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Morphology and cultural behavior of *Botryococcus protuberans* with notes on the genus

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Abstract: *The green alga Botryococcus protuberans was isolated from its natural environment and its morphology under different cultural conditions was examined. The alga was characterized by a high starch content and reddish oil drops as the assimilatory products. Photosynthetic pigments, Chl a, Chl b, carotenoids and xanthophylls are present. Modification of environmental conditions in modified Chu-10 medium resulted in optimum growth of the alga. Fatty acid composition revealed palmitic acid being the major component, while lauric acid, myristic acid and stearic acid were found in less quantity.*

Key words: *Cultural behavior, Systematic position, Botryococcus protuberans, Chlorococcales, Chlorophyceae*

Introduction

The colonial green micro alga *Botryococcus braunii* Kuetz has been much studied (Casadevall *et al*., 1985; Ahmad, 1988; Pal *et al*., 1998). This alga occupies a unique position in being a rich source for production of hydrocarbons and lipids. The cells of the alga produce and accumulate oily hydrocarbons called botryococcenes (36% of organic matter) and contain a high level of lipids amounting to 30-70% of its dry weight under different conditions of growth (Wolf, 1983; Yamaguchi *et al*., 1987). However, another *Botryococcus* strain *i.e.*, *B. protuberans* is widely distributed all around the world as minute green cells, generally held together by long tough and gelatinous strands. It is found suspended in freshwater lakes as well as in brackish water, but has not been properly investigated. This is mainly because of its extremely slow growth rate in laboratory culture as well as in the natural environment as the ecophysiological requirements for its bloom formation are not fully understood (Swale, 1968). The unusual morphology, behavior and chemical composition of this alga have been extensively studied, but no growth conditions have been defined which may be suitable for the large scale cultivation of the alga for its use as an efficient hydrocarbon producer. For cultivation of *B. braunii,* a modified improved Chu-10 medium has been developed (Rai *et al*., 1987), and the growth of the alga was also found to be increased with the application of growth promoting substances (Ahmad, 1988). Currently available data do not point optimistically to the widespread use of *B. braunii* as renewable fuel source, although a long-range potential for the production of a industrial feedstocks may exist (Bachofen, 1982). We have isolated and characterized *B. protuberans* from a man made water tank located at National Botanical Research Institute, Lucknow, Uttar Pradesh, India lying between the parallels 26° 30' and 27° 10' North latitude and 80° 34' and 81° 12' East longitude. The isolated strains (*B. protuberans*) grew frequently in the month of February and March 2001. Observations on the morphology and cultural characteristics of this alga are reported in this paper and compared with the fatty acid composition of *B. braunii*.

Materials and Methods

The alga was collected during the rainy season as periphyton on decaying leaves and twigs in a cement water tank at the National Botanical Research Institute, Lucknow (India). It was isolated and raised to a unialgal population in a modified Chu 10 medium (Safferman and Morris, 1964). Stock cultures were maintained in this medium by weekly transfers to fresh medium. All cultures were routinely harvested by centrifugation when they entered the stationary phase. The cultures were non-aerated but shaken thrice daily. Growth studies were conducted in the modified Chu 10 (Safferman and Morris, 1964); Chu 13 (Chu, 1942); Bold basal (Nicholas and Bold, 1965) and Hughes basal media (Hughes *et al*., 1958) with or without soil extract. Cultures maintained in Chu 10 medium served as control. Pigments were separated chromatographically using petroleum ether/acetone and identified according to Jensen (1978). Different conditions of light intensity and regime were maintained in a constant temperature room maintained at 24±2°C. To determine optimal pH for algal growth, pH of medium was adjusted, at various levels with 5.0 M. Tris-HCl buffer.

Growth was measured spectrophotometrically by the determining absorbency of algal suspensions at 680 and 740 nm. Measurement at 740 nm eliminated absorption by pigments. All experiments were conducted with five replicates and repeated thrice times. For fatty acid analysis, aliquots of the algal samples were saponified, methylated according to Cocks and Redec (1966) and analyzed by gas chromatography. The data presented

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are average of three replicates of three independent experiments conducted under identical conditions.

Results and Discussion

The alga forms irregular clusters of 4-8-16 or more cells held together with a tough gelatinous sheath. The clusters, 16-17 µm broad and 30-95 µm long, are mostly joined together by long tough mucilaginous hyaline strands (Fig. 1a). Cells of the alga in the colony are ovoid to ovoid-cunate with their narrower ends embedded into mucilaginous matrix and distal end projecting out of the colony as free ends. The cells are 6-10 µm broad and 11-20 µm long with double layered cell wall containing mucilaginous envelope (matrix) measuring 0.5-0.6 µm in thickness (Fig. 1c). The chromatophore is yellowish green to grass green, single, pariental, cup-shaped, laminated or reticulated, containing a single pyrenoid. Asexual reproduction is affected by fragmentation of colonies or by the formation of four autospores per cell (Fig. 1b).

The principal pigments were separated by chromatography using 80% acetone and partition between solvents. Two green zones and four yellow zones were found on the chromatogram *i.e.*, Chl.a (l, 663), Chl b (l, 645), a- carotene (l, 465), b-carotene (l, 451), g-carotene (l, 470) and fucoxanthin

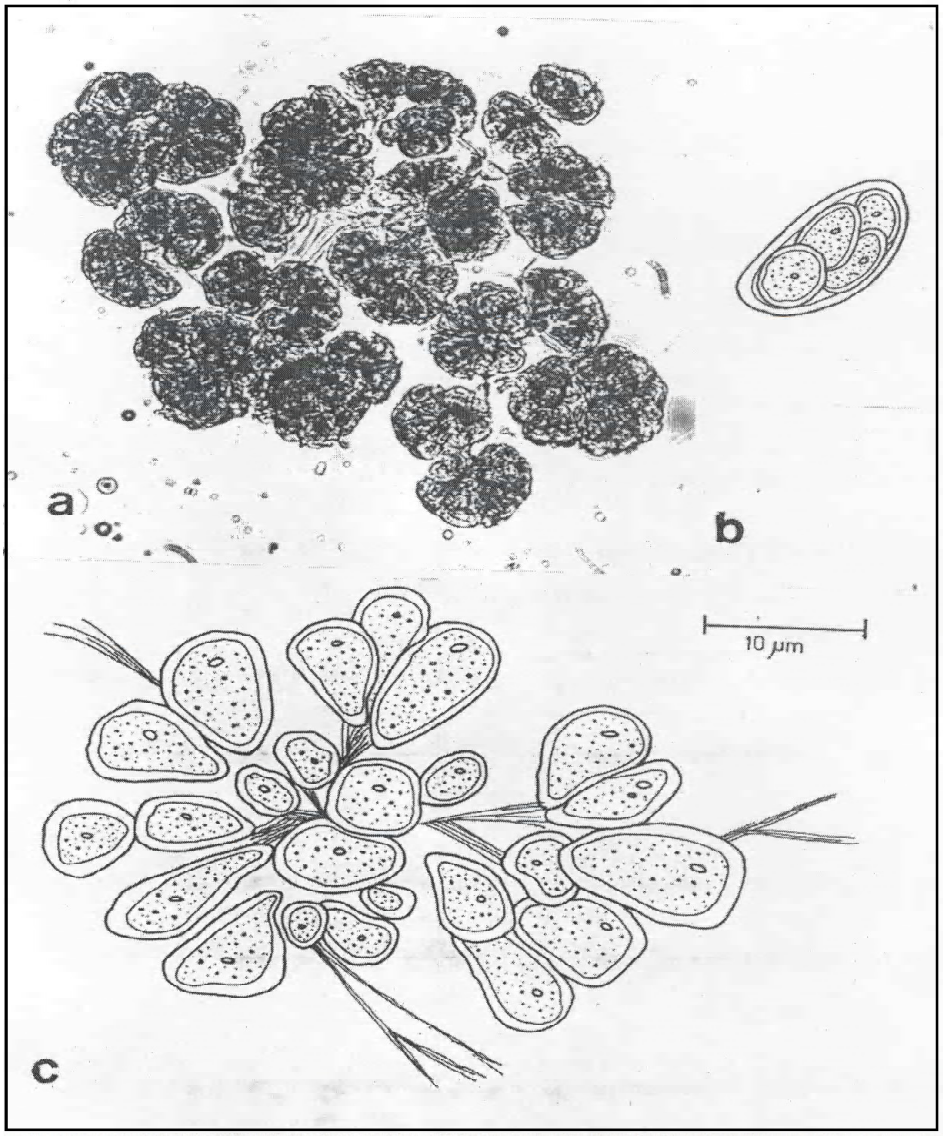

Fig. 1: Morphology of *Botryococcus protuberans*
- **a.** Colony showing cell arrangement and nature of mucilaginous matrix x 2400
- **b.** A cell showing four auto spores embedded with in the mother cell
- **c.** Camera Lucida drawing showing nature of mucilaginous threads

(I, 450). Determination of refractive index and absorption maxima of these pigments after extraction through paper chromatography confirmed their identity (Fig. 2).

Of all the media tried, maximum growth of *B. protuberans* was obtained either in modified Chu 10 or in Hughes basal medium supplemented with 5% soil extract at pH 8.5 and illuminated at 300 mmol/meter^2 tungsten light with 14/10 LD cycle at 24±2°C (Table 1). A comparison of the fatty acid composition of *B. braunii* and *B. protuberans* grown under similar cultural conditions has been shown in Table 2. Among the saturated fatty acid, lauric acid (12:0), myristic acid (14:0), and stearic acid (18:0) were in low amount while palmitic acid (16:0) was the major saturated fatty acid in both the algae.

The systematic position of the genus *Botryococcus* Kuetz. is quite uncertain. Smith (1920); Fritsch (1935); Skuja (1949) and Tiffany and Britton (1952) kept it under the Xanthophyceae (Order Heterochloridales). Prescott (1951, 1959); Philipose (1967); Bourrelly (1966) and Yamagishi and Kobayasi (1971), have placed in the order Chlorococcales, class Chlorophyceae. Several authors *viz*. Korshikov (1953); Pandey *et al.* (1983) and Prasad and Mishra (1992) after making critical observation on this alga have advocated the placement of this genus under order Chlorococcales (Family Oocystaceae) of class Chlorophyceae. The alga has long been regarded as aberrant in structure, behaviour and metabolism, and indeed it seems to have no close parallel in any algal group. The observations on the morphological features of the alga resembles closely those of *Botryococcus protuberans* W. et G.S. West as described by Korshikov (1953) and Philipose (1967). However, it differs from *B. braunii* in cell size, colony morphology, size and nature of colonial envelope, which only partly surrounds the colony leaving the distal end free.

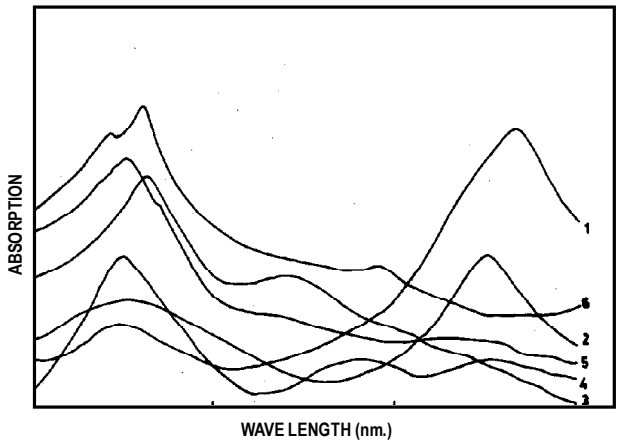

Fig. 2: Absorption spectra of individual pigments separated by chromatography. 1- Chlorophyll a, 2- Chlorophyll b, 3- g-carotene, 4- Fucoxanthin, 5- b-carotene and 6- a- carotene

The data obtained with various media supplemented with or without soil extract show that alga grows well either in modified Chu 10 or in Hughes basal medium supplemented with 5% soil extract. However, the growth of alga without soil extract is considerably slow. The results of experiments with various pH levels show that *B. protuberan* can tolerate pH levels ranging from 7.0 to 9.5, but appears to favour alkaline pH since maximum growth was obtained at pH 8.5. The members of Chlorococcales have been reported to prefer alkaline conditions (Philipose, 1967), although some members grow well under acidic condition (Prescott, 1951).

The fatty acid composition of the alga was found more or less in the same order as reported in case of *B. braunii* (Pal *et*

Table - 1: Effect of different conditions on the growth of *B. protuberans*

Medium		Optical density of culture after 20 days												
		Light quality		Light intensity (µmol m^{-2} s^{-1})			Photoperiod (L/D)					pH		
		Tung.	Flu.	90	115	175	10/14	12/12	14/10	16/8	Cont	7.0	8.5	9.5
Chu No. 10	+SE	0.36	0.28	0.15	0.22	0.32	0.09	0.13	0.36	0.21	0.05	0.21	0.33	0.28
	-SE	0.15	0.17	-	-	-	-	-	-	-	-	-	-	-
Chu No. 13	+SE	0.20	0.14	0.135	0.18	0.20	0.05	0.08	0.15	0.12	0.06	0.13	0.18	0.09
	-SE	0.12	0.9	-	-	-	-	-	-	-	-	-	-	-
Hughes	+SE	0.35	0.25	0.16	0.23	0.34	0.10	0.125	0.31	0.23	0.09	0.22	0.35	0.209
	-SE	0.31	0.15	-	-	-	-	-	-	-	-	-	-	-
Bolds	+SE	0.27	0.15	0.105	0.14	0.21	0.08	0.10	0.16	0.16	0.08	0.10	0.17	0.08
	-SE	0.12	0.11	-	-	-	-	-	-	-	-	-	-	-

Tung. = Tungsten lamp ; Flu. = Fluorescent lamp

Table - 2: Fatty acid composition of *Botryococcus braunii* and *Botryococcus protuberans* grown under similar cultural conditions

Fatty acid	% of total methylated fatty acid mixture	
	B. braunii	***B. protuberans***
Lauric acid (12:0)*	2.3±0.01	2.6±0.03
Myristic acid (14:0)	2.5±0.02	2.9±0.01
Tetradec-5-enoic acid (14:1)	0.2±0.01	0.4±0.001
Palmitic acid (16:0)	17.6±0.56	16.5±2.3
Hexadec-9-enoic acid (16:1)	1.8±0.04	1.4±0.04
Stearic acid (18:0)	1.4±0.02	1.8±0.07
Oleic acid (18:1)	38.5±2.8	38.0±4.7
Linoleic acid (18:2)	8.4±1.9	8.2±1.3
Linolenic acid (18:3)	20.7±2.1	20.9±2.9
Unidentified	6.5±0.07	6.6±1.2
Total unsaturated	69.2±3.6	69.5±4.1
Saturated: Unsaturated	0.37±0.001	0.39±0.03

* Number of carbon: number of double bonds
Values are mean ±SE (n=5)

al., 1998). While the biochemical composition of pigments was found similar as reported in case of chlorococcalean green algae (Strain, 1951). However, pigment fucoxanthin (l, 450), which is of trace occurrence seems to be rare but advocated to be present in few species of chlorococcalean green algae (Chapman, 1962). Therefore the taxonomic position of the genus has been a matter of ambiguity, but on the basis of auto spores formation and close relation with chlorococcalean members Prasad and Mishra (1992) kept in the class chlorophyceae. The saturated to unsaturated fatty acid ratio and total level of unsaturated fatty acids showed that lipid quality in *B. protuberans* is of high quality. However there was no difference with *B. braunii* grown under similar culture conditions. Therefore, results indicate that the green alga *B. protuberans* seems to offer an economically viable system for the production of hydrocarbons due to its fatty acid composition, which is similar to *B. braunii* and enhanced growth rate was obtained in modified Chu 10 or Hyghes basel medium supplemented with 5% soil extract. It may be concluded that *B. protuberans* may be utilized as a renewable source of energy, however, pilot scale studies are warranted to achieve the goal.

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