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Full Length Research Paper

Isolation, characterization and outdoor cultivation of green microalgae *Botryococcus* sp.

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Samples of the green, colonial, unicellular microalgae *Botryococcus* sp. were collected from freshwater ponds in Mahabalipuram, Tamil Nadu, India. Specimens were isolated and examined for morphological features using microscopic and scanning electron microscopic images and was found to be *Botryococcus* sp. The hydrocarbon analysis of its hexane extracts showed hexadecane (10.15%), heptadecane (17.82%) and pentacosane (18.74%) as its major hydrocarbons. Further, the 18S rRNA sequence (GU182893.1) analysis has confirmed its taxonomical relationship to the order Trebouxiophyceae and has shown similarities with the reported species of the genus *Botryococcus* and in particular to *Botryococcus braunii*. Based on morphological features and 18S rRNA sequence analysis, the Indian isolate was designated as *Botryococcus mahabali*. Biomass analysis of *B. mahabali* showed 19% proteins, 18% carbohydrates and 14% lipid. It was found that its oil comprised mainly of hexadecadienoic acid (16:2), oleic (18:1), linoleic (18:2), and linolenic acids (18:3) as its major fatty acids. Oleic acid is recognized to be the major fatty acid in most of the reported species of the genus *Botryococcus* and thus it can serve as another significant chemical signature for the Indian isolate. Its pigment profile exhibited lutein (41.57%) and β-carotene (37.96%) as major carotenoids. In view of its chemical profile, the algae was scaled up in open air raceway ponds in batch mode and the biomass yields were found to be 2 g/L (w/w) up on two weeks growth in outdoor raceway ponds. In conclusion, the results of the study reveal that an Indian isolate *B. mahabali* can be of relevance for its prospective applications from food to biodiesel feed stock.

Key words: Carotenoids, fatty acids, hydrocarbons, lipids, lutein, mass cultivation, raceway pond.

INTRODUCTION

There is an increasing quest all over the world for the exploration and exploitation of potential microalgae for various industrial applications from nutraceutical to bio-diesel feed stock. The green colonial lipid rich microalgae *Botryococcus* is predominant in freshwater, brackish lakes, reservoirs and ponds (Metzger and Largeau, 2005). *Botryococcus* is characterized by its ability to synthesize and accumulate very high levels of lipids. These lipid substances include numerous hydro-carbons, that is, highly reduced compounds comprising only carbon and hydrogen as elements (Brown et al., 1969). Being a photosynthetic organism, it has been reported to reduce CO_2 emissions by 1.5×10^5 tons/yr/8.4×10^3 ha (Sawayama et al., 1999) and thus it offers an eco-friendly process for production of lipid and other bioactive compounds along with its carbon dioxide mitigation credits.

The existence of *Botryococcus* sp. in USA, Ivory Coast, Portugal, Bolivia, Morocco, India, Philippines, Thailand, France and West Indies has confirmed its wide distribution (Chandra, 1964; Metzger et al., 1985; Wolf et al., 1985; Okada et al., 2000; Dayananda et al., 2010). Further, these geographical regions belong to different climatic zones like continental, temperate, tropical and alpine, indicating its ability to grow in varied climatic conditions (Tyson, 1995). Because of its potential to produce large amounts of lipids and hydrocarbons,

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Abbreviations: GC-MS, Gas chromatography-mass spectrometry; **HPLC,** high performance liquid chromatography; **rRNA,** ribosomal RNA**; SEM,** scanning electron microscope.

exploration for newer strains and species of the genus *Botryococcus* is increasing day by day and to date more than 60 *Botryococcus* strains were cultivated in laboratory (For a non-exhaustive list, see Metzger and Largeau, 1999) and the exploration is still on.

Most of the *Botryococcus* strains cultivated in laboratory and wild samples collected from lakes are analyzed for their lipid production (Metzger and Largeau, 1999) and are reported to produce 5 - 42% of lipids and 0.1 - 61.0% hydrocarbons on their dry weight (Metzger and Largeau, 1999, 2005; Dayananda et al., 2007a; Ranga Rao et al., 2007; Hai-Linh et al., 2009; Ela and Anastasios, 2010; Chiara et al., 2010). Therefore, the exploration for a suitable strain for biotechnological exploitation for lipid production is on in various algal research laboratories and industries of various countries across the world. However, there were no reports available regarding the mass cultivation of *Botryococcus* sp. either in raceway or in any other type of mass cultivation systems. In view of its importance, the present study aims to isolate the indigenous species of *Botryococcus* for production of lipids and other bioactive compounds of commercial importance and an attempt was also made to scale up in open air raceway ponds.

MATERIALS AND METHODS

Isolation and purification

The algal samples were collected from the blooms in freshwater ponds of Mahabalipuram (Latitude 12°37' in the North to Longitude 80°14' in the East.) Tamil Nadu, India. The samples were cultured in modified Chu 13 medium and subjected to purification by serial dilution. The individual colonies were microscopically observed for their colonial and morphological features. Pure culture was obtained from single colony and the culture was established in both liquid and agar slants of modified Chu 13 medium, incubated at 25 ± 1°C under 1.2 ± 0.2 klux irradiance with 16:8 h light dark cycle. The purity of the culture was ensured by repeated plating and by regular observation under microscope. The Indian isolate was analyzed for its 18S rRNA sequence along with hydrocarbons, carotenoids and fatty acids profiles.

Light microscopy and scanning electron microscopy

The algal cells were observed under light microscope for their morphological features and for other cellular details, the cells were further studied using scanning electron microscope (SEM) according to the method of Fowke et al. (1994). The samples were fixed in 2% glutaraldehyde in 0.2 M phosphate buffer (pH 6.8) for 12 h, dried in alcohol series up to 100%, sputter coated with gold and examined in a LEO Scanning Electron Microscope 435 VP (Leo Electron Microscopy Ltd. Cambridge UK).

Hydrocarbon extraction and analysis by GC-MS

Hydrocarbons were extracted by homogenizing the dry biomass with n-hexane for 30 min intermittently. Supernatant recovered was evaporated to complete dryness under the stream of nitrogen. Hydrocarbon content was measured gravimetrically and expressed as percentage dry weight (Sawayama et al., 1999). The hydrocarbon samples were analyzed on SPB-5 column (30 m × 0.32 mm ID × 0.25 µm film thickness) using GCMS equipped with FID and were identified by comparing their fragmentation pattern with standards (Sigma chemicals USA) and with NIST library (Dayananda et al., 2005).

Total Lipid estimation and fatty acid analysis

Total lipids were extracted with chloroform-methanol (2:1) and quantified gravimetrically. The fatty acid methyl esters (FAME) were prepared by following the procedure of Christie (1982). FAME were analyzed by GC-MS (PerkinElmer, Turbomass Gold, Mass spectrometer) equipped with FID using SPB-1 (poly (dimethysiloxane)) capillary column (30 m × 0.32 mm ID × 0.25 µm film thickness) with a temperature programming of 150°C(3') to 280°C (5') at a rate of 5°C/min. The FAMEs were identified by comparing their fragmentation pattern with authentic standards (Sigma) and with NIST library.

HPLC analysis of carotenoids

The acetone extract of the alga *Botryococcus mahabali* was analyzed by HPLC using a reversed phase C18 column (4.5 cm × 150 mm) with an isocratic solvent system consisting of acetonitrile/ methanol/dichloromethane (7:1:2) at a flow rate of 1.0 ml/min and the compounds were detected at 450 nm. Lutein, β-carotene were identified using standards (Dayananda et al., 2007b).

Extraction of genomic DNA and 18S rRNA amplification

Genomic DNA was extracted from the lyophilized algal biomass using the GenElute™ Plant Genomic DNA Mini prep kit (Sigma, St. Louis, USA). The RNA contamination was removed by digesting the extract with 10 µg of RNase-A (Bangalore Genei, Bangalore, India) for 30 min at 37°C. Quality and quantity of DNA preparations were checked by standard spectrophotometry and the samples were diluted to a concentration of 25 ng/µl and used for PCR reactions. The 18S rRNA gene specific primers for the *Botryococcus* sp. were designed from the reported sequences at NCBI data base, 5'-CTGTGAAACTGCGAATGGC-3'as FP and 5'CTCCAATCCCTAGTCGGCATCG-3' as RP. PCR reaction was performed in thermo-cycler using a PCR programme with 4 min initial denaturation at 94°C and 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 52°C and 30 s at 72°C for extension with final extension at 72ºC for 10 min. The PCR products were separated on agarose gels and stained with ethidium bromide and the gels were documented with a Hero Lab gel documentation system. The PCR was performed at least thrice to check the reproducibility and then the PCR product was purified by the purification kit supplied by Sigma and was again checked by gel electrophoresis. The PCR product was cloned in T/A cloning vector (kit supplied by Invitrogen) and was sequenced after confirmation by both PCR and restriction digestion. The sequence was submitted to NCBI data base and was accorded with an accession number GU182893.1.

Acclimatization of *Botryococcus* to outdoor conditions

B. mahabali established in agar plates were inoculated into 150-ml Erlenmeyer flasks and incubated for two weeks at 25 ± 1°C under 1.2 ± 0.2 klux light intensity with 16:8 h light dark photoperiod. The cultures were sub-cultured at an intervals of two weeks and such sub-culturing were done at least ten times prior to its scale up in

500, 1000 and 2000-ml Erlenmeyer flasks in modified Chu 13 medium.

The seed cultures were exposed to open air environments in Corbouy of 10 - 20 L capacity for 4 - 8 weeks. And then cultures were inoculated into glass tanks of size (26 cm height × 76 cm long × 26 cm wide) with a culture holding capacities of 10 - 15 L. The algae in glass tanks were subjected for adaptation to open air condition for more than 4 cycles (sub-culturing at an intervals of two weeks) at ambient temperature and were covered with transparent glass plates having four to five holes of size 1 - 2 cm to facilitate aeration and to avoid condensation. Cultures were observed under microscope periodically for any possible contaminants. Algal cultures in carbouys and glass tanks were used as starter cultures for open air circular (cultures in circular tanks were mixed twice a day) ponds which in turn served as a starter culture for 1000 L capacity raceway pond.

Cultivation in raceway pond

Modified Chu 13 media was prepared by using potable water supplied by CFTRI water facility, and the pH was tentatively adjusted to 7.0 - 7.5. The culture was inoculated at 30 - 35% (v/v) in to raceway pond of 1000 L capacity and the volume was made up to 800 ± 5 L. The paddle wheel was set to 15 rpm to provide the aeration from 10 am to 5 pm daily. Light irradiance, pH, chlorophyll, carotenoids and biomass yields were recorded on daily basis. Periodically cells were observed under microscope for any possible contamination. Strainer was used to remove any dust and particulate matters from raceway tanks. Cultures were monitored for their growth for two weeks in outdoor conditions and the biomass was harvested by online centrifugation and lyophilized biomass was analyzed for its chlorophyll, carotenoids and lipid yields.

Biomass estimation

The known volume of cultures was harvested by centrifugation at 5000 rpm for 5 min and the pellet was washed at least twice with distilled water and freeze dried. The dry weight of algal biomass was determined gravimetrically and growth was expressed in terms of dry weight gram per liter.

Harvesting of algal biomass

The algal culture in raceway tank was allowed to settle by density for 2 - 3 h and then the upper clear algal medium was removed. The biomass settled at the bottom was collected and was fed to bowl centrifuge used at a speed of 5000 rpm (M/s West Folia, Germany). Thirty liter of algal culture was manually fed at a time to the centrifuge. The rotor speed was 5000 rpm with flow of the culture adjusted to 5 L/h. Biomass collected in a cone shaped rotor was recovered, lyophilized and stored at -20°C for further use.

Freeze-drying

Algal biomass was spread uniformly in a stainless steel tray and was lyophilized using a freeze drier (Model-10XB, Lyophylization Systems Inc. USA). The freeze-dried samples were analyzed for chlorophyll, carotenoids and lipid yields.

Chlorophyll and carotenoids estimation

A known volume of culture was centrifuged (5000 rpm) for 5 min and the pellet was treated with known volume of methanol (1:1) and kept in water bath for 30 min at 60°C. Absorbance of the pooled extracts was measured at 450, 652 and 665 nm and total chlorophyll and total carotenoids were estimated using Lichtenthaler equations (Lichtenthaler, 1987).

Total protein analysis

Total protein of the lyophilized algal biomass was estimated by Kjeldahl method (AOAC, 2000).

Total carbohydrate estimation

Total carbohydrate from the lyophilized algal biomass was estimated spectroscopically using phenol-sulphuric method and glucose as the standard, as indicated by Dubois et al. (1956).

Estimation of moisture and ash

Moisture and ash contents were determined by following official AOAC methods (AOAC, 1984).

RESULTS AND DISCUSSION

Isolation and identification

Colony characteristics and morphological features of the Indian isolate have demonstrated its close similarity with the genus *Botryococcus*. The individual cells of the colonies were in the range of 3 - 11 µm and the colonies were found to be between 25 - 150 µm. However, even bigger aggregates of colonies were also observed in natural habitat as well as during their stationary growth phase (Figure 1). Cells are spherical in shape and the variation in colonial size of the Indian isolate is depending upon the daughter colonies which remain attached to one another. Cells are generally green to yellowish green, and under some stressed conditions, they also show orange yellow colouration as well. Similar observations were made by Chandra in Miocene lignites of Kerala, India (Chandra 1964). And further, the 18S rRNA sequence (GU182893.1) analysis was carried out to know the taxonomical identity of the Indian isolate and the studies revealed its taxonomical relationship to the order Trebouxiophyceae, and further the sequence analysis has shown more than 90% similarities with the reported 18S rRNA sequences of genus *Botryococcus* and in particular to the species *B. braunii*. The species of the genus *Botryococcus* were mainly distinguished based on colony size and details of cell shape. Therefore, based on its distinctive morphological and cellular features with that of the reported *Botryococcus* species, the Indian isolate was designated as *B. mahabali*.

The production of hydrocarbons is one of the characteristic features of *Botryococcus* species and hence hydrocarbons analysis was also carried out to understand the nature of hydrocarbons produced by the Indian isolate. Hydrocarbons of *B. mahabali* were

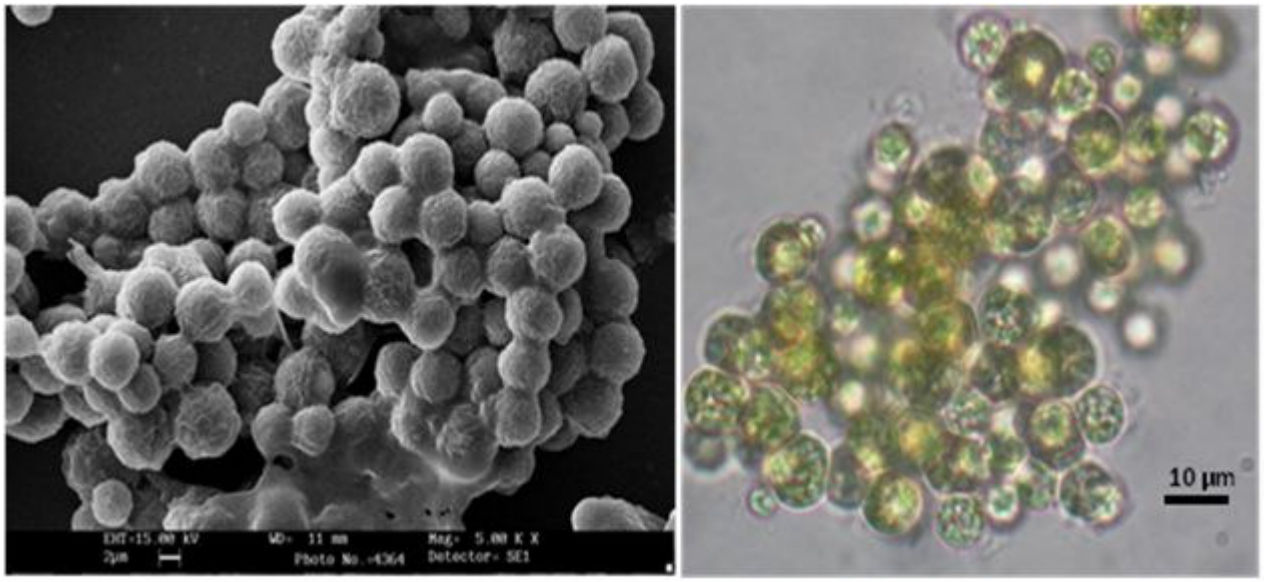

Figure 1. Scanning electron microscopic and light microscopic images of *B. mahabali*.

Table 1. Hydrocarbons of *B. mahabali*.

Hydrocarbons	Relative %
C_{13}	0.04
C_{14}	3.64
C_{15}	7.64
C_{16}	10.15
C_{17}	17.82
C_{18}	6.2
C_{19}	4.19
C_{20}	8.21
C_{21}	2.57
C_{22}	5.8
C_{23}	6.35
C_{24}	4.81
C_{25}	18.74
C_{26}	4.33

identified by comparing their mass spectra with standard hydrocarbons (Sigma) and also with the NIST library. The types of hydrocarbons produced by the Indian isolate were identified as saturated hydrocarbons in the range of C_{13} to C_{26} (Table 1). The Indian isolate has produced hexadecane (10.15%), heptadecane (17.82%) and pentacosane (18.74%) as its major hydrocarbons (Table 1). Similar types of saturated hydrocarbons (C21 to C31) were also reported by Yang et al. (2004) from the Chinese strain of *B. braunii* with heptacosane as its major constituent. Volova et al. (2003) has also reported the presence of saturated straight-chain, branched-chain (C_{14}–C_{28}) and long-chain linear aliphatic (C_{20}–C_{27}) hydrocarbons. Dennies and Kolattukudy (1992) have demonstrated the alkane biosynthesis by decarbonylation of aldehyde catalyzed reactions in the absence of oxygen in the microsomal preparations of *B. braunii*. Audino et al. (2000) identified the macrocyclic alkanes (ranging from C_{15} to C_{34}) and their methylated analogues (ranging from C_{17} to C_{26}) in *B. braunii* rich sediment (torbanite). These hydrocarbons served as another chemical marker for the Indian isolate *B. mahabali* to assign to the genus *Botryococcus*.

B. mahabali was evaluated for its lipid content and was found to be 14% on dry weight basis and its fatty acids analysis has revealed hexadecadienoic acid (16:2), oleic (18:1), linoleic (18:2), and linolenic acids (18:3) as its major fatty acids (Table 2). Similarly, Fang et al. (2004)

Table 2. Fatty acid composition* of *B. mahabali*.

Fatty acids	Relative %
14:0	0.05 ± 0.01
15:0	0.1 ± 0.08
16:0	28.9 ± 5.43
16:1	1.92 ± 0.32
16:2	2.9 ± 1.64
18:0	2.35 ± 1.33
18:1	22.81 ± 3.7
18:2	14.94 ± 3.71
18:3	25.28 ± 2.56
20:0	0.32 ± 0.37
20:1	0.1 ± 0.11
22:0	0.31 ± 0.19

*Values represents Mean ± SD of three replicates.

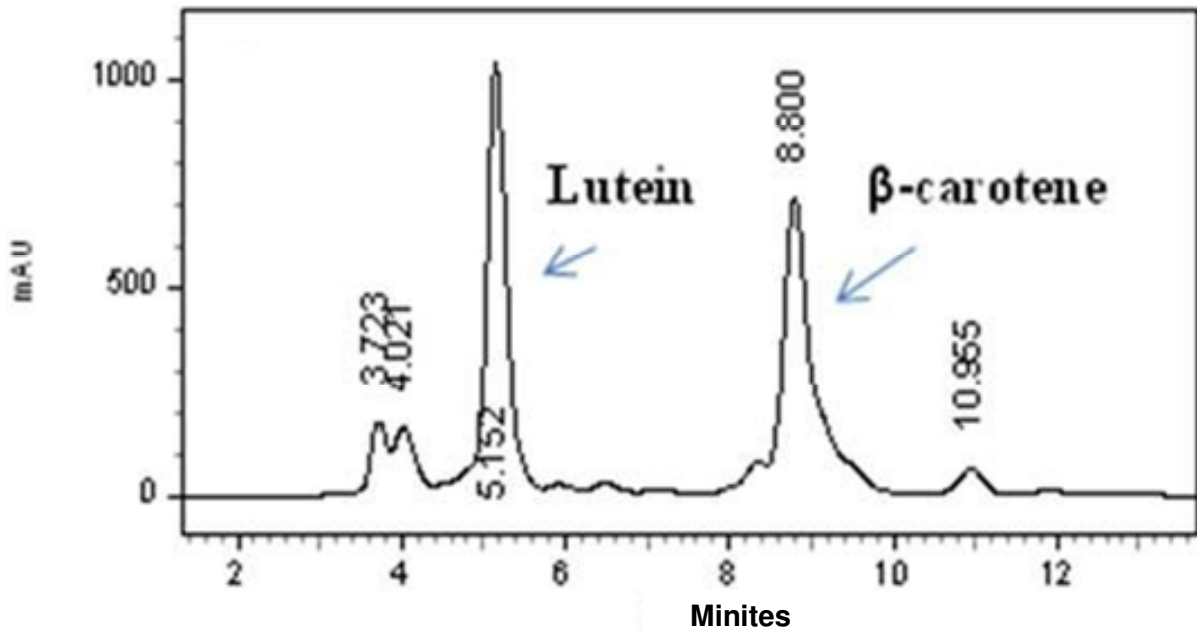

Figure 2. HPLC profile of carotenoids from *B. mahabali*.

have also reported palmitic acid and oleic acids as major components in the *Botryococcus* sp. (Fang et al., 2004). The algal biomass was also analyzed for total carotenoids contents (2.5 µg/g DW) and constituted predominantly lutein (41.57%) and carotene (37.96%) (Figure 2). These carotenoids are well known for their various pharmaceutical, nutraceutical and cosmetic applications.

Scale up of *B. mahabali* in raceway ponds

In view of its chemical profile, the algae was attempted for scale up in open air raceway ponds. Therefore, as a prerequisite, *B. mahabali* was gradually scaled up in 500-ml, 1 and 2-L flasks and then in 10 and 20 L carbouys. Rectangular glass tanks with a culture holding capacity of 10 - 15 L (26 cm height × 76 cm long × 26 cm wide) covered with a glass plate with 4 - 5 holes of size 1 - 2 cm to facilitate aeration as well as to avoid condensation were used to acclimatize the algae for outdoor conditions. The culture was observed under microscope periodically for any possible contaminants. Glass tanks with 10 -15 L algal culture were subjected to acclimatization for more than 4 cycles (sub-culturing at an intervals of two weeks) with open air conditions at ambient temperature. Thus

Figure 3. *B. mahabali* cultivated in raceway pond.

Figure 4. Online centrifuge showing the harvesting of *B. mahabali*.

developed starter cultures were inoculated into open air circular ponds (cultures were mixed twice a day) with a culture holding capacities of 100 - 200 L wherein the algae was gown for a week and that seed culture was utilized for raceway ponds cultivation studies (Figure 3).

Raceway ponds were maintained in natural environmental conditions with ambient temperature and light. 600 ± 15 L modified Chu 13 media was prepared with the potable water supplied by CFTRI water facility and inoculated with 200 ± 5 L of green culture developed in circular pond to get an at least initial OD of 0.5 - 580 nm. The cultures were daily mixed by paddle wheels with 15 rpm between 10 AM to 5 PM, in order to prevent settling of cells at the bottom of the tank. *B. mahabali* was grown for a period of two weeks, and the culture was allowed to settle by gravity, then the clear supernatant media was removed by HDPE tube by vacuum succession. The biomass was washed with potable water and was again allowed to settle; the process was repeated thrice and fed to online centrifuge (Figure 4). Wet biomass was dried by using a Freeze drier (Model-10XB, Lyophylization Systems Inc. USA). The freeze

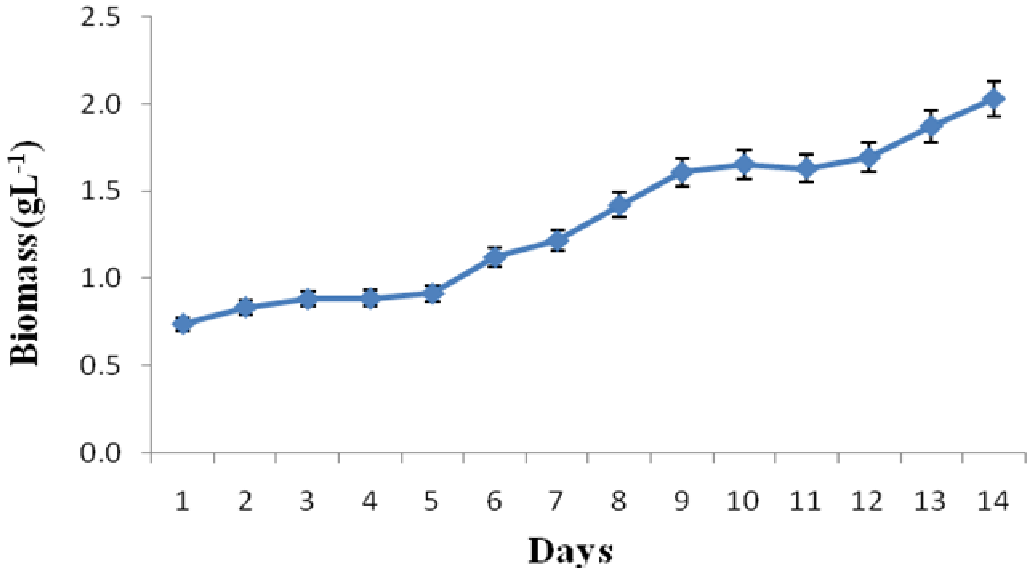

Figure 5. Biomass yields of *B. mahabali* cultivated in raceway pond.

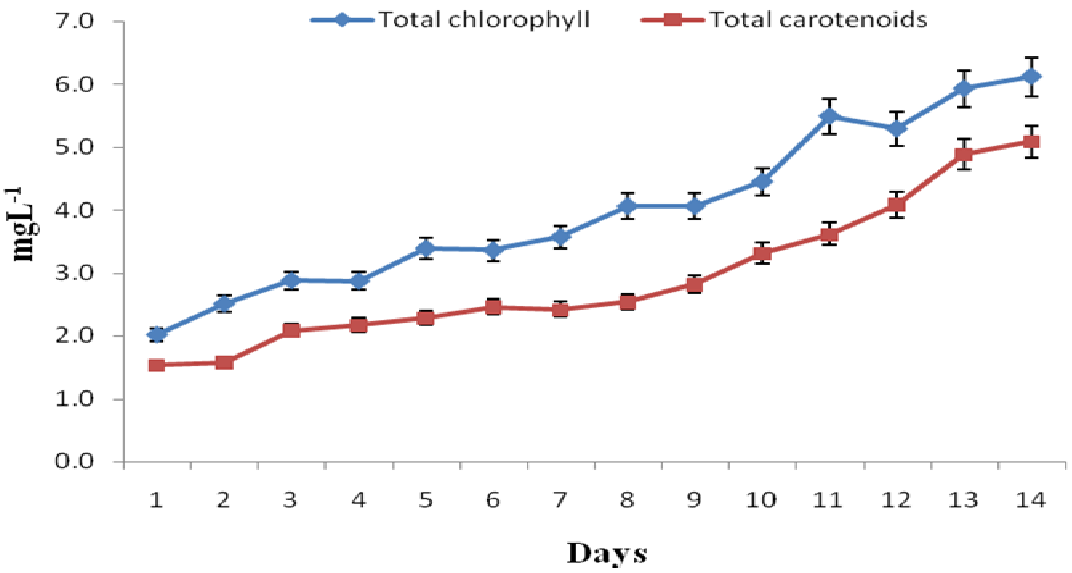

Figure 6. Total chlorophyll and total carotenoids yields of *B. mahabali* cultivated in raceway pond.

dried samples were analyzed for carotenoids and lipid yields. *B. mahabali* has shown exponential growth (Figure 5) up to 14 days and similar profiles were also observed by chlorophyll and carotenoids evaluation as well (Figure 6). The biomass yields were found to be 2.0 ± 0.09 g/L (w/w) up on two weeks growth in outdoor raceway ponds (Figure 5). During the first week of its growth, pH of the culture media was steadily increased from 7.5 to 9.3 and in the second week, it was increased up to 10.3. Contamination by any other algal species was checked periodically by microscopic observations, and no algal contaminations were witnessed. This may be due to the alkaline pH of the algal medium or may be due to other chemical profiles of the algae. The Indian isolate have shown relatively high contents of protein, carbohydrate and fat (Table 3).

Further, its fatty acids profile has shown relatively high levels of unsaturated fatty acids and hence it could be exploited for human or animal nutrition. However, further detailed studies are required to study the changes in its chemical profile using varied culture conditions, since various algae are reported to accumulate high levels of secondary metabolites under various stress conditions (Banerjee et al., 2002).

Table 3. Proximate composition[a] of *B. mahabali*.

Proximate composition	Outdoor	Indoor
Protein	19	20.5
Carbohydrate	18.74	18.78
Fat	14.3	13.7
Hydrocarbon	6.3	9.1
Moisture	5.41	4.93
Ash	39.73	33.15

[a]Means of triplicate determinations based on alga dry matter.

In conclusion the Indian isolate *B. mahabali* was cultivated for the first time in open air raceway ponds and the same can be exploited for its lipid and protein rich biomass for various applications from food to biodiesel. Indigenous species are most likely desirable for any successful industrial exploitation and hence the present investigation has used the new Indian isolate for outdoor cultivation. However, further studies are required to optimize culture parameters which are necessary for high yields of biomass and other secondary metabolites production for its industrial prospects.

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