

Effect of salinity on growth of green alga *Botryococcus braunii* and its constituents

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Abstract

Growth of *Botryococcus braunii* (race 'A') and production of its constituents viz, hydrocarbon, carbohydrate, fatty acid, and carotenoids were influenced by different levels of salinity. Under salinity at 34 mM and 85 mM, 1.7–2.25-fold increase in the relative proportion of palmitic acid and two fold increase in oleic acid were observed. A twofold increase in carotenoid content was noticed at 85 mM salinity with lutein (75% of total carotenoid) as the major carotenoid followed by β -carotene. The increase in biomass yields and changes in other constituents indicated the influence of salinity and the organism's adaptability to the tested levels of salinity (17 mM to 85 mM).

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1. Introduction

Botryococcus braunii is a green colonial fresh water microalga and is recognized as one of the renewable resource for the production of liquid hydrocarbons. *B. braunii* is classified into A, B and L races depending on the type of hydrocarbons synthesized. Race A produces C₂₃ to C₃₃ odd numbered *n*-alkadienes, mono-, tri-, tetra-, and pentaenes, which are derived from fatty acids (Metzger et al., 1990). Race B produces C₃₀ to C₃₇ unsaturated hydrocarbons known as botryococcenes and small amounts of methyl branched squalenes (Metzger and Largeau, 2005), whereas race L, produces a single tetraterpene hydrocarbon known as lycopadiene (Metzger et al., 1990). Hydrocarbons extracted from the alga can be converted into fuel such as gasoline and diesel by catalytic

cracking (Hillen et al., 1982). *B. braunii* (Races A and B) strains are also known to produce exopolysaccharides up to 250 gm⁻³, whereas L race produce up to 1 kg m⁻³ (Banerjee et al., 2002). However, the amount of exopolysaccharides production varies with the strains and the culture conditions.

Algae differ in their adaptability to salinity and based on their tolerance extent they are grouped as halophilic (salt requiring for optimum growth) and halotolerant (having response mechanism that permits their existence in saline medium). In either case, the algae produce some metabolites to protect from salt injury and also to balance as per the surroundings osmotica (Richmond, 1986). *Dunaliella*, the unicellular green alga is an example for its ability to survive extreme salt stress and serve as a useful model to comprehend the strategies of cell response to high salt concentration. The present study focused on the adaptation of *B. braunii* (race A) to varied range of saline conditions and their effect on the growth, hydrocarbon, carotenoid and carbohydrate production.

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2. Methods

2.1. Algal culture

The strain *Botryococcus braunii* (LB 572) was obtained from University of Texas, USA. This organism was identified as race-A based on its hydrocarbon profile. Stock cultures were maintained regularly on both liquid and agar slants of modified Chu 13 medium (Largeau et al., 1980).

2.2. Media and culture conditions

A set of 500 ml Erlenmeyer conical flasks were taken and 200 ml of modified Chu 13 medium was distributed and sodium chloride was added in the range of 17 mM to 85 mM to the flasks and inoculated. Two weeks old culture of *B. braunii* LB 572 grown in modified Chu 13 was used as inoculum at 20% (v/v). The culture flasks were incubated at 26 ± 1 °C temperature under 1.2 ± 0.2 klux light intensity and 16:8 h light dark cycle. All the experiments were carried out in triplicates.

2.3. Analytical methods

2.3.1. Biomass, chlorophyll and carotenoid estimation

The cultures were harvested by centrifugation at 5000 rpm and the cells were washed twice with distilled water. Then the pellet was freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight (g L^{-1}). To estimate chlorophyll, a known volume of *B. braunii* culture was centrifuged and the residue was extracted with methanol repeatedly. The chlorophyll content in the pooled extract was estimated spectrophotometrically by recording absorbance at 652 and 665 nm and quantified using the method of Lichtenthaler (1987). To estimate carotenoids, known amount of freeze dried algal biomass was extracted with acetone and absorbance was measured at 450 nm and the concentration of carotenoid was determined using Davies (1976) method.

2.3.1.1. HPLC analysis of carotenoids. The carotenoid profiles of stress induced cultures were analysed by HPLC using a reversed phase C18 column with an isocratic solvent system consisting of acetonitrile/methanol/dichloromethane (70:10:20) at a flow rate of 1.0 ml/min and detected at 450 nm. Lutein, β -carotene, violoxanthin and zeaxanthin were identified using authentic standards (Sigma).

2.3.2. Carbohydrate and protein estimation

Known amount of cell free (spent) medium was analysed for total carbohydrate by phenol-sulphuric acid method (Dubois et al., 1956). Protein content in the cell free (spent) medium was analysed by Bradford protein assay (Zor and Selinger, 1996).

2.3.3. Hydrocarbon extraction and analysis

The dry biomass was homogenised in mortar and pestle with *n*-hexane for 15 minutes and centrifuged. The extraction process was repeated twice and supernatant was transferred to pre-weighed glass vial and evaporated under the stream of nitrogen to complete dryness. The quantity of residue was measured gravimetrically and expressed as dry weight percentage (Dayananda et al., 2005). The crude extracts were purified by column chromatography on silica gel with *n*-hexane as an eluent. The eluent was concentrated and analysed by GC using BP-5 capillary column as described by Dayananda et al. (2005).

2.3.4. Fatty acid analysis

The lipids were extracted with chloroform-methanol (2:1) and quantified gravimetrically. The lipid sample was dissolved in benzene and 5% methanolic hydrogen chloride (95 mL chilled methanol + 5 mL of acetyl chloride) was added and shaken well. The mixture was refluxed for 2 h then 5% sodium chloride solution was added and the fatty acid methyl esters (FAME) were extracted with hexane. The hexane layer was washed with 2% potassium bicarbonate solution and dried over anhydrous sodium sulphate (Christie, 1982). FAME were analysed by GC-MS (Perkin-Elmer, Turbomass Gold, Mass Spectrometer) equipped with FID using SPB-1 (poly(dimethylsiloxane)) capillary column (30 m \times 0.32 mm ID \times 0.25 μm film thickness) with a temperature programming 130 °C to 280 °C at a rate of 3 °C/min. The FAME were identified by comparing their fragmentation pattern with authentic standards (Sigma) and also with NIST library.

3. Results

3.1. Effect of sodium chloride on growth

B. braunii was able to grow in all the tested concentrations of sodium chloride (17 mM to 85 mM). The biomass yields increased with increasing concentration of sodium chloride and maximum biomass was achieved in 17 mM and 34 mM salinity (Fig. 1). The variation in pH from 8.5 to 9.5 observed during the experimental period was independent of sodium chloride concentration. It was evident from the data (Fig. 1) that the decrease in phosphate was due to its utilization by the alga, which was not influenced by salinity concentration.

3.2. Effect of salinity on metabolite production

Hydrocarbon content in *B. braunii* was similar to growth pattern. The content varied in the range of 12–28% in different salinities and maximum hydrocarbon content was observed in 51 mM and 68 mM of salinity (Fig. 2). The hydrocarbon profile as analysed by GC

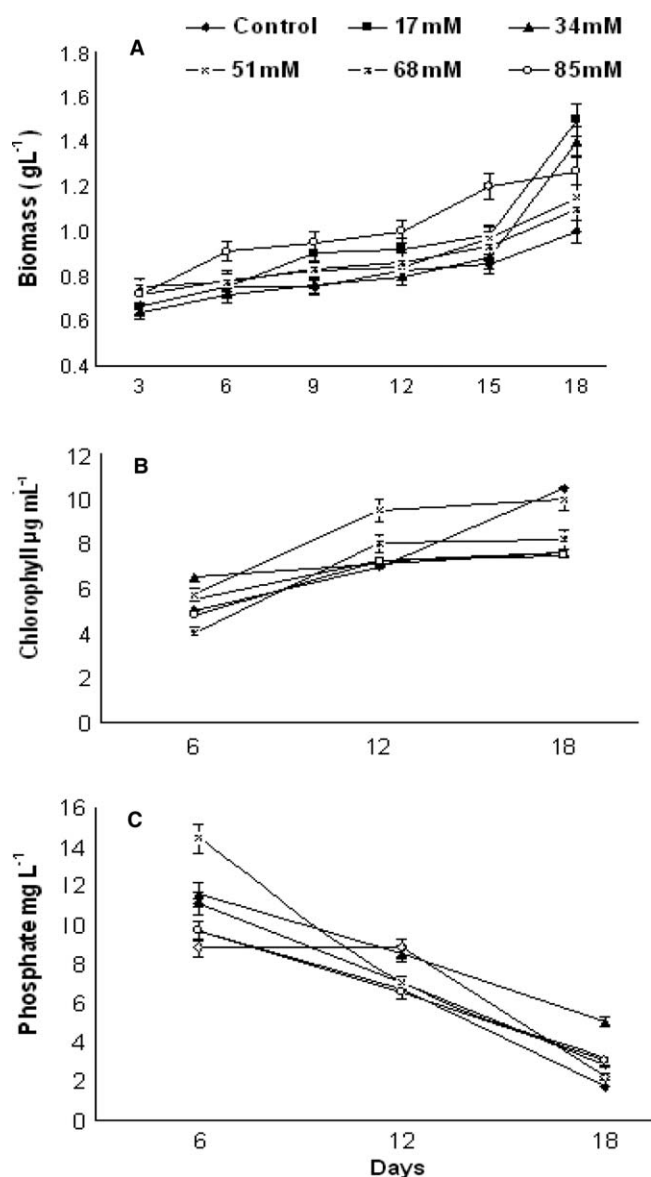


Fig. 1. Effect of salinity on (A) biomass yield, (B) chlorophyll content and (C) phosphate utilization by *B. braunii*. Data represents an average of three replicates. Bars indicate mean \pm SD.

indicated only a marginal influence on the relative proportion of hydrocarbons lower than C_{30} and higher than C_{30} at different salinity concentration. The proportion of higher chain (>30) constitutes $30 \pm 4\%$ at different salinity concentrations.

The carotenoid content in *B. braunii* grown at different salinity concentrations is shown in Fig. 2. The content increased with increase in salinity and maximum was observed at 85mM. The HPLC analysis of carotenoid extract from *B. braunii* revealed that lutein was the major carotenoid followed by β -carotene and low levels of violoxanthin, astaxanthin and zeaxanthin were identified against authentic standards.

The carbohydrate content in the medium as analysed in terms of total sugars increased with the growth of the alga.

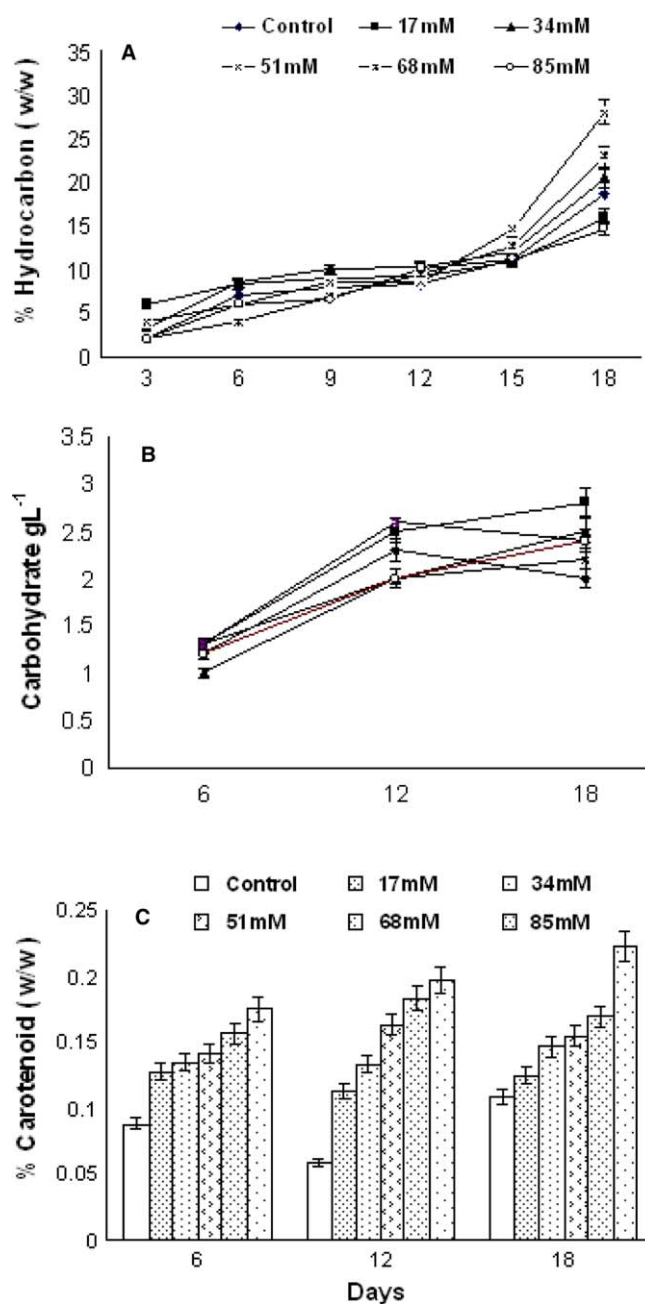


Fig. 2. Effect of salinity on (A) hydrocarbon, (B) carbohydrate and (C) carotenoid contents in *B. braunii*. Data represents an average of three replicates. Bars indicate mean \pm SD.

A marginal increase in carbohydrate content was observed at 17mM and 34mM salinity cultures over the control (Fig. 2).

The total fat content of the alga grown in different salinity varied in the range of 24–28% (w/w) whereas in control it was 20%. The fatty acid profile as shown in Table 1 indicated the presence of $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, $C_{22:0}$, $C_{22:1}$ and $24:0$ fatty acids. Stearic and linoleic acids were in higher proportion in control culture while palmitoleic and oleic acids were the major fatty acids in 34mM and 85mM salinity.

Table 1
Lipid profile of *B. braunii*

Fatty acids	Relative percentage		
	Control	34 mM NaCl	85 mM NaCl
16:0	15.14 ± 0.43	25.62 ± 0.96	33.76 ± 1.31
16:1	10.64 ± 0.26	14.84 ± 0.32	Trace
18:0	28.19 ± 2.04	5.91 ± 1.12	9.33 ± 0.42
18:1	13.35 ± 1.37	25.23 ± 0.86	28.28 ± 1.06
18:2	22.12 ± 1.42	19.15 ± 0.97	10.06 ± 0.65
22:0	Trace	1.88 ± 0.68	2.94 ± 0.47
22:1	10.54 ± 0.34	7.30 ± 0.92	Trace
24:0	Trace	Trace	15.61 ± 1.82

Data represent mean ± SD of three replicates. Data recorded on 18 days of culture.

4. Discussion

In the present study, biomass, carbohydrate and carotenoids were higher compared to the control under salinity (Figs. 1(A), 2(B) and (C)). This could be possibly due to the adaptation of the organism to the lower salinity (17 mM to 85 mM). However, Vazquez-Duhalt and Arredondo-Vega (1991) reported decrease in protein content and biomass yield while carbohydrates and lipids remain unchanged during haloadaptation of *B. braunii*. Hart et al. (1991) showed the reduced growth at higher salinities due to decrease in photosynthetic rate. Ben-Amotz et al. (1985) found low contents of protein, carbohydrates and pigments except lipid in *B. braunii* cells when grown in 0.5 M NaCl. The decreased yields of biomass as reported by Vazquez-Duhalt and Arredondo-Vega (1991) and Ben-Amotz et al. (1985) were probably due to non adaptability of the organism to higher salinity (0.1–0.75 M). The fat (24–28%) and hydrocarbons content (12–28%) varied in control and salinity cultures. The major difference was observed in fatty acid profile especially in the relative proportion of stearic, linoleic, palmitic and oleic acids (Table 1). In general, the fatty acid profile changes with the physiological state of the culture. In *B. braunii*, oleic acid was reported as the precursor of the non-isoprenoid hydrocarbons produced by the race A. Laureillard et al. (1988) reported that oleic acid was involved in the formation of very long chain fatty acid derivatives through chain elongation. It is also a precursor of PRB A and PRB B resistant biopolymer present in the outer cell wall of the A and B races, respectively. Differences in the relative proportion of different fatty acids in control and salinity cultures could be attributed to the physiological state of the alga as reported by Templier et al. (1984). High levels of salinity (>170 mM) were lethal for fresh water green alga, *Haematococcus*, and there was induction of carotenoid accumulation at lower salinity of 0.43 mM (Sarada et al., 2002). Recently, Fazeli et al. (2005) also reported that 0.1 M to 0.5 M salinity favoured the total carotenoid production in *Dunaliella tertiolecta*. The alga *B. braunii* produced carbohydrate as an osmoprotectant as reported by Vazquez-Duhalt and Arredondo-Vega

(1991) at higher salinity was also in accordance with the present results (Fig. 2B). From the results, it could be concluded that the *B. braunii* was adaptable to lower levels (17 mM to 85 mM) of salinity with an increased production of biomass, hydrocarbon, fat, carbohydrate and carotenoids.

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