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Photosynthetic Activity, and Lipid and Hydrocarbon Production by Alginate-Immobilized Cells of *Botryococcus* in Relation to Growth Phase

SINGH, YASHVEER*

Department of Botany, Govt. Raza P.G. College, Rampur - 244 901, (U.P.) India

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Abstract Whole-cell immobilization of the hydrocarbon rich microalgae, *Botryococcus braunii* and *B. protuberans*, in alginate beads under air-lift batch cultures resulted in a significant increase in chlorophyll, carotenoid, dry weight, and lipid contents at stationary and resting growth phases, as compared to free cells. Photosynthetic activity in both the species of *Botryococcus* was enhanced, relative to free cells, at any growth phase of cultures. Immobilization exerted a protective influence on ageing of the cultures as reflected by higher chlorophyll and dry weight contents. Entrapment also stabilized the chlorophyll and carotenoid contents even at stationary and resting phases as compared to free cells in both the species.

Key words: *Botryococcus*, growth phase, hydrocarbon immobilization, photosynthesis

Microalgae are still a relatively untapped resource for lipids and hydrocarbons. Algal lipids are highly reduced hydrocarbons, and are similar to those of vegetable oils in composition. Immobilization of microalgal biomass by entrapment in polyurethane foams and alginate has been suggested to produce organic compounds by fermentation [3, 7] In comparison to free cells, immobilized cells offer physical stabilization of algal cells, biomass retention, and prevention of overgrowth and easy separation of cells [15]. Immobilized cells have many potential applications including biocatalysts for biotransformations and biosynthesis. Inspired by such considerations, many workers have used this technology to produce certain chemicals. A preliminary study on biotransformations by immobilized microalgae includes the production of sulfated polysaccharides by *Porphyridium* [8], and glycolate and glycerol from *Chlorella* and *Dunaliella*, respectively [11]. An earlier study showed that entrapment of algal cells and organelles generally results in a great improvement in photosynthetic capacity [1]. Growth and hydrocarbon production by *B. braunii* entrapped and adsorbed in polyurethane foam have been investigated [3]. The promising green microalga, *B. braunii* is exploited for unusually large production of extracellular hydrocarbons [6, 15]. Free cells of *Botryococcus* species exist in three different physiological (green exponential, yellow stationary, and brown resting) states, which are characterized by varying growth rates, and lipid and hydrocarbon production [20, 17].

In the present investigation, *B. braunii* and *B. protuberans* were used for whole-cell immobilization in alginate beads. Besides studying the effect of immobilization on growth, photosynthetic activity, chlorophyll and carotenoid contents, lipids and hydrocarbon production in batch cultures at various physiological states were also investigated.

Materials and Methods

Test Organisms

Microalga *Botryococcus braunii* was isolated from a local pond (pH 7.8) near Varanasi, India, while *B. protuberans* was obtained from the University of Texas Culture Collection, Austin, U.S.A. Three physiological states were observed in both the species. *B. braunii* grew exponentially upto 22 days after a lag of 3 days, whereas *B. protuberans* grew upto 24 days after a lag of 5 days. Yellow stationary state lasted in *B. braunii* from the 23rd day to 35th day and in *B. protuberans* from the 25th to the 35th days. From the 35th to 50th day, in both the species there was a brownish-orange resting state [17].

Culture Conditions

Both the species of *Botryococcus* were grown in improved Chu-13 medium [16]. Standard methods were followed for maintenance of the batch culture in axenic conditions. The culture vessels were kept in a culture room at 27±1°C with

*Corresponding author
Phone: 91-595-2350515/2340111;

relative humidity (in room) of about 60% and a 18 h light and 6 h dark cycle. Illumination was provided by cool white fluorescent tube lights giving 16 Wm^{-2} light at the surface of vessels. For air-lift condition, sterile air enriched with 0.4% (v/v) CO_2 was bubbled through the cultures for 6- 8 h daily. Unless otherwise stated, exponentially growing cells from standard free cultures were used as inocula. In each experiment, the same quantity of algal inoculum and the biomass were used for initiating the immobilized culture, as were used in the control.

Immobilization

A known volume of concentrated homogeneous algal suspension was mixed with 4.0% (w/v) sodium alginate (Fluka AG, Chemische Fabrik, Switzerland) solution with continuous shaking for 20 min. Sodium alginate mixed cultures were taken into a 10 ml syringe fitted with a needle of 1.0 mm bore. The mixture was extruded dropwise through the syringe from a height of about 10 cm into a glass container having excess of 0.2 M $CaCl_2$. Sodium alginate beads were allowed to harden in sterilized $CaCl_2$ solution for 30 min. Stabilized beads of about 3 mm in diameter were washed with sterilized double distilled water and then transferred into basal medium for growth. Immobilized cells were suspended and released by addition of K_2CO_3 solution (50 g/100 ml distilled water) for the estimation of chemical contents.

Dry Weight

Dry weight of *Botryococcus* biomass was determined by filtration of known volumes of cultures through a tared Whatman No. 1 filter paper. The filter paper with algae was dried in a vacuum oven at 80°C for 24 h, cooled, and weighed.

Pigments

Pigments (chlorophyll a+b and carotenoids) were determined by the method of Myers and Kratz [13]. The chlorophyll and carotenoid contents were calculated and expressed as percent of dry weight.

Photosynthetic Activity

Uptake of $NaH^{14}CO_3$. Photosynthesis was assayed indirectly by measuring the uptake of ^{14}C from $NaH^{14}CO_3$. Cultures were grown in the medium containing sodium bicarbonate (^{14}C) obtained from Bhabha Atomic Research Centre, Bombay, India with specific activity of 5 µCi ml^{-1}. Cultures were removed at indicated time intervals for the determination of ^{14}C uptake by free and immobilized cells. The ^{14}C uptake was stopped by adding 0.2 ml 50% acetic acid. Counting was done in a Beckman model LS-7000 liquid scintillation counter using scintillation cocktail (3 parts ethanol+4 parts toluene supplemented with 0.8% PPO and 0.01% POPOP).

O_2 - Evolution. Photosynthetic O_2 evolution by free and entrapped cells was measured with a Polarographic oxygen electrode fitted in 10 ml airtight reaction vessel and connected to an oxygen analyzer (Universal Biochem., model $M_{76}T$, India). Known volumes (2 ml) of concentrated algal suspension of different growth states were transferred to the reaction vessel on indicated days and oxygen evolution was recorded with the help of O_2 analyzer and expressed as mmole O_2 evolved mg $chl^{-1}a$ h^{-1}. The temperature (27°C) of the vessel was maintained by a thermocirculator.

Lipid and Hydrocarbon Estimation

Total lipids were assayed by the method of Kates [10]. The lipids were phase separated by adjusting the resolution ratio to 10:10:9 (methanol-chloroform-water, by volume). The chloroform phase was evaporated under a stream of N_2 at 42°C, and weight was determined gravimetrically.

The total lipid extracts were fractionated in a glass column (1×20 cm) packed with heat activated silicic acid (Silica gel $60F_{254}$ E. Merck) using chromatography grade hexane, benzene, chloroform, and methanol in order to isolate hydrocarbons, nonpolar lipids with hydrocarbons, other nonpolar lipids, and polar lipids, respectively. The isolated lipids in each eluate were measured gravimetrically after evaporation of the solvent.

RESULTS AND DISCUSSION

Growth data of free and immobilized cells of *B. braunii* and *B. protuberans* are shown in Fig. 1. In terms of biomass (dry weight), the yield of immobilized cells was higher after exponential state (25 days) in both the species. After 30 days of incubation, a rapid bleaching took place

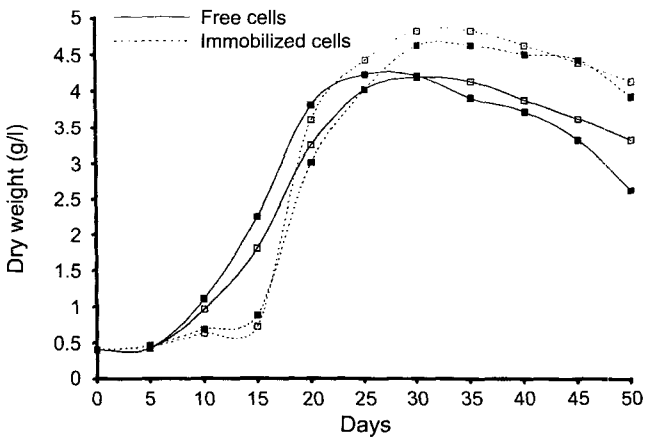

Fig. 1. Growth of free and immobilized cells of *Botryococcus* species.
B. braunii (■—■), *B. protuberans* (□—□).

Table 1. Influence of immobilization of cells on photosynthesis, chlorophyll, carotenoid, lipid, and hydrocarbon contents at the end of each physiological state.

Species	Contents (% of dry weight)	Exponential (25th day)		Stationary (35th day)		Resting (50th day)	
		Free	Immobilized	Free	Immobilized	Free	Immobilized
B. braunii	Chlorophylls	1.48±0.06	1.60±0.08	1.12±0.05	1.85±0.06	0.81±0.04	1.62±0.05
	Carotenoids	1.10±0.04	1.00±0.02	1.26±0.03	1.46±0.06	2.24±0.12	2.96±0.14
	Lipids	37.62±1.80	37.80±2.00	39.50±2.20	42.48±1.84	43.92±2.00	45.80±2.40
	Hydrocarbons*	20.15±1.16	19.82±1.20	47.65±2.40	52.45±2.62	68.15±2.80	72.50±2.86
	Photosynthesis**	0.30±0.02	0.39±0.03	0.28±0.01	0.39±0.04	0.16±0.01	0.38±0.04
B. protuberans	Chlorophylls	1.38±0.08	1.47±0.06	1.10±0.05	1.66±0.06	0.76±0.04	1.42±0.06
	Carotenoids	1.00±0.06	0.96±0.02	1.20±0.04	1.40±0.06	2.10±0.08	2.74±0.10
	Lipids	35.80±1.30	36.12±1.25	37.84±1.38	40.72±2.00	41.25±2.20	44.60±2.00
	Hydrocarbons	21.30±1.80	20.64±1.60	45.86±1.84	60.75±2.50	63.25±2.64	70.34±2.90
	Photosynthesis	0.34±0.02	0.42±0.04	0.30±0.02	0.42±0.02	0.19±0.06	0.38±0.02

Results are the means±SD of three independent replicates. Cultures were harvested on indicated days.

* The value of hydrocarbon is % of total lipid.

** The rate of photosynthesis is expressed in mmole O_2 evolved mg $chl^{-1}a$ hr^{-1}.

in suspended cultures, while immobilized algae were still deep green even at the end of stationary state (35th day). It is an established fact that immobilized cells offer biomass retention in microorganisms.

Immobilization stimulated and stabilized photosynthetic O_2 evolution, chlorophyll, and carotenoid contents even at the end of stationary (35th day) and resting (50th day) states (Table 1). Carotenoid content in entrapped cells increased significantly in both the species after the exponential state, and this increase was maintained upto the resting state. Regarding chlorophyll content, the same pattern was observed in immobilized and free cells. At all stages, however, entrapped cells of both the species had a higher chlorophyll content than controls and the final decrease in chlorophyll was also delayed in alginate upto the 45th day of the culture. Maximum lipids (46% in *B. braunii* and 45% in *B. protuberans*) and hydrocarbons (72.5% in *B. braunii* and 70% in *B. protuberans*) were recorded in immobilized cells of resting state, followed by stationary state in both the species (Table 1). Immobilization activates enzymic activity in cells [1]. High levels of chlorophyll are retained during dark storage of *Euglena gracilis* [18] entrapped in alginate gel, while they decrease faster in free cells. Concerning functional stability, when *Scenedesmus obliquus* cells in alginate are provided with light and nutrients, they show an increased chlorophyll per bead [4]. In the present study, it was observed that immobilization of *B. braunii* and *B. protuberans* in alginate was conducive to higher chlorophyll and carotenoid content under functional conditions, relative to suspended cultures. The present results are in accordance with the earlier observations [2, 18]. Degradation of the chlorophyll and an increase in certain carotenoid pigments often accompany lipid synthesis during stationary and resting states in *Botryococcus* species [15]. The observed increase in lipid and hydrocarbon accumulation is in agreement with the findings of Bailliez *et al.* [2, 3]. The synthesis of secondary metabolites by microalgae entrapped in polyurethane foams is also reported [9]. Algae immobilized in alginate beads can transform exogenous oleic acid, a fatty acid, into hydrocarbons. Immobilization of cells may create anaerobic conditions and enhance the cellular enzymic activity. Anaerobiosis appears to trigger lipid synthesis by activating an oxygen-sensitive pyruvate dehydrogenase, which is located in mitochondria [15]. Hydrocarbons are the end product of the reduction of organic compounds derived from various reactions of fatty acids [19].

Figure 2 shows the photosynthetic activity of *Botryococcus* species at exponential growth state in terms of the $^{14}CO_2$ uptake by immobilized and free cells. $^{14}CO_2$ uptake pattern in both the species was similar in both free and entrapped

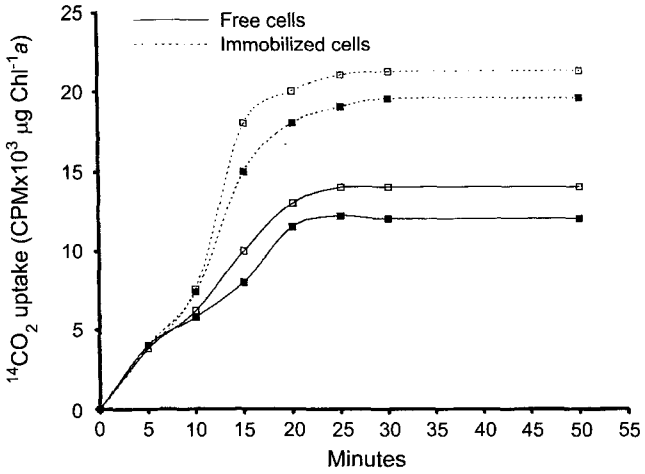

Fig. 2. Effect of immobilization on CO_2-fixation in *Botryococcus* species at exponential growth state.
B. braunii (■—■), *B. protuberans* (□—□).

cells. However, the values were comparatively higher in *B. protuberans*. The activity was higher at each growth state in entrapped cells (data not shown) than in the suspended cultures. Moreover, the decrease in activity of cultures was also delayed in the immobilized cells. After 30 min of incubation, the uptake of $^{14}CO_2$ almost ceased and showed linear activity in both free and entrapped cells.

Comparison of photosynthetic activity in terms of oxygen evolution by immobilized and free cells of *B. braunii* and *B. protuberans* at three physiological states is shown in Table 1. Oxygen evolution by entrapped cells was markedly higher in both the species at each state and the values were comparatively higher in *B. protuberans*.

The possible causes of stabilization of photosynthetic activity ($^{14}CO_2$ uptake and O_2 evolution) by immobilization are not well established. In some cases, partition of damaging metabolites by the gel could be involved [5]. In fact, entrapped cells are subjected to a microenvironment in which ionic properties or chemical composition may be different from those of the bulk medium surrounding the free cells [14]. Thus, entrapment probably increases the local concentration of some ions in the cell environment, and it is well known that various ions are capable of stabilizing photosynthetic systems both during storage and active growth phases [18]. Long-term photosynthesis and N_2-fixation have been studied in an immobilized system with rates 75% higher than those in free-living cyanobacteria [12].

In summary, when the indices of early degradation (fast bleaching, sharp decreases in biomass, and photosynthetic activity) are considered, degradation of *B. braunii* and *B. protuberans* under air-lift conditions was significantly reduced by immobilization in alginate. In both free and entrapped cells of *Botryococcus* species, lipids and hydrocarbons accumulation occurred mainly during the stationary and resting states, and the hydrocarbon productivities were higher during exponential and early stationary states.

In conclusion, the entrapment of *B. braunii* and *B. protuberans* in alginate beads increases lipid and hydrocarbon production as well as chlorophyll content and photosynthetic activity. The stimulating and protective effects are probably related to higher enzymic activity in chloroplast and mitochondria and increase in the concentration of some ions, including Ca^{2+}, within the microenvironment of immobilized cells.

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