustion of fossil fuels can be recycled in the form of biochemical and biomasses.

The micro alga *Botryococcus* has drawn more attention in recent decades due to high accumulation of different types of hydrocarbons (Borowitzka, 1988). The *Botryococcus braunii* Kutzing is a green colonial micro alga occurs in fresh water, brackish water, reservoirs and ponds which are cosmopolitan in nature (Metzger and Largeau, 2005; Banerjee *et al.*, 2002).

The hydrocarbon fraction of *Botryococcus braunii* has three different types of races such as (A, B and L) Squalene synthase, lycopadiene and botryococcene. The *Botryococcus* race B from Sumatran crude oil comprises of 1.4 % of botryococcene and available as completely hydrogenated form (Moldowan and Seifert, 1980). The Brauniaceae, a

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new isoprenoid hydrocarbon ( $C_{33}H_{54}$ ) membrane fused botryococcene family was the first time reported from the Berkeley strains of *B. braunii* and constituted about 28-34 % from dry biomass (Huang *et al.*, 1987). Both oleic acid and palmitic acid are the fundamental components of lipid and as a precursor for hydrocarbon accumulation in *B. braunii* (Yamaguchi *et al.*, 1987).

## Materials and Methods:-

### Fresh water sample collection:-

Sample collection were made by random sampling method from fresh water bodies at Mylapore and Triplicane temple tanks Chennai, Tamil Nadu, India. The predominant criterion for sample collection was the evident abundance of microalgae in the water, 1000 mL of water samples was collected from the each sampling site.

### Shake Flask Culture:-

At this stage 25 mL culture in Erlenmeyer Flasks was shaken manually twice a day in a full strength medium where sub cultured for further analysis. Most samples were enriched by this way for dominant strains until the samples taken for serial dilution or streaking on to solid media used for strain isolation. The isolated microalgae were studied under a compound microscope (OLYMPUS CH20i) and photographs were taken. The micro algae were identified based on the morphological characteristic features using 'Microalgae Identification for Aquaculture', by Rosen (1990).

### Growth kinetics of the microalga *Botryococcus braunii*:-

The green microalga *Botryococcus braunii* isolated and pure culture was maintained in the Algal culture collection laboratory, Department of Plant Biology and Plant Biotechnology, Presidency College (Autonomous), Chennai-600005. The microalga *Botryococcus braunii* was allowed to grown in a Bold Basal Medium (BBM) liquid broth (400 mL in 1l Erlenmeyer flask with 4 % inoculums) and incubated for 16 days under 12:12 hrs of dark and light (Crompton 40 W, Cool day light 6500 K at an intensity of 2000 lux.) at room temperature (28°C). The optical absorbance values were analyzed for 16 days at 680 nm in an UV-Vis. Spectrophotometer (Hitachi U-2900). The Growth kinetics (K) and generation times (G) of all the microalga *Botryococcus braunii* was calculated by the following equations 1 and 2 (Qin, 2005).

Equation 1:

$$K = (\log OD_f - \log OD_i) / T \times 3.322$$

Where,

OD<sub>f</sub> : final optical density, OD<sub>i</sub> : initial optical density, T : time in days

Equation 2: Following equation was used for Calculation of generation time (G) in days:

$$G = 0.301 / K$$

### Harvest and estimation of dry biomass:-

The biomass after *in vitro* mass cultivation was harvested by centrifugation at 8000 rpm for 5 min. at 20°C. The concentrated biomass in the pellet was obtained and mixed using a cyclomixer and transferred to a watch glass, which was then incubated in an incubator at 35°C for overnight. The dry biomass was wiped using a sharp sterilized surgical blade and weighed gravimetrically using an electronic weighing balance.

## Biochemical constituents:-

### Estimation of total Chlorophyll a and Chlorophyll b

The green photosynthetic pigment Chlorophyll a and Chlorophyll b were estimated by Jeffrey and Humphrey (1975) method and its equation given below.

$$\text{Chlorophyll } a = 11.93E664 - 1.93E647 \mu\text{g mL}^{-1}$$

$$\text{Chlorophyll } b = 20.36E647 - 5.50E664 \mu\text{g mL}^{-1}$$

The absorbance values at 664 and 647 nm were recorded daily for about 16 days using a UV-Vis. Spectrophotometer.

### Estimation of total carotene:-

The total carotene content of the green microalga *Botryococcus braunii* was determined everyday up to 16 days based on the formula is given below.

$$\text{Carotene } (\mu\text{g/mL}) = A_{450} \text{ nm} \times 25.2$$



**Extraction and estimation of carbohydrate:-**

The total carbohydrate of green algae *B. braunii* was determined based on the method well defined by Dubois *et al.* in the year 1956 (Phenol Sulphuric acid method). About 100 µl of the crude sample was subjected to treat with 5% phenol and 2.5 mL of Conc. Sulphuric acid. Then the whole content was incubated for 10-15 min. at room temperature. Then the optical absorbance values were measured at 490 nm using a UV-Vis. Spectrophotometer. The total carbohydrate content was determined by comparing the absorbance values with the standard graph using D-glucose as a known carbohydrate sample.

**Extraction and estimation of protein:-**

The estimation of protein of green algae *B. braunii*, in this present study was done based on the method described by Bradford (1976). The protein content of the unknown sample was determined in comparison with the standard graph of the known sample. The standard graph for protein was constructed using Bovine serum albumin as a standard known protein. About 1 mL of the crude sample used to determine the total protein content with 5 mL of the Bradford reagent. After incubation for 15 min. the content was subjected to determine the absorbance values at 595 nm in a UV-Vis. Spectrophotometer.

**Extraction and estimation of lipid:-**

The total lipid constituent of green algae *B. braunii* was determined based on the method described by Folch *et al.* in 1957. A 6 mL of chloroform and methanol (2:1 ratio) was mixed with the extract and vortexed for 3 min. at room temperature. The extract was then centrifuged at 8000 rpm for 5 min. The resultant liquid phase was washed with 6 mL of 0.9% of NaCl and vortexed for 5 min. The entire content was then centrifuged at 2000 rpm for 5 min. which resulted to form two distinct phases. The upper phase conserved due to its richness in lipid and the lower aqueous phase was discarded. The total lipid content was determined gravimetrically after the complete evaporation of the solvent (Upper phase).

**Extraction and analysis of fatty acid methyl esters (FAME) by gas chromatography:-**

About 20 mL of green algae *B. braunii* sample was subjected to direct Transesterification. About 1 mL of reagent 1 was poured to the fresh algal samples and vortexed for 5-10 sec. using a cyclomixer. The mixture was incubated in a water bath at 100°C for 5 min. and again vortexed for 5-10 sec. Then after incubated at 100°C for 25 min. reagent 2 was added and vortexed for 5-10 min. followed by thermal incubation at 80°C and rapidly cooled down to 4°C. Reagent 3 was added the mixture and mixed gently up to 10 min. Two phases were seen blatantly from which, the lower phase was conserved. To the content 3 mL of reagent 4 was added followed by mixing for 5 min. and obtained upper phase was removed. The lower phase rich in fatty acid methyl esters were stored in a vial at 4°C in a refrigerator.

The tranesterified fatty methyl esters were analyzed with the help of a gas chromatography equipped with flame ionization detector (FID) (Perkin Elmer, USA). A SP-2560 column (100 m × 0.25 mm I. D., 0.20 µm) (Sigma, Germany) along with standard fatty acid Supelco 37 Component FAME mix from Supelco (Bellefonte, PA, USA) was employed. About 5 µl of the sample was injected and the GC conditions were injector temperature: 260°C; Column temperature: 140°C and detector temperature: 260°C. Helium was used as a carrier gas with the flow rate of 1 mL/min. The unknown FAMES were determined in comparison with the retention times of the standard FAMES (Supelco) using a mass spectra from NIST library.

**Fourier transforms infra red spectrometric (FT-IR) analysis of FAME:-**

The FAME sample were analyzed under infra red (Perkin Elmer model spectrum – I PC). The FT-IR spectra with the resolution of 4 cm<sup>-1</sup>, Scan Number: 3 were performed after the evaporation of the lipid fraction on Thallium bromide tablets. The FT-IR spectrums of all the FAME samples were obtained as a percentage of transmission ranged from 450 cm<sup>-1</sup> to 4000 cm<sup>-1</sup>.

**Molecular identification of *Botryococcus braunii*:-****Isolation of RNA:-**

About 300 µl of the culture was ground into fine powder in liquid nitrogen using a mortar and pestle. A 2 mL of solution I was added while grinding to make homogenous mixture and allowed to thaw completely with intermittent grinding. Then 800 µl of nuclease free water was added by mixing and grinding. The whole content was transferred to 2 mL microcentrifuge tubes and kept undisturbed place for 5 min. at room temperature. Chloroform (200 µl) was added to each tube and vortexed briefly for less than 10 seconds and kept undisturbed for 10 min. at room



temperature. Then the whole mixture was allowed to centrifuge for 10 min. at 4°C and the upper aqueous phase was transferred into fresh microcentrifuge tubes. Then about 0.6 volumes of isopropanol were added and vortexed briefly less than 10 seconds and left for 10 min. at room temperature. Then after centrifugation at 13,000 rpm for 10 min. at 4°C the supernatant was discarded and the obtained pellet with RNA was washed with 70 % ethanol, air dried and dissolved in 50 µl of DEPC-treated water. The extracted RNA was separated and analyzed using 1.5 % Agarose in submarine gel electrophoresis.

**Solution I:-** Phenol saturated with tris (hydroxymethyl) aminomethane buffer to a pH of  $6.7 \pm 0.2$ . To this sodium dodecyl sulphate [SDS; 0.1% (w/v)], sodium acetate [NaOAc; 0.32 M (w/v)] and ethylenediaminetetra acetic acid (EDTA; 0.01 M final concentration from a stock solution of 0.5 M, pH 8.0) were added.

#### cDNA synthesis:-

The first strand of cDNA was synthesized using 3 µl of RNA sample with SMART IV oligonucleotide and CDS/3' PCR primer 1 µl each. After thorough mixing the content was incubated at 72°C for 2 min. and cooled on ice for 2 min. After spinning briefly, 5X first strand buffer (2 µl), DTT (20mM) (1µl), dNTP mix (10mM) (1µl) and Power script RT (1µl) were added and mixed. After incubation for 1 hr at 42°C, the content was stored at -80°C in an ice.

#### PCR amplification:-

A PCR was performed in a total volume of 30µl containing 15µl master mixture, 1µl of µM each of primer and 1 µl of cDNA template. PCR conditions were as follows: denaturation at 94°C for 5 min; 35 cycles of 94°C for 1 min, primer-specific annealing temperature at 45 sec and extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The PCR products were resolved by electrophoresis in a 1% agarose gel in  $1 \times$  TAE buffer. The gels were pre-stained with 10 mg/mL ethidium bromide.

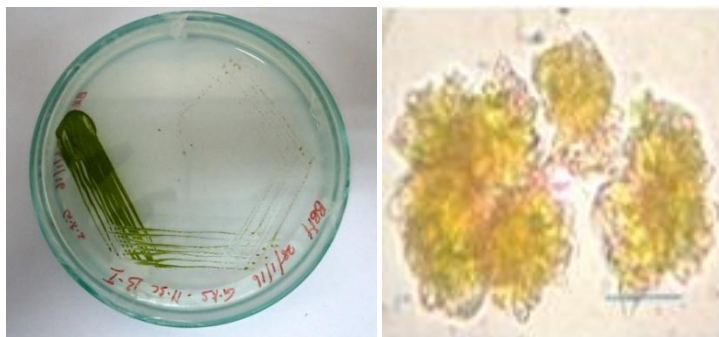
#### Gene sequence and submission to NCBI GenBank:-

Amplified PCR product purified using Qiaquick PCR purification kit (QIAGEN, USA). Sequencing reactions were carried out in both directions using same forward and reverse primers used for amplification with BigDye Version 3.1 kit (Applied Bio-systems) on an ABI-PRISM 3730 DNA Sequencer (Applied Bio-systems). Ambiguous sequences from the base called sequences were corrected with Chromas (Version 2.01) and the sequences were assembled with Bio-Edit (Version 7.0.9.0). The search for sequence homolog of potential gene product was made using the BLASTn and BLASTx program (NCBI).

#### Results:-

##### Microscopical identification of *Botryococcus braunii*:-

In the present investigation, *Botryococcus braunii* was microscopically identified from the collected fresh water samples. The identified *Botryococcus braunii* was presented in Fig.1. The identification of microalgae was based on their distinctive characteristics (Butcher, 1959; Carmelo and Grethe, 1997) and the instruction for microscopical identification was suggested by the Krishnamurthy Institute of Algology, Chennai.



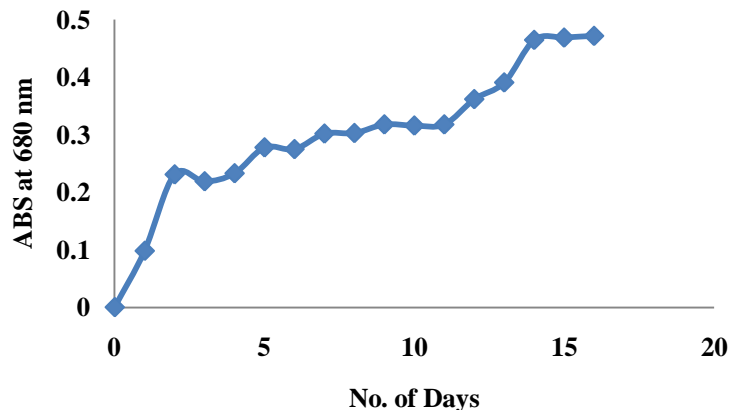
**Fig. 1:-** *Botryococcus braunii*.

##### Growth kinetics of the microalga *Botryococcus braunii*:-

The growth curve has blatantly shown that the log phase was initiated from the second day and attained stationary phase from third day till the sixteenth day (Graph 1). The death stage was not occurred up to sixteen days instead all



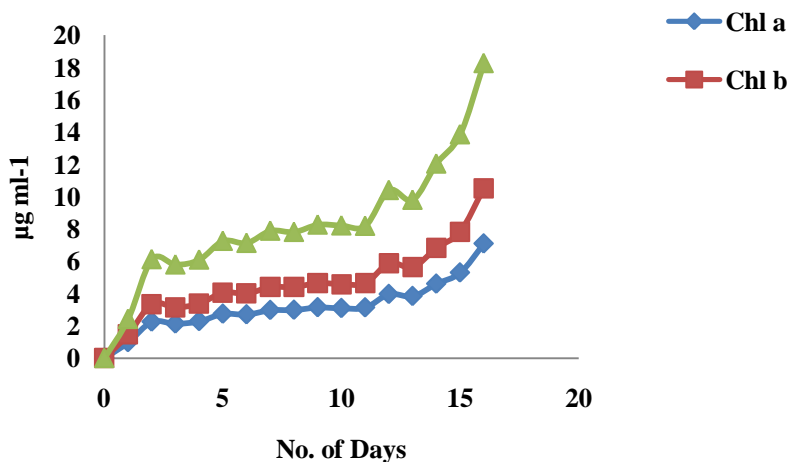
the biomass was settled at the bottom of culture flask. The microalga *Botryococcus braunii* attained the growth kinetic (K) of 0.099 and the generation time (G) was 3.09.



**Graph 1:-** Growth curve of the green microalga *Botryococcus braunii*.

#### Estimation of pigments:-

The important photosynthetic pigments of green microalgae *B. braunii* including chlorophyll a and b were found to be  $7.09 \mu\text{g ml}^{-1}$  and  $10.51 \mu\text{g ml}^{-1}$  respectively. The total carotene was found high with  $18.27 \mu\text{g ml}^{-1}$ , when compared with the chlorophyll pigments. The production of pigments seems to be correlated with the proliferation of the microalga *Botryococcus braunii*. Where, hike in the pigment production starts from the third day and found approximately stable in the stationary phase and propelled further during the last 14<sup>th</sup>, 15<sup>th</sup> and 16<sup>th</sup> days (Graph 2).

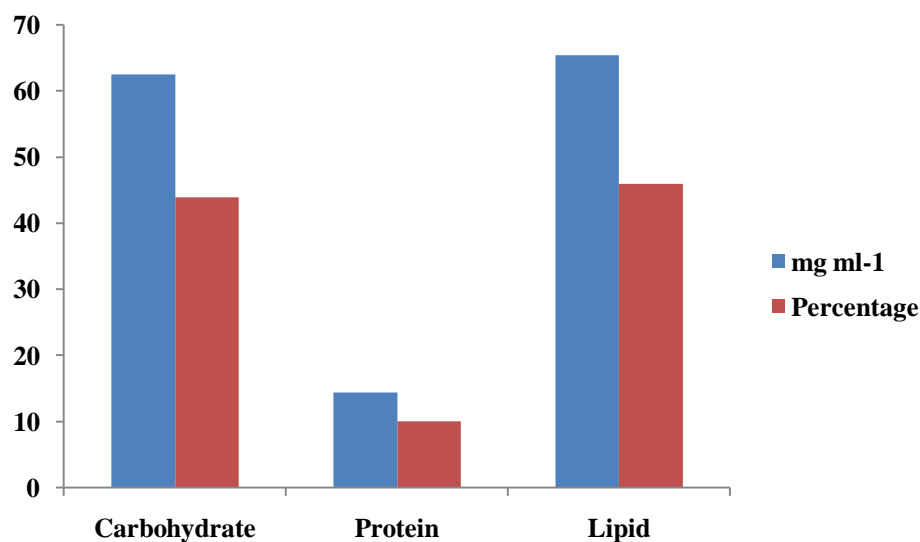


**Graph 2:-** The figure shows the production of the three important pigments by *Botryococcus braunii*

#### Biochemical constituents:-

After *in vitro* mass cultivation, the harvested biomass was dried and the dried biomass estimated in an electronic weighing balance which was about  $236 \text{ mg ml}^{-1}$ . From the obtained biomass, total carbohydrate, total protein and total lipid content was estimated. The total carbohydrate content was  $62.57 \text{ mg ml}^{-1}$  which engulfs about 43.93 % of total biochemical composition. The total protein comprises 10.11 % of the total biochemical constituents with  $14.4 \text{ mg ml}^{-1}$ . The total lipid content was  $65.46 \text{ mg ml}^{-1}$  and holds 45.95 % of the total biochemical component (Graph 3). On the whole, it is evident that the lipid and carbohydrate content was produced high by the green microalga *Botryococcus braunii*, but the protein content was approximately and comparatively half when compared with the total lipid content.

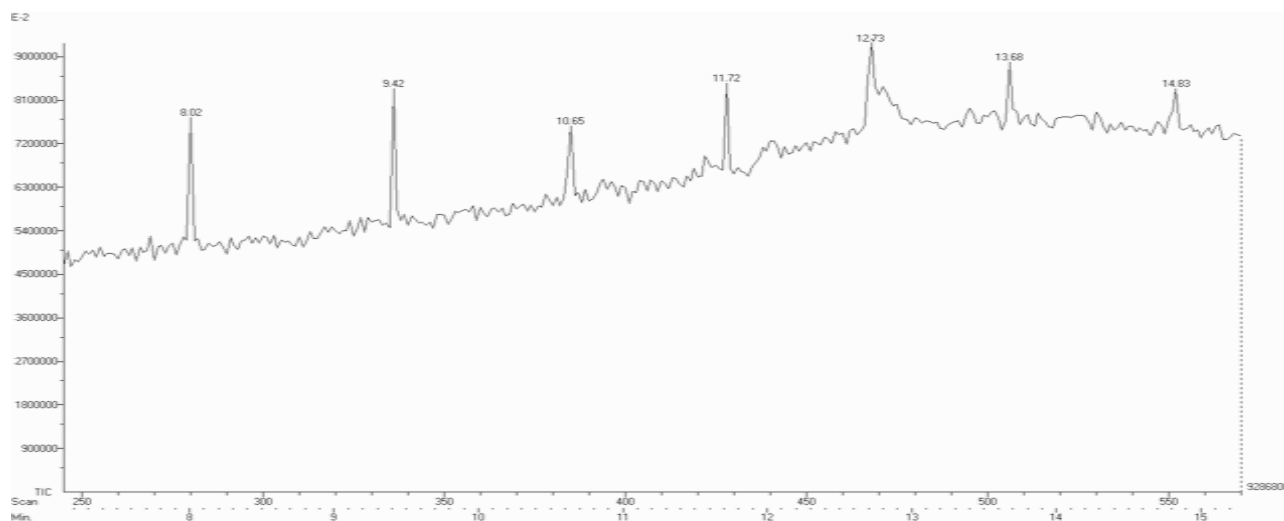




**Graph 3:-** Biochemical constituents of *Botryococcus braunii*.

**Analysis of fatty acid methyl esters (FAME) by gas chromatography and mass spectrometry (GC-MS):-**

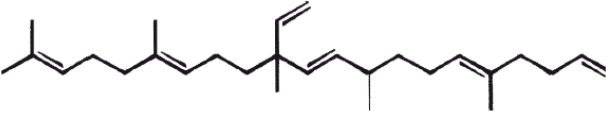
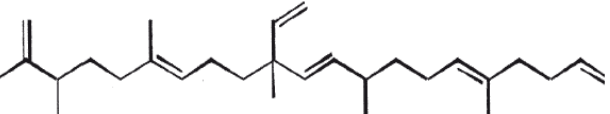
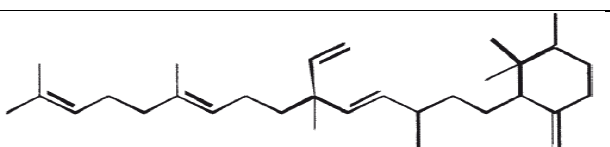
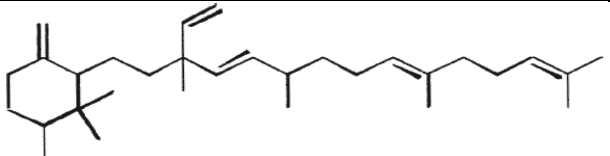
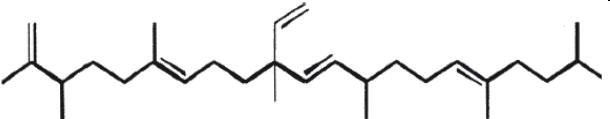
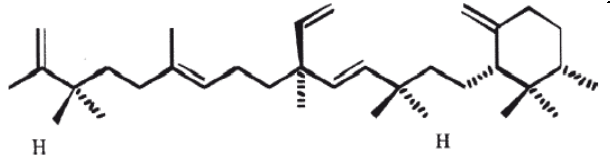
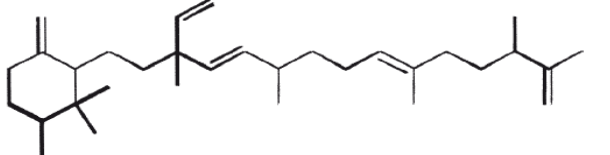
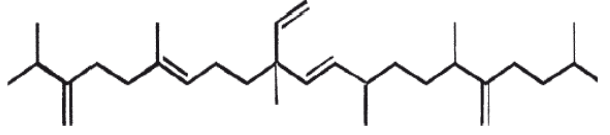
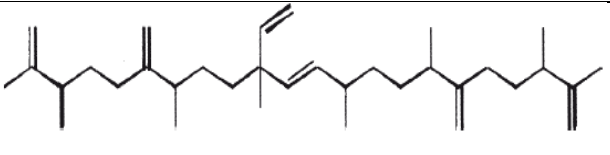
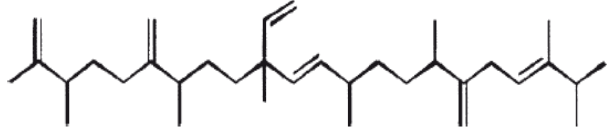
The total fatty acid methyl esters obtained in the *Botryococcus braunii* strain was about 64.26 % of the total lipid content (Fig. 5). The fatty acid methyl esters include Propanoic acid C27:0 (22.60 %); Propanoic acid C27:0 (15.65 %); 9-octadecenoic acid C38:0 (15.21 %) and Carotenoic acid C27:0 (10.8 %) (Table: 1) (Graph: 4). The presence of high cetane number compounds in the FAME of *Botryococcus braunii* represents the occurrence of long chain fatty acids and hydrocarbon.



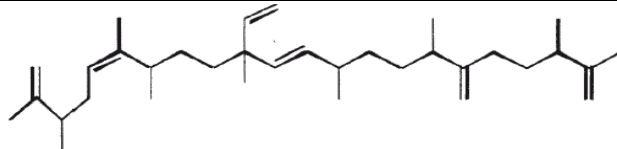
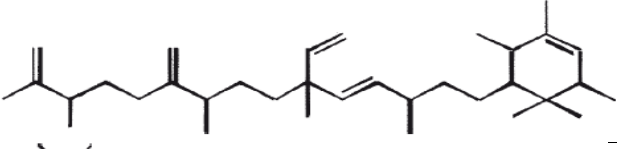
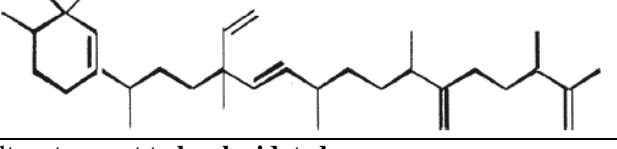
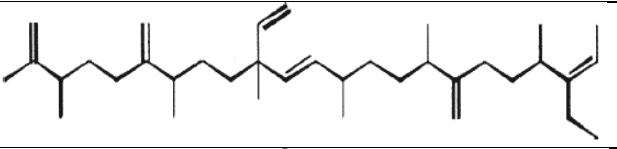
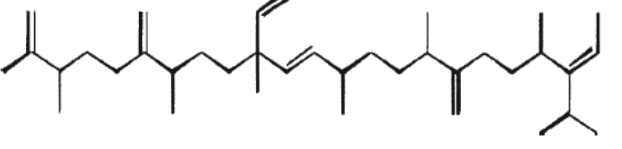
**Graph 4:-** GC Chromatogram of FAME of *Botryococcus braunii*.



**Table 1:-** Some of the important hydrocarbons and its molecular structures produced by *B. braunii* race B (Banerjee *et al.*, 2002)

S. NO.	HYDROCARBONS	MOLECULAR STRUCTURE
1.	$C_{30}H_{50}$	
2.	$C_{31}H_{52}$	
3.	$C_{31}H_{52}$ (Wolfine)	
4.	$C_{31}H_{52}$ (Isowolfine)	
5.	$C_{32}H_{54}$	
6.	$C_{32}H_{54}$ (Meijicoccene/Braunicene)	
7.	$C_{32}H_{54}$ (Isobraunicene)	
8.	$C_{33}H_{56}$	
9.	$C_{34}H_{58}$	
10.	$C_{34}H_{58}$ (Isobotryococcene)	



11.	$C_{34}H_{58}$	
12.	$C_{34}H_{58}$	
13.	$C_{34}H_{58}$	
14.	$C_{35}H_{60}$ (strain isolated from Darwin lake, Australia)	Structure yet to be elucidated
15.	$C_{36}H_{62}$ (Darwinene)	
16.	$C_{37}H_{64}$	

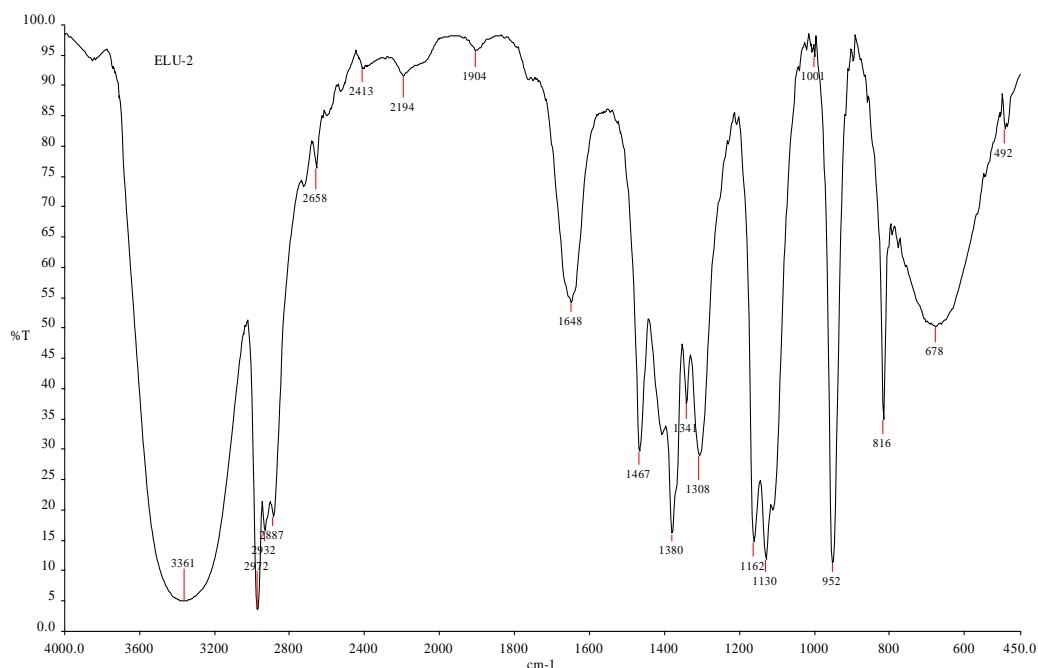
#### Fourier transforms infra red spectrometric (FT-IR) analysis of FAME:-

The alkane (C-H) and alkyne ( $C\equiv C$ ) groups were determined in the FAME samples of *Botryococcus braunii* at 2972, 2932, 2887, 1467, 1380  $cm^{-1}$  and 2194  $cm^{-1}$  respectively. The presence of alkenes ( $C=C$ ) were determined by the occurrence of peak at 952  $cm^{-1}$ . The esters were also reported at 1308  $cm^{-1}$  and as a result the FAME is rich in long chain hydrocarbon compounds (Table 2) (Graph. 5).

**Table 2:-** FT-IR of Functional Groups of Constituents from *Botryococcus braunii*.

Peaks	Bonds	Functional groups
3361 $cm^{-1}$	N-H	1°, 2° amines, amides
2972, 2932, 2887 $cm^{-1}$	C-H	Alkanes
2658, 2413 $cm^{-1}$	H-C=O: C-H	Aldehydes
2194 $cm^{-1}$	$C\equiv C$	Alkynes
1904 $cm^{-1}$	C=O	Carbonyls (general)
1648 $cm^{-1}$	N-H	1° amines
1467, 1380 $cm^{-1}$	C-H	Alkanes
1341 $cm^{-1}$	N-O	Nitro compounds
1308 $cm^{-1}$	C-O	Alcohols, carboxylic acids, esters, ethers
1162, 1130 $cm^{-1}$	C-H $-CH_2X$	Alkyl halides
1001 $cm^{-1}$	C-N	Aliphatic amines
952 $cm^{-1}$	$C=C$	Alkenes
816 $cm^{-1}$	C-Cl	Alkyl halides
678, 492 $cm^{-1}$	C-Br	Alkyl halides



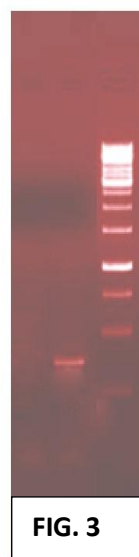


**Graph 5:-** FT-IR spectrum of the FAME of *Botryococcus braunii*

### Molecular identification:-

#### Isolation of RNA and cDNA synthesis:-

The total RNA was extracted from the green microalga *Botryococcus braunii* was then separated and analyzed under submarine agarose gel electrophoresis. Two different bands in the gel shows mixed of RNA where the major RNAs are 28S and 18S rRNA (Fig. 2). The cDNA was synthesized using the SSL-3 primer (Table 3) already mentioned above and were qualitatively confirmed by 1.5 % agarose gel electrophoresis. Where, the single band shows the cDNA synthesized and were allowed to sequence based on the Sanger's method of dideoxy sequencing (Fig. 3). The SSL-3 gene sequence was submitted to NCBI through BankIt, GenBank and the temporary BankIt submission number was retrieved (BankIt submission no. 1897142) and the accession number yet to be received.



**Fig. 2:-** The bands in the agarose gel image showing the extracted RNA from *Botryococcus braunii*.

**Fig. 3:-** The single band in lane 1 shows the cDNA of the gene Botryococcene synthase



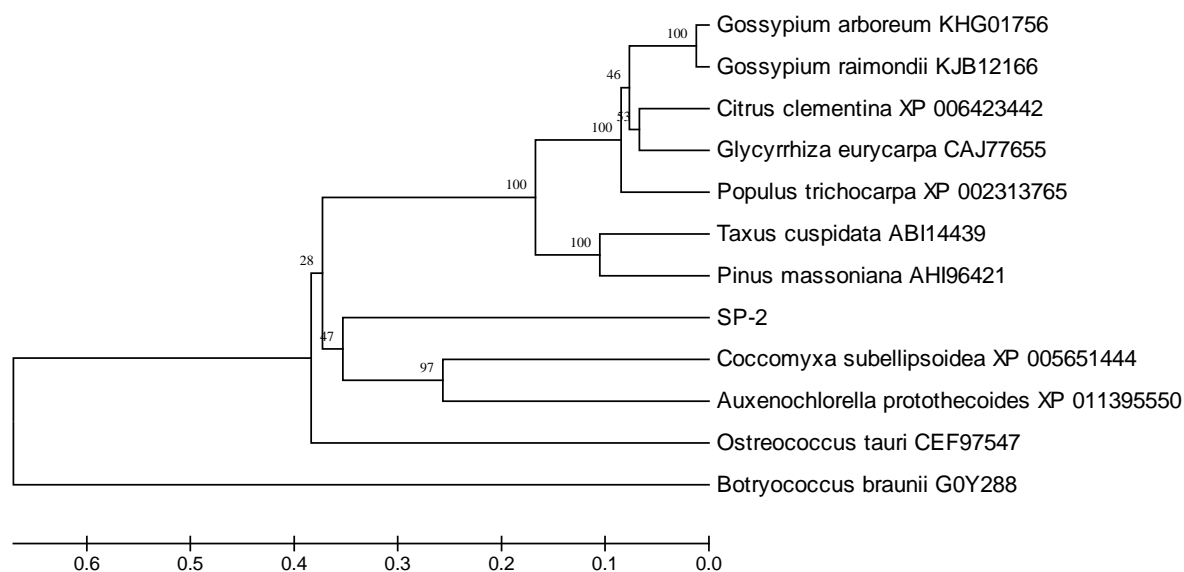
**Table 3:-** Primer details used for amplification.

Gene (Accession no)	Primer sequence (5'-3')	Annealing temperature °C	Size (bp)
<b>Squalene synthase-like 3 (HQ585060)</b>	F:TCGGGAAGTCTTGCAGCACC R:AAGCACCTTAGCTGAAACCTT	54.9	250

**Phylogenetic analysis:-**

The dendrogram represents the phylogenetic relationship of the microalga *Botryococcus braunii* with other group of organisms using the gene SSL-3 (Squalene Synthase like – 3 or Botryococcene synthase) (Fig. 4). In which, the SP-2 is the sequenced SSL-3 gene of *Botryococcus braunii* placed in a separate clade along with the green microalgae. Another phylogenetic tree was constructed using the NCBI BLAST Pair wise alignment of Neighbour joining method with maximum sequence difference of 0.005. Where, a unique clade was formed (1CI|Query\_224699) between the similar strains of *Botryococcus braunii* and which molecularly confirms that the gene belongs to SSL-3 and the strain belongs to *Botryococcus braunii* (Fig. 5).

The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 2.34483239 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown above the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [3] and are in the units of the number of amino acid substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 12 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 408 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

**Fig. 4:-** Evolutionary relationships of Botryococcene synthase (SSL-3) obtained from *Botryococcus braunii*.





**Fig. 5:-** The phylogenetic tree of the gene Botryococcene synthase gene from *Botryococcus braunii* constructed using NCBI.

### Discussion:-

Mogilevskii and his associates investigated the microbial Petroleum prospecting incited the interest of petroleum geochemists worldwide. Initially proposed and applied the microbial surveys in the USSR (Mogilevskii, 1940). It has been shown that out of 20 microbial anomalies, 16 were proved successful drilling. These investigations initiated the interest of petroleum geochemists worldwide. In USA microbial prospecting surveys showed positive correlation about 85.7% respectively (Sealy, 1974). In oil field of microbial survey at USA indicated that good contrast nearby dry area (Miller, 1976).

Microalgae are the major sources of lipids in lacustrine and marine environments. Volkman *et al.* (1989) provides a review of some recent advances in our knowledge of the wide variety of lipid types that have been isolated from microalgae with an emphasis on those likely to be useful biomarkers for identifying sources of organic matter in sediments. In many numbers of microalgae species were found among these *Botryococcus* sp. is a remarkable due to its capacity for synthesizing major amount of unsaturated hydrocarbons (up to 75 % from dry biomass). Similar to those from oil deposits (Moldowan and Seifert, 1980), physical and chemical analysis of kerogen from boghead coals identified in different geographical regions, presented a similar composition with *B. braunii* hydrocarbons.

Over recent years, many new compounds have been identified in sediments deposited in marine and lacustrine environments. Despite the fact that our knowledge of algal lipids is still far from comprehensive, microalgal sources have now been identified for many of the lipids that are widely distributed in recent sediments (Volkman *et al.*, 1995; Conte *et al.*, 1994). Volkman *et al.* (1998) provides a review of some recent advances in our knowledge of the wide variety of lipid types that have been isolated from microalgae with an emphasis on those likely to be useful biomarkers for identifying sources of organic matter in sediments. From the obtained biomass, total carbohydrate, total protein and total lipid content was estimated. The total carbohydrate content was 62.57 mg ml<sup>-1</sup> which engulfs about 43.93 % of total biochemical composition. The total protein comprises 10.11 % of the total biochemical constituents with 14.4 mg ml<sup>-1</sup>. The total lipid content was 65.46 mg ml<sup>-1</sup> and holds 45.95 % of the total biochemical component (Fig. 4.3). On the whole, it is evident that the lipid and carbohydrate content was produced



high by the green microalga *Botryococcus braunii*, but the protein content was approximately and comparatively half when compared with the total lipid content.

Microalgae are major source of fatty acids in most sedimentary environments. The contribution from different microalgal classes can often be discerned from characteristic differences between the distributions, especially if the positions of double bonds in polyunsaturated fatty acids are considered (Volkman and Johns, 1977). Some microalgae contain high concentrations of certain long-chain essential polyunsaturated fatty acids such as 20:5n-3 and 22:6n-3 (Volkman *et al.*, 1989). For example, marine eustigmatophytes such as *Nannochloropsis* spp. contain 20:5n-3 but little 22:6n-3, whereas haptophytes (prymnesiophytes) such as *Pavlova* spp. contain both 20:5n-3 and 22:6n-3. Chlorophytes rarely contain significant amounts of these fatty acids, but instead have a predominance of C<sub>18</sub> polyunsaturated fatty acids such as 18:2n-6 and 18:3n-3. Dinoflagellates have high levels of 20:5n-3 and 22:6n-3 and many contain the unusual fatty acid 18:5n-3. However, the latter is not unique to dinoflagellates and has been found in algae as diverse as haptophytes, raphidophytes and some prasinophytes (Volkman *et al.*, 1989). The total fatty acid methyl esters obtained in the *Botryococcus braunii* strain was about 64.26 % of the total lipid content. The fatty acid methyl esters include Propanoic acid C27:0 (22.60 %); Propanoic acid C27:0 (15.65 %); 9-octadecenoic acid C38:0 (15.21 %) and Carotenoic acid C27:0 (10.8 %) (Table 3) (Fig.7 to 12). The presence of high cetane number compounds in the FAME of *Botryococcus braunii* represents the occurrence of long chain fatty acids and hydrocarbon. Fourier transform infra red spectroscopy (FT-IR) was studied in *B. braunii*. The presence of esters, alkanes and alkenes confirms the presence of hydrocarbons from the FAME.

Botryococcene represents a particularly diagnostic biomarker for the assessment of paleoenvironments. This C<sub>34</sub> isoprenoidal alkane is considered to be derived from botryococcene, a compound known to exist only in *Botryococcus braunii*, which is widely distributed in freshwater lakes. Thus the identification of botryococcene in oils and sediments indicates the contribution of *B. braunii* to the depositional environment. However despite the quite widespread presence of *Botryococcus* remains in type I kerogen, this specific biomarker was only detected in a few extracts and oils, such as Sumatran oils (Seifert and Moldowan, 1985), Australian coastal bitumen (McKirdy *et al.*, 1985) and Maoming oil shale (Brassell *et al.*, 1986). This observation may be explained, at least in part, by the fact that of the three modern chemical races of *B. braunii* (A, B and L; for a review see Metzger *et al.*, 1991), only race B produces botryococcene. Indeed, the fully hydrogenated derivatives, botryococcanes and tetrametylsqualane, are specific markers of *B. braunii* which are found in petroleum and oil shales, sometimes in high amounts (McKirdy *et al.*, 1986; Summons *et al.*, 2002). The SSL-3 gene sequence of *Botryococcus braunii* was translated into amino acids and the molecular structure of the protein Botryococcene synthase (SSL-3) was predicted and illustrated. The predicted molecular structure of botryococcene synthase has shown about 14 helical structures with the absence of  $\beta$ -pleated sheets. The superimposed study on the obtained molecular structure of botryococcene synthase protein of *Botryococcus braunii* with the Human Squalene synthase protein resulted that the former protein has more conserved amino acid sequence, thus the structure resembles with the human squalene synthase protein. But there are some conformational differences in their quaternary structure.

### Conclusion:-

The green microalga *Botryococcus braunii* has been already reported widely to be the efficient candidate for the production of biodiesel due to the high accumulation of lipid molecules. The biodiesel production from the microalga *Botryococcus braunii* is a cost effective, ecofriendly, efficient and intensive method for the oil production. In this present study, a new micro algal strain *Botryococcus braunii* was isolated from the temple tanks. The biochemical constituents and occurrence of different fatty acid methyl esters (FAME) proves the present microalgal strain *Botryococcus braunii* a potent candidate for the production of biodiesel.

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