Influence of Different Culture Conditions on Yield of Biomass and Value Added Products in Microalgae

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ABSTRACT

Microalgae *viz*., *Botryococcus*, *Haematococcus* and *Dunaliella*, were studied for their adaptability to different culture conditions. *Botryococcus* strains of indigenous origin and culture collection centres were studied for their growth, hydrocarbon and lipid profile and for their biomass yields under different bicarbonate concentrations. Growth of *Haematococcus* was compared in autotrophic (ambient and enriched CO_2 atmosphere) and heterotrophic culture conditions. Influence of ammonium salts, urea and commercial fertilizers as nitrogen source under autotrophic conditions and different amino acids as nitrogen source under heterotrophic culture conditions, was studied on growth and astaxanthin content of *Haematococcus*. Influence of different salinities was studied on growth and β-Carotene content of *Dunaliella* in AS100 and De Walnes media. Results indicated that *Botryococcus* strains varied in their biomass yields (0.25-2 g L^{-1}), hydrocarbon content (15-60% w/w) and lipid content (15-30%, w/w). An indigenous strain was able to grow at all the tested concentrations of bicarbonate with maximum biomass yield at 0.25 g L^{-1}. The growth rate of *Haematococcus* was higher in heterotrophic medium with a cell count of 41-44 × 10^4 on 5th day after inoculation. The supplementation of autotrophic medium with 2% CO_2 led to a 4-fold increase in cell count. Use of DAP as nitrogen source showed a 20% increase in yield of encysted biomass. Both DAP and Suphala led to astaxanthin productivity of 46-48 g L^{-1}. *Haematococcus* was able to utilize all the amino acids at tested concentrations in heterotrophic culture conditions. Enhanced growth of *Dunaliella* was observed at 1.0 M NaCl concentration in both the AS 100 and De Walnes media while 2.0M NaCl in De Walnes medium showed higher β-carotene content (45.6 mg g^{-1}).

INTRODUCTION

Microalgae are known as a source of value added compounds of nutraceutical, pharmaceutical and biochemical applications. Although several micro algal species are known taxonomically, their biochemical evaluation for high value metabolites is limited to very few species and when it comes to commercial exploitation, only a few of the organisms like *Spirulina*, *Chlorella*, *Dunaliella*, *Haematococcus*, *Porphyridium*, *Phaeodactylum*, *Crypthecodinium*, etc. are being cultivated at large scale for food supplements, feed ingredients and for cosmetic and pharmaceutical applications. In recent years exploration of microalgae for hydrocarbon and oil production has gained importance due to rise in crude oil prices and limited fossil fuel resources (Chisti 2008; Raja *et al.* 2008; Milledge *et al.* 2011).

Although algae are known to grow in waste water and some species are used in treatment of waste water, their potential as energy source has not been exploited. In the present scenario of energy crisis and global warming, microalgae are gaining importance as renewable resource for energy and CO_2 sequestration. In view of this, attempts were made to evaluate microalgal strains of *Botryococcus*, *Haematococcus* and *Dunaliella* for production of hydrocarbon, lipid and value added products. The strains were evaluated for their adaptability to varying concentrations of bicarbonate in growth media, heterotrophic growth conditions, different organic and inorganic nitrogen sources and salinity conditions.

Botryococcus braunii, a colonial chlorophycean is known to produce large amounts of hydrocarbons (Metzger

et al. 2005; Dayananda *et al*. 2010; Ranga Rao *et al*. 2012). *B.braunii* species are grouped into A, B and L races based on the type of hydrocarbons they produce (Metzger *et al*. 2005). Race A produces C_{23} to C_{33} odd numbered *n*-alkadienes, mono-, tri-, tetra-, and pentenes and are derived from fatty acids. The B race produces two types of triterpenes called botryococcenes of $C_{30} – C_{37}$ as major hydrocarbons and small amounts of methyl branched squalene (Achitouv *et al*. 2004). The L race produces a single hydrocarbon C_{40-78}, a tetraterpene known as lycopadiene. *B. braunii* is a promising renewable resource for the production of hydrocarbons and it has been reported that on hydrocracking, the distillate yields 67% gasoline, 15%, aviation turbine fuel, 15% diesel fuel and 3% residual oil (Hillen *et al*. 1982). *B. braunii* is also known to produce varying amount of ether lipids, the quantity and composition of which varies with species and also among the races (Metzger and Largeau 2005). Strains of *B. braunii* Races are known to produce exopolysaccharides which may be up to 250 g m^3 in strains of 'A' and 'B' race and up to 1 kg m^3 in strains of 'L' race (Banerjee *et al*. 2002; Niehaus *et al*. 2011). Large scale cultivation of *B. braunii* will also contribute towards CO_2 sequestration (Sawayama *et al*. 1999; Tanoi *et al*. 2011). *B. braunii* strains have been shown to be adaptable to outdoor culture conditions in circular and raceway ponds indicating the potential for their mass cultivation. However, improving the hydrocarbon content in outdoor conditions remains a challenge (Ranga Rao *et al*. 2012). The focus of present study is to evaluate growth, hydrocarbon and fatty acid profile of different strains of *B. braunii*, in response to different concentrations of bicarbonate.

Original Research Paper

Haematococcus pluvialis, the fresh water green alga produces a ketocarotenoid – astaxanthin, which accumulates in lipid globules under stress conditions (Kobayashi *et al.* 1993; Sarada *et al.* 2002). *Haematococucus* has two distinct phases in its life cycle. Motile vegetative and stationery encysted phase. The motile vegetative cells are shown to transform to astaxanthin containing encysted cells under environmental and nutritional stress conditions such as nitrogen and phosphorus limitation, high temperature, high salinity and high irradiance (Del Campo *et al.* 2007). In the stationary encysted phase the alga accumulates carotenoids with 85-90% of astaxanthin and its esters followed by other carotenoids (Maio *et al.* 2006). *Haematococcus* is capable of growth under autotrophic and heterotrophic conditions. Dalay *et al.* (2007) reported the ability of *H. pluvialis* strains to adapt to media comprising of commercial fertilizers. Closed photobioreactors of various designs like airlift, bubble column and tubular have been used for cultivation of *H. pluvialis* (Ugwu *et al.* 2008). Owing to high antioxidant activity of astaxanthin it has gained nutraceutical and pharmaceutical importance besides use as pigmentation source in farmed aquaculture (Kamath *et al.* 2008; Ranga Rao *et al.* 2011). Astaxanthin from *Haematococcus* was approved by US FDA and some European countries for its use in humans as dietary food supplement (Lorenz and Cysewski 2000). In the present study, a comparison between autotrophic and heterotrophic cultivation was made using different nitrogen sources.

Dunaliella is a halotolerant microalga which is known to accumulate higher amounts of β-carotene, up to 10% (w/w) in intrachloroplastid lipid globules under stress conditions such as nutrient starvation, high salinity, high temperature and high illumination (Ben-Amotz and Avron 1990; Borowitzka and Siva 2007; Lamers *et al.* 2008). Outdoor cultivation of *Dunaliella* for β-carotene has been successfully established and such commercial plants are in operation in Australia, Israel, China and India (Borowitzka and Siva 2007). *Dunaliella* is also gaining importance for its potential exploitation as a host for expression of foreign proteins (Barzegari *et al.* 2010). In the present work, growth profile and β-carotene content of *D. salina* was investigated in two different media (AS100 and De walnes) under varying salinities.

MATERIALS AND METHODS

Microalgal cultures, maintenance and experimental studies

1. Botryococcus braunii

Botryococcus braunii strains were obtained from National Institute for Environmental Studies, Tsukuba, Japan, *B. braunii* (N-836); SammLung von Algen Kulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany, *B. braunii* (SAG 30.81) and UTEX culture collection, U.S.A., *B. braunii* (LB 572). Indigenous strains were isolated from water bodies of Kodaikanal and Mamallapuram, Tamil Nadu, India and the strains were designated as CFTRI-Bb-1 and CFTRI-Bb-2. The stock cultures were maintained in modified Chu 13 medium, both in agar slants and liquid medium (Dayananda *et al.* 2005). For experiments, the cultures were grown in Erlenmeyer flasks of 150 mL capacity, containing 40 mL modified Chu 13 medium, inoculated with 20% (v/v) inoculum and incubated at $25 \pm 1°C$ under 1.7647 erg m^{-2} s^{-1} light intensity with 16-h photo period for 4 weeks. At the end of incubation period, biomass, lipid and hydrocarbon yields were estimated. The effect of bicarbonate on *B. braunii* strains was studied at 0.25-1.0 g L^{-1}.

2. Haematococcus pluvialis

Haematococcus pluvialis was obtained from SammLung von Kulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany and was maintained in heterotrophic (Kobayashi *et al.* 1991) and autotrophic Bold's Basal Medium (Kanz and

Bold 1969), both in agar slants and liquid medium.

Comparison of *H. pluvialis* growth in autotrophic and heterotrophic media

Haematococcus culture was inoculated in to autotrophic medium with ambient carbon dioxide and 2% CO_2 atmosphere (in two tier flasks as detailed below) and heterotrophic medium independently. Growth was monitored by following cell count at regular intervals.

Influence of different nitrogen sources on growth and astaxanthin production in *H. pluvialis* under autotrophic conditions

Bold's Basal Medium (BBM) containing ammonium chloride (0.25-1.0 g L^{-1} or 4.67-18.68 mM in terms of nitrogen), urea (0.15-1.0 g L^{-1} or 4.99-33.27 mM in terms of nitrogen) ammonium carbonate (0.15-1.0 g L^{-1} or 2.86-19.07 mM in terms of nitrogen), ammonium sulphate (0.15-1.0 g L^{-1} or 2.27-15.33 mM in terms of nitrogen) and ammonium acetate (0.25-1.0 g L^{-1} or 3.24-12.96 mM in terms of nitrogen), as nitrogen sources were independently prepared and *Haematococcus* cells in log phase were inoculated with an initial cell count of 12×10^4 cells/mL. BBM with sodium nitrate (0.249 g L^{-1} equivalent to 2.93 mM of nitrogen) was considered as control. The experiments were carried out in 150 mL Erlenmyer flasks containing 50 mL medium. Effect of commercial fertilizers i.e., Suphala (N-P-K mixture, i.e., nitrogen, phosphorus, potassium mixture) and diammonium phosphate (DAP) was also studied on *H. pluvialis* growth and astaxanthin production in the presence of 2% CO_2 using two tier flasks. While adding commercial fertilizers, the concentration of nitrogen and phosphorus were estimated using APHA (1989) and AOAC procedures (1999) and the amounts were adjusted accordingly equivalent to BBM. Suphala was obtained from Rashtriya Chemicals and Fertilizers Ltd. Mumbai, India and DAP from Gujarat State Fertilizers Ltd. Gujarat, India.

Two-tier vessels consisting of two 250-mL narrow neck Erlenmeyer flasks were used for enriching the culture environment with CO_2. The lower compartment of the flask contained 100 mL of 3M buffer mixture (KHCO$_3$/K$_2$CO$_3$) at specific ratio, which generated a partial pressure of CO_2 at 2% in the two-tier flask (Usha *et al.* 2001). The upper chamber contained 50 mL of Bold's basal medium with different nitrogen sources. A 10-day old autotrophic culture was used as inoculum for all the experiments. All the flasks were incubated at $26 \pm 1°C$ in a culture room under light intensity of 2.2058×10^7 erg m^{-2} s^{-1}. After two weeks of growth, one set of flasks were harvested for cell count and biomass estimation while second set of flasks were incubated further for two weeks under high light intensity of 5.1468×10^7 erg m^{-2} s^{-1} for carotenoid accumulation.

Influence of different amino acids as nitrogen source on growth and astaxanthin production in *H. pluvialis* in heterotrophic medium

Heterotrophic medium (Kobayashi *et al.* 1991) containing amino acids such as Leucine, Isoleucine, Valine, Methionine, Threonine, Serine, Tyrosine, Tryptophan, Histidine, and Proline as nitrogen sources were independently prepared and inoculated with 10% (v/v) inoculum. The concentration of the individual amino acids was calculated in terms of nitrogen equivalent to L-Asparagine in the heterotrophic medium. All the flasks were incubated at $26 \pm 1°C$ in a culture room under light intensity of 2.2058×10^7 erg m^{-2} s^{-1}. After 8 days of growth, culture flasks were subjected to stress by adding 0.25% NaCl with 14 mM sodium acetate and were incubated at high light intensity of 5.1468×10^7 erg m^{-2} s^{-1} for a period of 12 days. At the end of 12 days the cultures were harvested and estimated for biomass, total carotenoid and astaxanthin contents.

3. Dunaliella salina

Dunaliella salina strain V101 was obtained from Centre for Advanced Studies (CAS) in Botany, University of Madras, Chennai, India. *D. salina* was cultivated in AS100 (Vonshak 1986) and De walnes (Orset and Young 1999) media with varying salinities (0.5,

Table 1 Hydrocarbon profile of *B. braunii* grown in autotrophic medium.

B. braunii	$< C_{20}$	C_{20}-C_{24}	C_{24}-C_{30}	$> C_{30}$
SAG 30.81	3.12 ± 0.03 d	14.52 ± 0.13 b	2.77 ± 0.11 d	79.59 ± 3.20 b
LB-572	2.50 ± 0.04 c	3.30 ± 0.14 c	37.70 ± 0.40 a	56.50 ± 2.59 d
N-836*	2.20 ± 0.02 c	3.35 ± 0.05 c	4.45 ± 0.11 c	90.00 ± 3.96 a
CFTRI -Bb-1	8.94 ± 0.16 b	22.99 ± 1.28 a	2.67 ± 0.07 d	65.40 ± 3.14 c
CFTRI -Bb-2	10.25 ± 0.32 a	23.66 ± 1.98 a	10.50 ± 1.74 b	55.59 ± 2.98 d

* C_{33} and C_{34} botryococcenes constituted the major proportion of hydrocarbons (70± 5%) (Identified by GCMS) while around 20 ± 4 % are higher than C_{34} hydrocarbons which are not identified. Values are expressed as mean ± SD (n=3). Values not sharing a similar letter within the same column are significantly different ($P < 0.05$) as determined by ANOVA.

1.0 and 2.0 M). The AS100 medium was modified by replacing Tris with $NaHCO_3$. The liquid cultures were maintained at 25±1°C in Erlenmeyer flasks under 12-h photo period at a light intensity of 1.7647×10^7 erg m^{-2} s^{-1}. The cultures were mixed manually once a day. After 20 days of incubation cultures were harvested and total carotenoid and β-carotene content were estimated.

Estimation of algal growth

Algal growth of *H. pluvialis* and *D. salina* was monitored by cell count using a haemocytometer (Thoma neu, Menzelglaser, Germany). Since *Botryococcus* is a colonial form, growth was monitored in terms of biomass on a dry weight basis. *Botryococcus* cultures were harvested by centrifugation at 5000 rpm for 10 min and the harvested cells were washed twice with distilled water and resultant pellets were freeze dried. The dry weight of algal biomass was determined gravimetrically and expressed as g L^{-1}.

Extraction and analysis of metabolites

1. Estimation of total carotenoid, β-carotene and astaxanthin

Carotenoids were extracted from known quantity of biomass with acetone and the absorbance was measured spectrophotometrically at 450, 470 and 480 nm. Total carotenoid was estimated using equations of Lichtenthaler (1987). Astaxanthin was determined at 480 nm using an absorption coefficient, A1% of 2500 by the method of Davies (1976). For estimation of β-carotene, acetone extract from *Dunaliella* biomass was analyzed using HPLC (Shimadzu LC-10 AT liquid chromatography instrument) with reverse phase C18 column (Supelco, 25 cm × 4.6 mm) at a wavelength of 445 nm. Gradient solvent system consisting of acetone and methanol (90%) (v/v) at a flow rate of 1.25 mL min^{-1} was used. The separated carotenoids were identified by comparing retention times and spectra against known standards. The peaks were integrated by Class VP version 6.14 SPI software (Shimadzu, Singapore) at 445 nm to quantify carotenoids. β-carotene was identified and quantified with authentic standard (Sigma-Aldrich, Bangalore).

2. Hydrocarbon extraction

Hydrocarbon was extracted by homogenizing the dry biomass with *n*-hexane for 30 min and supernatant recovered after centrifugation was evaporated to complete dryness under the stream of nitrogen gas. Hydrocarbon content was measured gravimetrically and expressed on a dry weight basis (Sawayama *et al*. 1992).

3. Hydrocarbon analysis by GC/GC-MS

Hydrocarbon extract was purified by column chromatography on silica gel. The hydrocarbon sample was analyzed on GC/GC-MS (Perkin Elmer Instruments, USA) using Elite-5 (cross bond 5% diphenyl -95% dimethyl polysiloxane) capillary column (30 m × 0.25 mm ID × 0.25 μm film thickness, Perkin Elmer Instruments, USA) with a temperature gradient of initial temperature of 130°C for 5 min followed by 8°C per min rise to 200°C and retained for 2 min followed by 5°C per min rise to final temperature of 280°C retained for 15 min with nitrogen as carrier gas at a flow rate of 1 mL min^{-1}. The injector and detector port temperatures were maintained at 240 and 250°C, respectively (Dayananda *et al*. 2005). The mass spectra were recorded under electron impact ionization at 70 eV electron energy with a mass range from 40-600 at a rate

of one scan/s. Hydrocarbons were identified by their M$^{+•}$ ions and comparison of the mass spectra with those of the standards as reported by Wake and Hillen (1980). Comparison of the botryococcenes major daughter ions and their relative abundances afforded a high degree of (>96%) match. Further, relative retention data of these compounds conformed with respect to squalene with those reported in literature (Metzger *et al*. 1990).

4. Fatty acid methyl esters analysis

Lipids were extracted with chloroform: methanol (2: 1) and quantified gravimetrically. The fatty acid methyl esters (FAME) were prepared following the procedure of Christie (1982). FAME were analyzed by GC (Dayananda *et al*. 2006) and GC-MS (Perkin Elmer, Turbomass Gold, mass spectrometer) equipped with FID using SPB-1 (poly(dimethylsiloxane)) capillary column (30 m × 0.32 mm ID × 0.25 μm film thickness) with a temperature programming from 130 to 280°C at a rate of 2°C min^{-1}. The FAME were identified by comparing their fragmentation pattern with authentic standards (Sigma) and also with NIST library (Dayananda *et al*. 2006).

Statistical analysis

Experiments were conducted in triplicates and the data represented are the averages of mean of three independent experiments with standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel XP (Microsoft Corp., Redmond, WA), and the *post-hoc* mean separations were performed by Duncan's multiple range test (DMRT) at $P < 0.05$.

RESULTS AND DISCUSSION

Growth and hydrocarbon profile in *B. braunii* strains

B. braunii strains were grown in modified Chu 13 medium and their growth and hydrocarbon content were estimated (**Fig. 1**). Among the strains studied, LB-572 was found to be the best in terms of biomass yields (2 gL^{-1}) followed by CFTRI-Bb-1and 2, SAG-30.81 and N-836. The hydrocarbon content in N-836 strain was significantly high (60% w/w) when compared to the other strains, although its biomass yield was very low (**Fig. 1**). The strains SAG-30.81 and LB-572 were identified as 'A' race based on their
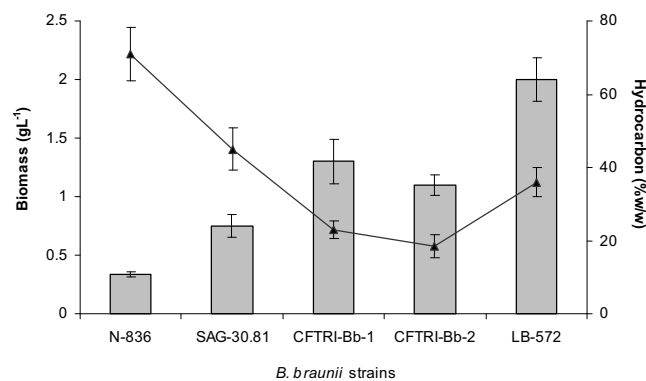
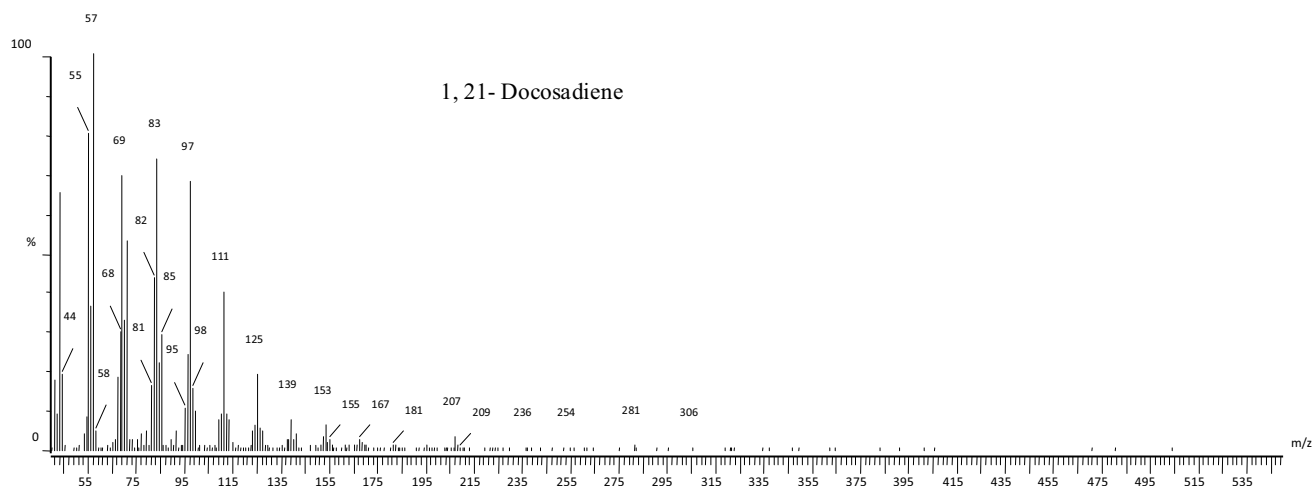**Fig. 1 Biomass and hydrocarbon yields of different *Botryococcus braunii* strains.**

Fig. 2 Mass spectra of unsaturated hydrocarbon produced by the alga *Botryococcus braunii* (SAG 30.81 and LB-572) strains.
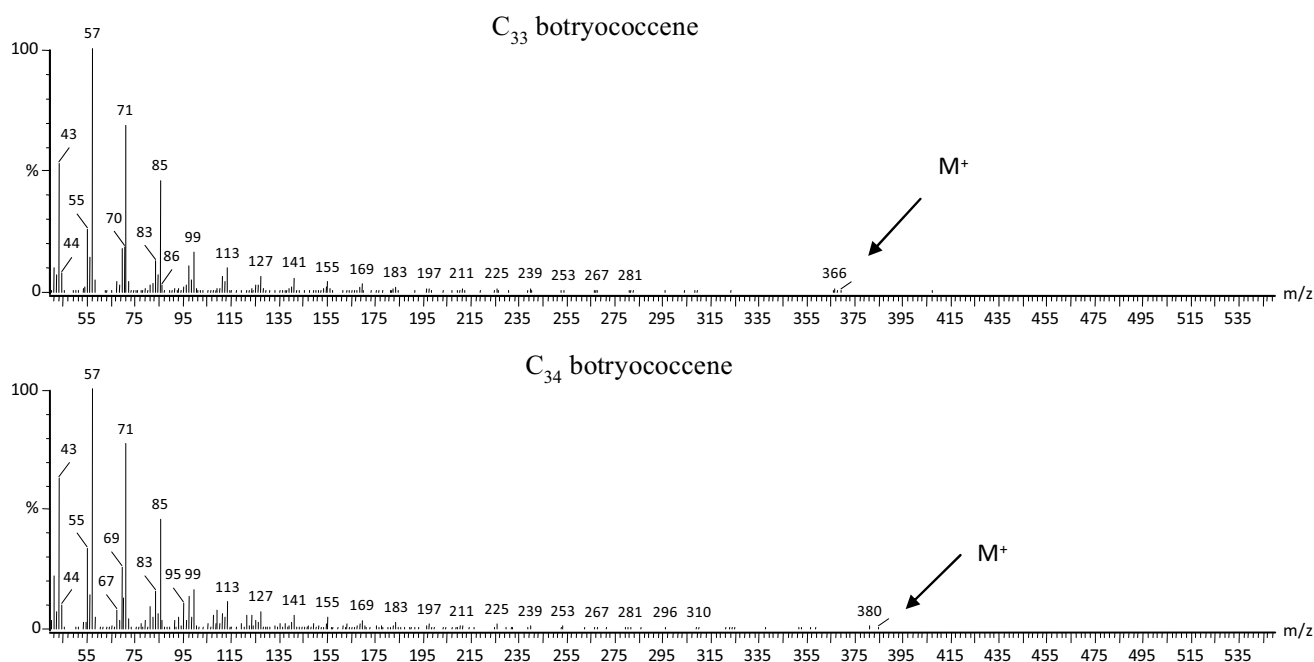

Fig. 3 Mass spectra of C$_{33}$ botryococcene and C$_{34}$ botryococcene hydrocarbons of *Botryococcus braunii* (N-836).

hydrocarbon profile (**Fig. 2**), however they differed in biomass yields and hydrocarbon productivity (**Fig. 1**). The strains SAG-30.81 and LB-572 also differed in their hydrocarbon profile as analysed by GC and presented in **Table 1**. The strain N-836 was identified as 'B' race on the basis of its hydrocarbons, and found to contain C$_{33}$ and C$_{34}$ botryococcenes (**Fig. 3; Table 1**) as the major hydrocarbons. The CFTRI -Bb-1and 2 produced saturated hydrocarbons in the range of C$_{20}$ to C$_{32}$ (**Fig. 4**) with 15-18% (w/w) hydrocarbon content (**Fig. 1**). There are reports on *B. braunii* producing saturated hydrocarbons in the range of C$_{21}$ to C$_{31}$ (Yang *et al.* 2004). Dayananda *et al.* (2006) also reported production of saturated hydrocarbons by a botryococcene-producing strain of *B. braunii*.

The hydrocarbon content varied (15-60% w/w) among the different strains studied. Largeau *et al.* (1980) reported the variation of hydrocarbon content in the range of 14-40% among the strains based on culture conditions and their natural habitats. Casadevall *et al.* (1985) studied the growth of *B. braunii* in "air-lift" reactors in batch and continuous mode with various dilution rates and reported 27% (w/w) hydrocarbon under batch culture and 22% under continuous culture in 'A' race of *B. braunii*. Dayananda *et al.* (2005) reported 50% of hydrocarbon production by the 'A' race of *B. braunii* under optimized culture conditions. Frenz *et al.*

(1989) reported 1.5 g L^{-1} biomass yield with 21% (w/w) of hydrocarbon production in 'B' race of *B. braunii*. The biomass yield and hydrocarbon production in various species of *B. braunii* under indoor and outdoor culture conditions are reported by Ranga Rao *et al.* (2007, 2012).

Fatty acid profile of *B. braunii* strains

The lipid content (w/w) varied among the strains of *B. braunii* (SAG-30.81, 30%; LB-572, 24%; N-836, 15%). The strains also differed in their fatty acid profile (**Table 2**), especially in the relative proportion of stearic, linoleic, palmitic and oleic acids. Oleic acid was found to be the major fatty acid in SAG 30.81, LB 572 and N-836 strains. Palmitic acid was a major fatty acid in indigenous strains, CFTRI -Bb-1 and CFTRI Bb-2. Palmitic acid was also a major fatty acid in N-836 strain. The fatty acid profile in both indigenous strains, CFTRI-Bb-1 and 2, was found to be similar. In general, the fatty acid profile changes with the physiological state of the culture. In *B. braunii* species, oleic acid was reported as the precursor of the non-iso-prenoid hydrocarbons produced by the race A. Oleic acid involvement in the formation of very long chain fatty acid derivatives through chain elongation was reported by Lareillard *et al.* (1988). Templier *et al.* (1984) reported
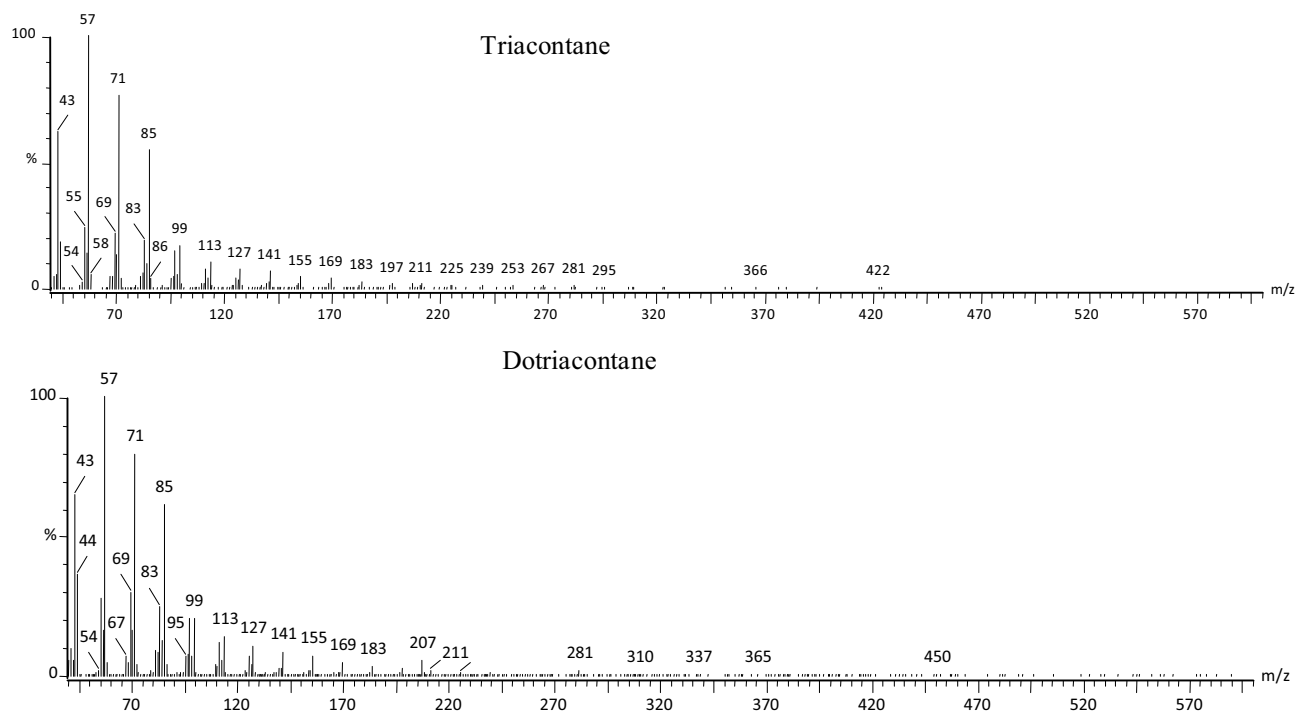
Triacontane

Dotriacontane

Fig. 4 Mass spectra of saturated hydrocarbons produced by the CFTRI-Bb1 and2 strains.

Table 2 Fatty acid profiles of different *B. braunii* strains.

Fatty acids	Relative (%)				
	*SAG 30.81	+LB-572	**N-836	CFTRI -Bb-1	CFTRI -Bb-2
12:0	0.67 ± 0.06 e	0.78 ± 0.29 f	0.48 ± 0.28 g	2.35 ± 0.56 e	1.98 ± 0.08 h
14:0	8.40 ± 0.64 d	2.48 ± 0.39 e	1.68 ± 0.45 f	2.69 ± 0.91 e	3.65 ± 1.12 f
15:0	0.48 ± 0.04 f	0.60 ± 0.15 g	ND	ND	ND
16:1	2.89 ± 0.32 d	0.87 ± 0.28 f	1.79 ± 0.56 f	ND	ND
16:0	13.28 ± 1.04 b	10.61 ± 0.39 c	34.89 ± 7.90 b	36.09 ± 4.35 a	41.64 ± 6.89 a
17:0	ND	ND	0.28 ± 0.12 h	ND	ND
18:3	0.66 ± 0.02 e	20.04 ± 0.74 b	5.06 ± 0.94 e	9.03 ± 2.02 d	12.18 ± 3.73 d
18:2	8.63 ± 0.48 c	Trace	7.56 ± 1.02 d	18.28 ± 3.71 c	13.55 ± 1.09 c
18:1	56.21 ± 2.16 a	53.19 ± 5.23 a	38.78 ± 7.18 a	26.02 ± 3.41 b	23.02 ± 4.24 b
18:0	2.20 ± 0.40 d	3.48 ± 2.9 d	9.93 ± 4.56 c	3.25 ± 1.12 f	4.52 ± 3.30 e

ND: not detected

* 19:0, 19:1, 20:0, 20:1, 24:0 26:0 and 28:0 fatty acids are present in traces, + 19:0, 19:1, 20:0, 20:1, 24:0 26:0 and 28:0 fatty acids are present in traces, **19:0, 19:1, 20:0, 20:1, 22:0, and 22:1 fatty acids are present in traces. Values are expressed as mean ± SD (n=3). Values not sharing a similar letter within the same column are significantly different ($P < 0.05$) as determined by ANOVA.

changes in the concentration of oleic acid with the physiological state of the alga. Lee *et al.* (2010) have also reported the oleic acid content to be high in *B. braunii* making it suitable for biodiesel. Therefore the changes observed in the fatty acid profile in different species are in accordance with the earlier reports.

Effect of bicarbonate on growth profile of *B. braunii* strains

The strain, LB-572 and two of the indigenous strains, CFTRI Bb-1 and CFTRI Bb-2, were evaluated for their ability to utilize bicarbonate as a carbon source. The growth profile of these strains is shown in **Fig. 5**. Of the three strains studied, indigenous strain (CFTRI-Bb-2) was able to grow at all the tested concentrations of bicarbonate with maximum biomass yield at 0.25 g L^{-1} (w/v). The strain LB-572 showed lesser yields of biomass compared to indigenous strains.

Growth profile of *H. pluvialis* in autotrophic and heterotrophic media

It is evident from **Fig. 6** that the growth phase of *Haematococcus* extended to 18 days at relatively slow growth rate in autotrophic medium compared to heterotrophic medium.

Supplementation of 2% carbon dioxide significantly enhanced the growth rate (4-fold increase in cell count) in autotrophic medium (**Fig. 6A**). In heterotrophic medium, the algal growth rate was higher and for a short duration (4-6 days). Maximum cell count of 41-44 × 10^4 cells mL^{-1} was observed on 5th day after inoculation (**Fig. 6B**). Enhanced growth rate in heterotrophic medium leads to early onset of nutrient limitation causing the cells to enter stationary phase
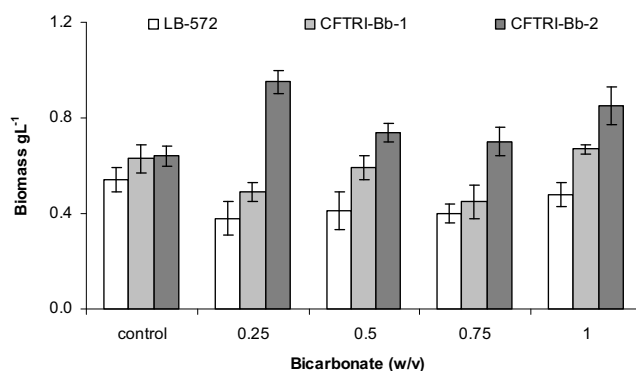

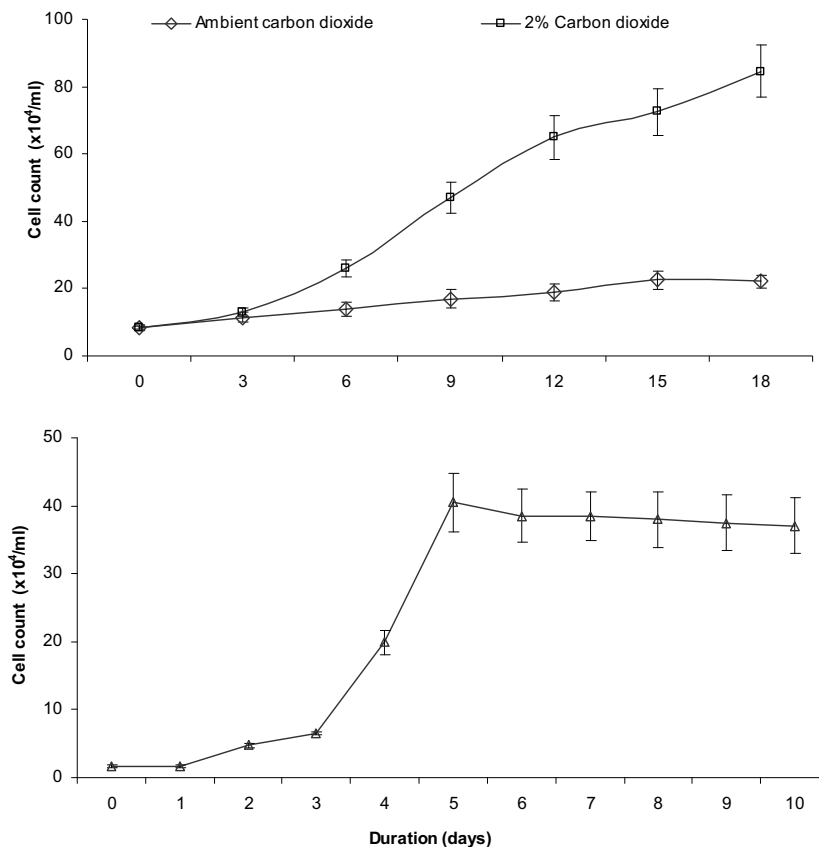**Fig. 5 Effect of bicarbonate on biomass yield and growth in *Botryococcus braunii* strains.**

Fig. 6 Growth profile of *Haematococcus pluvialis* in autotrophic (top) and heterotrophic (bottom) medium.

early and undergo encystment accompanied by carotenoid accumulation.

Influence of ammonium salts, urea and commercial fertilizers under autotrophic conditions on *H. pluvialis* growth and astaxanthin production

H. pluvialis was grown in modified Bold's Basal Medium with ammonia salts and commercial fertilizers, in both ambient and enriched environment of CO_2. In ambient environment of carbon dioxide, *H. pluvialis* cells were found to be motile up to 48 h at all concentrations of ammonia salts studied (except ammonium acetate), whereas at higher concentrations (>0.25 g L^{-1}) of ammonia salts, 50% of the cells lost motility after 48 h. The growth in terms of cell count (21×10^4 cells/mL) was comparable to control only at 0.25 g L^{-1} and at higher concentrations the cell count per mL decreased (data not shown).

In CO_2 enriched environment, cell count of $80-100 \times 10^4$ cells mL^{-1} was obtained with different ammonia salts (ammonium carbonate, ammonium acetate) which is 8-10-fold increase over initial cell count (12×10^4 cell mL^{-1}). The ammonia concentration in the *Haematococcus* culture was decreased to 9% of the initial level, which shows 90% of NH_3 utilization. The cells grown in urea, turned brownish yellow colour after 6 days indicating early induction of carotenoid formation (data not shown).

In the second set of CO_2 enriched flasks, which were exposed to high light the algal cells were found to be encysted with carotenoids accumulation. The encysted biomass yield was found to be 2.6 to 2.8 g L^{-1} which was comparable to the control (**Fig. 7A**) except in urea (0.15 g L^{-1}) and ammonium sulphate (0.15 g L^{-1}) where the biomass yield was significantly less. In the case of DAP there was 20% increase in biomass yield compared to control. Total carotenoid content in all treatments was found to be in the range of 2.0-2.2%, except in ammonium acetate (1.57%) and urea (1.02%). However, astaxanthin constituted 85-88% of the total carotenoid in all the cultures. Astaxanthin pro-

ductivity which is a sum of astaxanthin content and biomass yield was high in control (BBM) and commercial fertilizers (46-48 g L^{-1} of Suphala and DAP) followed by ammonium carbonate and ammonium acetate and least in urea (**Fig. 7B**). Total carotenoid and astaxanthin productivities were found to be 15 and 13% higher in DAP culture when compared to control and suphala cultures respectively. Dalay *et al.* (2007) reported that the commercial fertilizer (N-P-K 20:20:20) maximized the cell concentration to 0.90 g/l with a growth rate of 0.150 d^{-1}. Tocquin *et al.* (2012) reported the use of low-cost hydroponic fertilizers for achieving high cell density of vegetative cells of *H. pluvialis*. Our results also suggest that commercial fertilizers could be used as nutrient source in formulation of autotrophic growth medium for cultivation of *H. pluvialis*.

Algae in general have the ability to use different organic and inorganic nitrogen compounds as nitrogen source. Some microalgae were shown to be sensitive to ammonia as a nitrogen source by Abeliovich and Azov (1976), who reported unionized ammonia to be more toxic to various photosynthetic organisms with swelling and osmotic lysis of cells in the case of *Prymnesium parvum*. Swelling and cell lysis was also observed in the present study when ammonium acetate and urea were used at higher concentrations. The results indicated that *H. pluvialis* can utilize ammonia salts as nitrogen source in the presence of CO_2 for photoautotrophic growth and it could not utilize ammonium acetate without CO_2. It was of interest to know that the transfer of *Haematococcus* culture to medium containing DAP significantly increased the biomass yield (3.5 gL^{-1}) and carotenoid production (65 mg L^{-1}) with 85% astaxanthin (**Fig. 7B**). The importance of modulation of nitrogen concentration in the medium to promote growth and astaxanthin accumulation has been reported by Tocquin *et al.* (2012) showing 5 mM nitrate to promote vegetative cell growth and lower levels to induce astaxanthin accumulation.
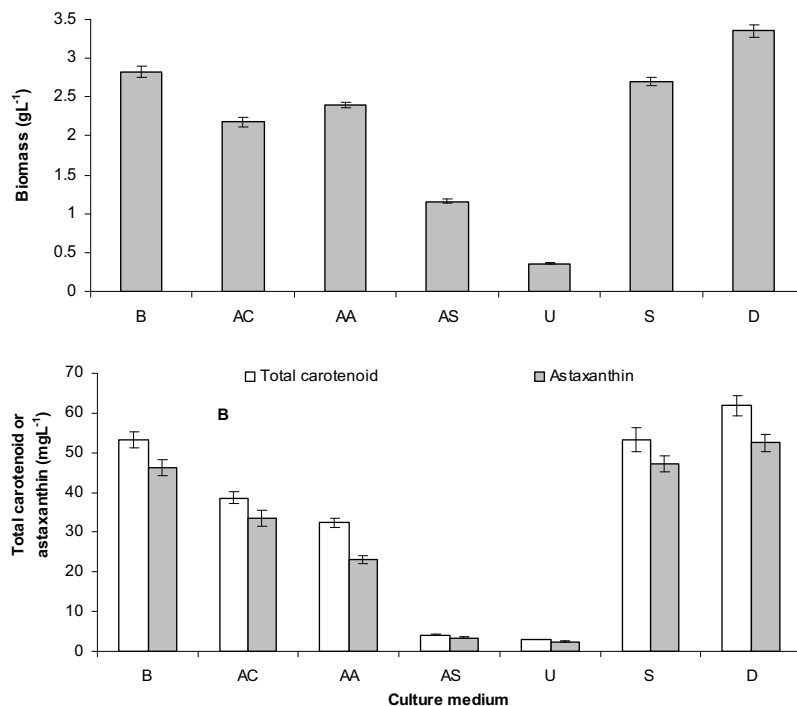
Fig. 7 Biomass yield of *Haematococcus pluvialis* (top) and total carotenoids and astaxanthin production (bottom) in the presence of 2% CO_2. B - BBM, AC - ammonium carbonate (0.15 gL⁻¹), AA - ammonium acetate (0.25 gL⁻¹), AS - ammonium sulphate (0.25 gL⁻¹), U - urea (0.15 gL⁻¹), S - Suphala (N-P-K mixture) (1.0 gL⁻¹), D - diammonium phosphate (0.25 gL⁻¹).

Influence of different amino acids on growth and astaxanthin production in *H. pluvialis*

The initial cell count in the heterotrophic medium was maintained at 2.2×10^4 cells mL⁻¹. The multiplication rate in 4 days duration ranged between 13-25 times in different amino acids studied. Growth in terms of cell count on 4th day was found to be same in leucine, methionine and valine (45 to 49×10^4 cells mL⁻¹) while it was marginally high in isoleucine and threonine (53 to 54.5×10^4 cells mL⁻¹) compared to L-asparagine (37.5×10^4 cells mL⁻¹). Low cell count (29 to 30×10^4 cells mL⁻¹) was observed in tyrosine and tryptophan. The organism was not able to utilize histidine at the tested concentration and the cells were bleached. The biomass yield in all amino acids was in the range of 0.74 to 0.96 g L⁻¹ except in L-asparagine where it was 0.54 g L⁻¹.

Total carotenoid and astaxanthin contents varied in different amino acids containing media. Total carotenoid production was found to be 7.7 to 7.8 mg L⁻¹, respectively in L-asparagine-, isoleucine- and threonine-treated cultures (**Fig. 8**). Irrespective of the productivities of total carotenoids, all amino acid treated cultures showed high content of astaxanthin (90-95% of total carotenoids) under high light and stress conditions except for serine and tyrosine where astaxanthin constituted only 84%.

The increase in cell count and biomass yields (**Fig. 8**) indicates that *Haematococcus* is able to utilize all the amino acids studied as nitrogen source. The differences in the biomass yields and carotenoid production in spite of high cell counts (especially in serine and proline) suggest the inter relationship of various metabolic activities in the cell. Sarada *et al.* (2002) reported that stress is a cumulative factor of the physiological state of the culture and the culture conditions. Therefore the variations in the yields of biomass and carotenoids under stress conditions with different amino acids as nitrogen source is as expected. In general the amides, urea, glutamine and asparagine are good sources of nitrogen. Algae are known for their ability to utilize amino acids as source of nitrogen (Berman and Bronk 2003).

The results clearly indicated that the organism can tolerate ammonia salts in the range of 3-4.7 mM concentration in autotrophic conditions and can use all amino acids as
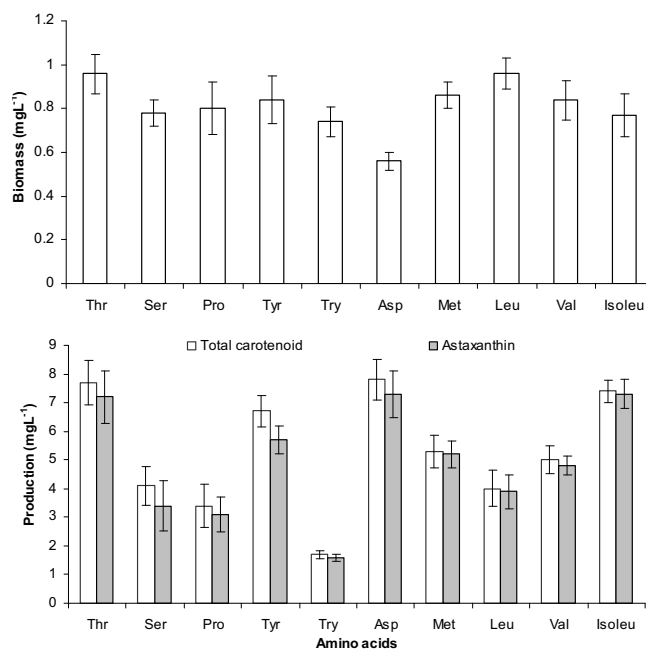
Fig. 8 Biomass yields (top) and carotenoid production (bottom) in *H. pluvialis* grown in different amino acids with 0.25% NaCl and 14 mM sodium acetate at high light intensity of 5.1468×10^7 erg m⁻² s⁻¹. Thr - Threonine, Ser - Serine, Pro - Proline, Tyr - Tyrosine, Try -Tryptophan, Asp - Asparagine, Met - Methionine, Leu - Leucine, Val - Valine, Isoleu - Isoleucine.

nitrogen source in heterotrophic conditions. The results also substantiated that cultivation of *Haematococcus* in autotrophic conditions with CO_2 supplementation resulted in higher yields of biomass, total carotenoid and astaxanthin. The significant increase in biomass and astaxanthin production achieved with DAP could be exploited for the batch production system.
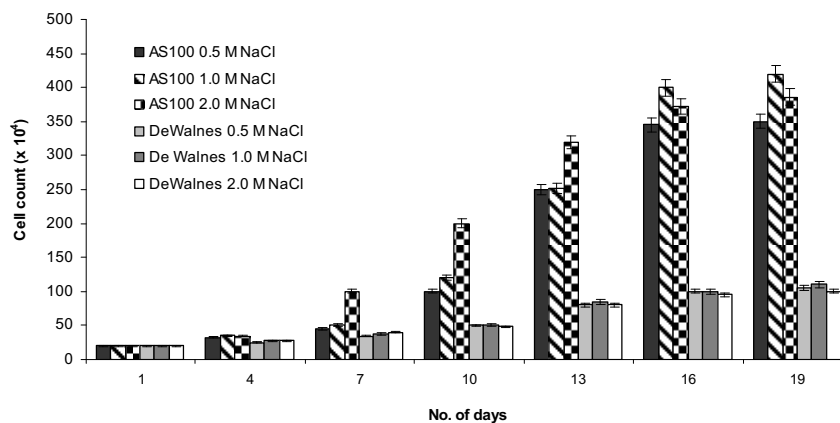
Fig. 9 Growth profile of *Dunaliella salina* in AS100 and De Walnes media at different salinities.

Influence of different salinities on growth and β-carotene content of *Dunaliella salina*

The growth profile of *D. salina* indicates AS100 medium to be better for growth than De Walnes at all the sodium chloride concentrations studied. Better growth was observed at 1.0 M NaCl in both media (**Fig. 9**) with AS 100 medium showing a 4-fold increase in growth rate compared to De Walnes medium. At 2.0 M NaCl, cell count was lower than 1.0 M in both media. NaCl concentrations above 3.0 M resulted in bleaching of the cells. Borowitzka *et al.* (1990) also reported low growth of alga at higher salinities. The lag phase was observed until 9 days in AS100 after which the cell count was increased up to 17 days. From the 18th day the cell count remained almost stationary. However in De Walnes medium, lag phase lasted for 13 days followed by growth phase. Although AS100 medium was better for growth, β-carotene content was found to be higher in De Walnes medium (**Fig. 10**). At 2.0 M NaCl, *D. salina* had a β-carotene content of 45.6 mg g^{-1} in De Walnes medium whereas in AS100 medium the β-carotene content was 16.5 mg g^{-1}. The results indicate that *Dunaliella* can grow over a range of salinity and accumulate β-carotene at higher salinity. Wide range of salinities is reported to be tolerated by *Dunaliella* spp. with hypersaline/halophilic species having salinity optima of >6% NaCl for growth (Borowitzka and Siva 2007). Algal species accumulate secondary metabolites mainly carotenoids under various stress conditions (Ranga Rao *et al.* 2007, 2010; Orosa *et al.* 2000) and carotenoid content of *Dunaliella* has been reported to be salinity dependent (Borowitzka *et al.* 1990) with higher levels of carotenoids accumulated at higher salinity (Ben-Amotz and Avron 1990). Fazeli *et al.* (2006) have also studied the effect of various salt concentrations (0.05 to 3.0 M NaCl) on growth and carotenoid accumulation in a *Dunaliella tertiolecta* isolated from Urmia salt lake of Iran and have reported similar results where growth and carotenoid production were salinity dependent.

CONCLUSIONS

The study clearly establishes the potential of microalgae – *Botryococcus*, *Haematococcus* and *Dunaliella* – in utilization of varied concentrations of ammonia salts and commercial fertilizers as source of nitrogen, carbon di-oxide and organic nutrients and ability to grow at different salinity, in autotrophic and heterotrophic conditions to generate biomass containing value added products. The effluents from urban, agricultural, municipal and industrial origin are known to have concentrations of nitrogen and phosphorus higher than the natural water bodies and also contain organic nutrients and microalgae have been identified to have potential to provide tertiary treatment of such waters (Noue *et al.* 1992; Wang *et al.* 2010). The microalgae would convert these nutrients and provide biomass enriched with value added metabolites that could be used for fuel, feed
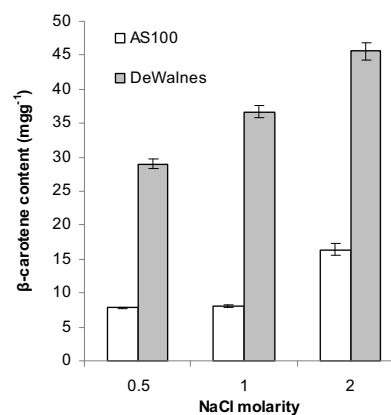

Fig. 10 β-Carotene content of *Dunaliella salina* in AS100 and De Walnes media at different salinities.

and other commercial applications. The current study establishes the potential of microalgae for such applications. The protocols developed at our lab on outdoor cultivation of microalgae, especially *Botryococcus* and *Dunaliella*, provide an adaptable model to cultivate microalgae. A detailed study towards development of cultivation protocols utilizing wastewaters of various origins for microalgal cultivation would be an important area of study.

ACKNOWLEDGEMENTS

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