ISSN: 2634 - 8845

Journal of Earth and Environmental Science Research

SCIENTIFIC Research and Community

Research Article

Open Access

Effect of Incubation Temperature, Light Intensity and Salinity on Growth, Lipid Content and Composition of Two Egyptian Race a of Botryococcus Braunii

Mohammad I Abdel-Hamid[1]*, Yehia A Azab[1], Mervat H Hussain[1] , Eman I Abdel-Aal[2] and M Abdel-Mogib[3]

[1]University of Mansoura, Faculty of Science, Botany Department, Mansoura, Egypt

[2]National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt

[3]University of Mansoura, Faculty of Science, Chemistry Department, Mansoura, Egypt

ABSTRACT

Effects of the incubation temperature, light intensity and NaCl salinity on growth in addition to lipid content and composition of two isolates of Botryococcus braunii, (JN580448.1 and JN580451.1) were investigated. The light to dark period was 16h: 8h. Cultures were incubated for 7 weeks and harvested with GF/C filters for dry weight determination. Lipids were extracted by n-hexane and analyzed by GC/MS. Compared to control cultures, the temperature of 30oC induced significant ($P \leq 0.05$) increase in biomass by 14.89% and 29.89% and lipids by 16.15% and 20.29% for isolates EG-Bb01 and EG-Bb04, respectively. The light intensity 48.6 µ mol photons $m\text{-}2s^{-1}$ induced very high significant ($P \leq 0.001$) increase in biomass (88.71% and 100.7%) and lipids (118.63% and 94.61%) of EG-Bb01 and EG-Bb04, respectively. Similarly, 17 mM NaCl salinity induced high significant ($P \leq 0.01$) increase in biomass (23.82% and 17.71%) and lipids (32.92% and 24.51%) of EG-Bb01 and EG-Bb04, respectively. Considerable numbers of short C6-C15 chain compounds were detected in biomass of cultures grown under light intensity of 48.6 µ mol photons $m\text{-}2s^{-1}$ and in cultures supplemented with 17mM NaCl. These results may indicate that the Egyptian isolates of Botryococcus are potential source of hydrocarbon biofuel.

***Corresponding author**

Mohammad I Abdel-Hamid, University of Mansoura, Faculty of Science, Botany Department, Mansoura, Egypt. E-mail: mhamid@mans.edu.eg

Received: October 26, 2019; **Accepted:** November 11, 2019; **Published:** November 14, 2019

Keywords: Botryococcus braunii, hydrocarbon, incubation temperature, light intensity, NaCl salinity.

Introduction

Production of microalgae biomass for biofuel must be economically feasible and cost competitive with liquid fuels. Consequently, the success of biofuel production from microalgae depends on high biomass productivity and considerable lipid yield [1,2,3]. It has been widely reported that certain growth controlling factors may involve distinct stimulatory effects on biomass and lipid production of microalgae. These growth controlling factors include, but not limited to; nitrogen concentration, silicon deficiency, phosphate limitation, Fe^{3+} concentrations, salinity, photoperiod and light intensity and the incubation temperature [2, 5-18].

Botryococcus braunii Kützing is a green colonial microalga belongs to the class Trebouxiophyceae, widespread in fresh and brackish waters of all continents [19,20]. This alga is characterized by the ability to produce high level of hydrocarbons in the range of 15-76% dry weight [21,22]. The absence of clear morphological differences between the *Botryococcus braunii* strains producing different types of hydrocarbons led to their categorization into three chemical races namely, A, B and L depending on the type of hydrocarbon synthesized [23]. Race A produces (C23–C33, CnH2n-2) straight-chain odd carbon-number n-alkadienes [24]. Race B produces unsaturated triterpenoid hydrocarbons (CnH2n–10, n = 30–37), referred to as botryococcenes, and small amounts of methyl branched squalenes [22,24,25]. The L race of *B. braunii* produces a single hydrocarbon tetraterpene ($C_{40}H_{78}$) known as lycopadiene [24].

Accordingly, *B. braunii* would be useful for hydrocarbon biofuels production. For commercial production of hydrocarbon biofuel, locally adapted algae strains and optimized cultivation conditions are required. Based on literature survey, it became evident that certain parameters including medium composition, in addition to certain physical and chemical factors affect, to a varying extent, both the biomass and hydrocarbon production of *B. braunii*. Accordingly, some specific potential growth controlling factors e.g. NaCl salinity, light intensity and temperature on biomass and lipid of *Botryococcus braunii* were selected and their effects on biomass and hydrocarbon production of two Egyptian isolates EG-Bb01 (JN580448.1) and EG-Bb04 (JN580451.1) were investigated.

Materials and methods

Isolates of B Braunii

Botryococcus braunii isolates EG-Bb01 and EG-Bb04 were isolated from Nile River at the Delta region, Egypt by using streaking agar plate method [26]. The isolated microalga was identified as *Botryococcus braunii* Kützing according to morphological properties and molecular properties by analysis of 5.8S ribosomal RNA gene (isolate EG-Bb01 (JN580448.1) and isolate EG-Bb04 (JN580451.1)) [27-29].

Nutrient Medium and Growth Conditions

The isolates were cultured in modified Chu 13 medium [30] composed of (gl^{-1}) 0.2 g KNO_3, 0.04 g K_2HPO_4, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.08 g $CaCl_2 \cdot 6H_2O$, 0.01g ferric citrate, 0.1 g citric acid, 0.5 mg H_3Bo_3, 0.5 mg $MnCl_2.4H_2O$, 0.02 mg $CuSO_4.5H_2O$, 0.02 mg $Co(NO_3)_2.6H_2O$, and 0.02 mg $Na_2MoO_4.2H_2O$. The pH is adjusted at 7.5 before sterilization. The growth experiments were carried out in 250 ml Erlenmeyer flasks, containing 80 ml modified Chu 13 medium [30]. The control culture flasks were inoculated with 20ml of two weeks old B. braunii culture containing approximately 0.05 gl^{-1} dry biomass (dried at 60°C) and incubated for 7 weeks at 25 ± 1ºC under 16.2 ± 2.7 µmol photons m-2 s-1 light intensity with 16:8 hrs light: dark cycle [31].

The investigated parameters

The investigated parameters include incubation temperature, light intensity and NaCl salinity. On studying the effect of a single variable, all other parameters were kept constant at their control levels [31]. For statistical analysis all growth experiments were carried out on triplicates.

Effect of incubation temperature (°C)

Four different incubation temperatures (20°C, 25°C (control), 30°C and 35°C) were selected to study the effect of temperature on biomass and lipid yields. At the end of the 7 weeks incubation period, dry weight biomass (gl^{-1}) and lipid (gg^{-1}) were determined.

Effect of light intensity

Different incubation light intensities including 8.1 ± 2.7, 16.2 ± 2.7 (control), 24.3 ± 2.7, 32.4 ± 2.7, 40.5 ± 2.7, 48.6 ± 2.7 and 56.7 ± 2.7 µ mol photons m^{-2} s^{-1} were selected to evaluate the effect of light intensity on biomass and lipid production of *B. braunii* isolates. Cool white fluorescent tubes were used as light source to avoid any drastic temperature changes. At the end of the 7 weeks incubation period, dry weight biomass (gl^{-1}) and lipid (gg^{-1}) were determined.

Effect of Salinity

The salt NaCl is not a constituent of the standard Chu 13 medium. The idea to investigate the effect of NaCl salinity based on reports indicating the stimulatory effects of certain levels of salinity on lipid accumulation and production of certain microalgae including *B. braunii* [12, 13]. To study the effect of salinity on *B. braunii* growth and lipid production, chemically pure sodium chloride (NaCl) was added to the modified Chu 13 medium to obtain salinity levels of 17 mM (0.994 gl^{-1}), 34 mM (1.987 gl^{-1}), and 51 mM (2.981 gl^{-1}). Culture flasks were incubated for 7 weeks under 25 °C, 16.2 ± 2.7 µmol photons m^{-2} s^{-1} with 16 h light: 8 h dark photoperiod. At the end of incubation period, the culture flasks were harvested for determinations of dry weight biomass (gl^{-1}) and lipid content (gg^{-1}). It is relevant to mention that the NaCl salinity of the control modified Chu 13 medium is 0.0 g l^{-1}.

Biomass determination

Algal cultures were filtered through dry clean pre-weighed Whatman No 1 filter papers. The cells were washed twice with distilled water and dried at 60°C to a constant weight. The dry weight of algal biomass was determined gravimetrically and expressed as g l^{-1} [32,33].

Lipid extraction

The lipid content of algal biomass dried at 60 °C was determined according to soxhlet solvent extraction method using n-hexane as extraction solvent. The lipid content was expressed as % gg^{-1} of algal biomass [33,34].

Gas chromatography and mass spectroscopy (GC/MS) analysis of lipid extracts

The lipid composition of n-hexane extracts of the parameters which give the highest biomass and lipid content were analysed by GC/MS. Qualitative and quantitative lipid composition was determined using Aglient 6890 gas chromatograph equipped with an Aglient mass spectrometric detector, with a direct capillary interface and fused silica capillary column HP-5MS (30m × 0.32mm × 0.25 µm film thicknesses). Lipid samples were injected under the following conditions; Helium was used as carrier gas at approximately 1.0 ml min^{-1}, pulsed split-less mode. The solvent delay was 3 min. and the injection size was 1.0 µl. The mass spectrometric detector was operated in electron impact ionization mode with an ionizing energy of 70 e.v. scanning from m/z 50 to 500. The ion source temperature was 230 ºC. The electron multiplier voltage (EM voltage) was maintained 1250 v above auto tune. The instrument was manually tuned using perfluorotributyl amine (PFTBA). The GC temperature program was started at 60ºC (2 min) then elevated to 280º Cat rate of 8ºC min^{-1}. The detector and injector temperature were set at 300 ºC and 280ºC, respectively. Lipids were identified by comparing their mass spectra with the standards of NIST/EPA/NIH mass spectral library (NIST11; http://www.gcimage.com/nist11.html), matching with literature spectral data, molecular weight and polarity sequences. GC/MS analysis was carried out at The Central Agricultural Pesticide Laboratory (CAPL), Dokki, Giza, Egypt (http://www.capl.sci.eg/CAPL.html).

Statistical analysis

Average values of the biomass concentration and lipid content of three replications and their standard deviations were calculated. Significant differences were determined using analysis of variance (STATGRAPHICS, ver. 4.2) with 95% confidence (probability limit of $p < 0.05$, $p < 0.01$ and $p < 0.001$).

Results and discussions

Effect of the incubation temperature (ºC)

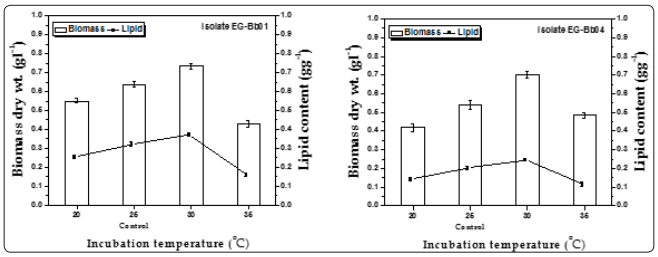

Figure 1: Effect of temperature on biomass yield and lipid content of isolates EG-Bb01 and EG-Bb04 of *Botryococcus braunii*

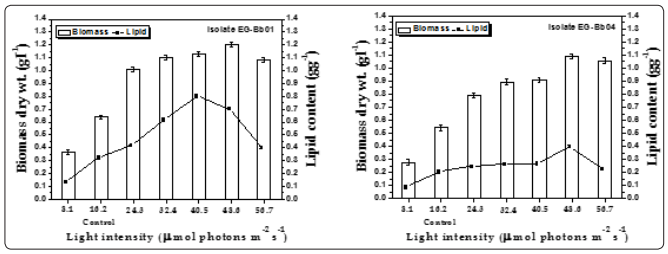

Figure 2: Effect of light intensity on biomass and lipid content of isolates EG-Bb01 and EG-Bb04 of *Botryococcus braunii*

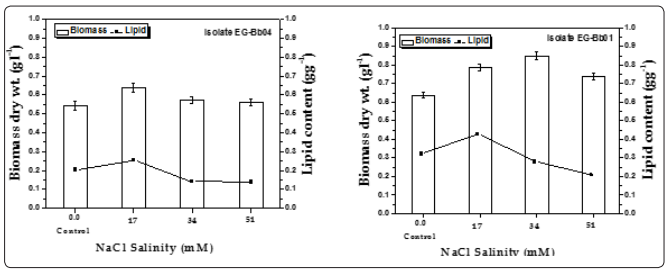

Figure 3: Effect of NaCl salinity on biomass yield of isolates EG-Bb01 and EG-Bb04 of *Botryococcus braunii*

Variations in the incubation temperature resulted in either obvious decrease or increase in biomass yield and lipid content of the two test isolates (Table 1). The incubation temperature of control cultures was 25±2 °C. The decrease of incubation temperature to 20°C decreased significantly the growth development and lipid content of *B. braunii* cultutres. However, The incubation temperature of 30±2 °C induced very high significant ($P \leq 0.001$) increase in biomass yield and lipid content the isolates EG-Bb01 (0.733 gl^{-1} biomass and 0.374 gg^{-1} lipid) and EG-Bb04 (0.704 gl^{-1} biomass and 0.246 gg^{-1} lipid) (Figure 1). Compared to the control condition (25±2 °C), the incubation temperature of 30±2 oC increased the biomass yield by 14.89% and 29.89% and lipid content by 16.15% and 20.29% of isolates EG-Bb01 and EG-Bb04, respectively (Table 1). Further increase of incubation temperature up to 35 °C exhibited very high significant decrease in biomass and lipid production. These results are in agreement with those of Casadevall et al., Fernandes et al. [35,36,37] and Lupi et al. who agreed that *B. braunii* grow well at ambient temperatures ranged between 25°C and 32°C with 30 °C as an optimal growth temperature [16].

Effect of light intensity

Light is the most important factor controlling the growth of algae. Low light intensity causes a reduction in dry weight while high intensity can cause biochemical damage to the photosynthetic machinery [38]. The ambient light intensity of the control cultures was 16.2 ± 2.7 µmol photons m^{-2} s^{-1}. In this study, increasing light intensity gave very promising results evidenced by very high significant ($P \leq 0.001$) increase in both biomass production and lipid contents of the test isolates of *B. braunii* (Table 2). In contrary, reducing the incubation light intensity by 50% (8.1 ± 2.7 µmol photons m^{-2} s^{-1}) resulted in very high significant ($P \leq 0.001$) decrease in biomass and lipid content (Table 2). The light intensity of 48.6 ± 2.7 µ mol photons m^{-2} s^{-1} induced very highly significant ($P \leq 0.001$) increase in biomass yield and lipid content of the isolates EG-Bb01 (1.2 gl^{-1} biomass and 0.704 gg^{-1} lipid) and EG-Bb04 (1.09 gl^{-1} biomass and 0.397 gg^{-1} lipid) (Figure 2). Compared to control, this light intensity increased the biomass yield between 88.71% and 100.7% and the lipid content between 118.63% and 94.61% of isolates EG-Bb01 and EG-Bb04, respectively (Table 2). Therefore, the light intensity of 48.6 ± 2.7 µmol photons m^{-2} s^{-1} is considered optimal for the growth and lipid production of the Egyptian isolates of B. braunii. In this context, Yeesang and Cheirsilp reported that the lipid contents in B. braunii strains KB, SK, TRG and PSU increased with increasing light intensity from 33 µ mol photons m^{-2} s^{-1} to 49 µ mol photons m^{-2} s^{-1}, but decreased when the light intensity was increased up to 82.5 µ mol photons m-2 s-z [39]. Ruangsomboon reported that the highest biomass of B [40]. braunii Strain KMITL 2 was obtained when incubated at 87.5 µ mol photons m^{-2} s^{-1}, while, the highest lipid yield obtained at 538 µ mol photons m^{-2} s^{-1}. Surprisingly, Metzger and Largeau reported that hydrocarbon synthesis is favored by relatively very high light intensity between 365 µ mol photons m^{-2} s^{-1} (40 Wm^{-2}) and 824 µ mol photons m^{-2} s^{-1} (90 Wm^{-2}) [22]. In general, Cepák and Lukavsk´y reported that B [41]. braunii could survive a wide range of light intensity from 135 µ mol photons m^{-2} s^{-1} (15 Wm^{-2}) to 1620 µ mol photons m^{-2} s^{-1} (180 Wm^{-2}). Niyogi and Solovchenko et al [42]. reported that the high lipid content of algae at high light intensity can be attributed to the production of excessive photo-assimilates that can be stored in the form of lipid as a mean to convert excess light to chemical energy with avoidance of photo-oxidative damage [43]. The light intensity of 48.6 ± 2.7 µ mol photons m^{-2} s^{-1} does not only affect biomass and lipid content of the Egyptian isolates of B. braunii, but also affect the hydrocarbon profile. Considerable numbers of C6-C15 short chain compounds -mostly hydrocarbons- were only detected in lipid contents of *B. braunii* cultures under this light intensity (Table 4).

Effect of salinity

The *Botryococcus braunii* test isolates EG-Bb01 and EG-Bb04 were able to grow in all tested concentrations of sodium chloride ranged between 17 mM (0.994 gl^{-1}) and 51 mM (2.981 gl^{-1}). The concentrations 17 mM and 34 mM salinity induced the highest biomass yields of the isolates EG-Bb04 (0.638 gl^{-1}) and EG-Bb01 (0.85 gl^{-1}), respectively (Figure 3). In a statistical term, cultures of both test isolates grown at 17 mM NaCl salinity maintained very high significant ($P \leq 0.001$) increase in lipid content (Table 3). However, further increases in NaCl salinity resulted in very high significant ($P \leq 0.001$) decrease in lipid content of both isolates (Table 3). In this context, Rao et al. reported that NaCl salinity in the range between 17mM and 34 mM is most favourable for relatively higher biomass and hydrocarbon production of B [13]. *braunii* LB 572, race A. Also, Ben-Amotz et al. reported that the lipid content of B [44]. *braunii* grown in 0.5 M NaCl salt concentration was higher than that without salt. However, Rafael and Bertha reported that *Botryococcus braunii* can even survive at 3M NaCl [35]. This salinity level does not only affect biomass and lipid content of the Egyptian isolates of *B. braunii*, but also affect the hydrocarbon profile. Considerable numbers of C6-C15 short chain compounds-mostly hydrocarbons- were only detected in lipid contents of *B. braunii* cultures grown in Chu 13 medium supplemented with 17 mM NaCl (Table 5). These short chain compounds contributed heavily, by 59.61% and 35.3% (w/w) to the n-hexane extracts of isolates EG-Bb01 and EG-Bb04, respectively. Authors of this article did not find any published data about the effect of NaCl salinity on lipid composition of *B. braunii*, to compare with.

GC/MS analysis of n-hexane extracts of control cultures

A total of 21 C16-C31 and 19 C16-C31 were identified from n-hexane extract of the two isolates EG-Bb01 and EG-Bb04 cultures on control condition, respectively (Table 4). Fourteen C16-C26 compounds in addition to the compound C28 octacosadiene (C28H54) were quantitatively minor constituents maintaining < 1.0 wt. % (w/w) of n-hexane extract of the isolate

EG-Bb01. Three monoenes including octacosene (C28H56), heptacosene (C27H54), and nonacosene (C29H58) exhibited minor.

Table 1: Effect of different incubation temperatures on biomass yield and lipid content of the test isolates EG-Bb01 and EG-Bb04 of *Botryococcus braunii*. Variations between the treated and control values were statistically evaluated by means of a simple t-test

Parameters	Temp., °C	Isolate EG-Bb01			Isolate EG-Bb04		
		% increase (+) or decrease (-)[1]	t-value	Significance level[2]	% increase (+) or decrease (-)	t-value	Significance level
Dry Biomass yield (gl^{-1})	20	-13.8	+30.14	*	-22.5	+52.39	***
	30	+14.89	-1.228	**	+29.89	-40.33	***
	35	-32.8	+3.878	***	-10.7	+1.73	*
Lipid content (gg^{-1})	20	-21.74	+132.79	***	-28.9	+62.78	***
	30	+16.15	-22.39	**	+20.59	-33.85	***
	35	-50.93	+52.28	***	-44.6	+36.25	***

Table 2: Effect of different light intensities on biomass yield and lipid content of the test isolates EG-Bb01 and EG-Bb04 of *Botryococcus braunii*. Variations between the treated and control values were statistically evaluated by means of a simple t-test

Parameters	light intensity ($\mu mol\ photons\ m^{-2}\ s^{-1}$)	Isolate EG-Bb01			Isolate EG-Bb04		
		% increase (+) or decrease (-)[1]	t-value	Significance[2]	% increase (+) or decrease (-)	t-value	Significance
Dry Biomass yield (gl^{-1})	16.2	-42.9	+472.9	***	-48.7	+75.9	***
	24.3	+57.99	-321.3	***	+45.39	-85.6	***
	32.4	+72.57	-267.9	***	+64.58	-202.6	***
	40.5	+76.8	-283.5	***	+67.53	-105.9	***
	48.6	+88.71	-982.1	***	+100.7	-135.4	***
	56.7	+69.59	-385.4	***	+95.02	-178.7	***
Lipid content (gg^{-1})	16.2	-60.25	+414.3	***	-55.9	+36.93	***
	24.3	+29.193	-35.77	***	+20.1	-26.94	***
	32.4	+91.925	-100.87	***	+28.43	-78.61	***
	40.5	+149.07	-163.36	***	+30.88	-48.19	***
	48.6	+118.63	-108.75	***	+94.61	-146.1	***
	56.7	+24.534	-19.52	***	+10.29	-42.34	**

Table 3: Effect of different NaCl salinity on biomass yield, lipid content and EPS production of the test isolates EG-Bb01 and EG-Bb04 of *Botryococcus braunii*. Variations between the treated and control values were statistically evaluated by means of a simple t-test

Parameters	NaCl (mM)	Isolate EG-Bb01			Isolate EG-Bb04		
		% increase (+) or decrease (-)[1]	t-value	Significance level[2]	% increase (+) or decrease (-)	t-value	Significance level
Dry Biomass yield (gl^{-1})	17	+23.82	-22.84	***	+17.71	-4.75	*
	34	+33.23	-17.16	***	+5.72	-2.09	NS[3]
	51	+15.99	-16.67	*	+3.14	-2.14	NS
Lipid content (gg^{-1})	17	+32.92	-15.05	***	+24.51	-11.74	***
	34	-12.11	+1.53	NS	-30.39	+2.67	***
	51	-35.71	+12.04	***	-32.35	+2.87	***

(1) Compared to the corresponding values of the control cultures of the same isolate.
(2) Differences between mean values of treated and control cultures, of the same isolate, are considered significant at $P \leq 0.05$ (*), highly significant at $P \leq 0.01$(**) and very highly significant at $P \leq 0.001$(***).
(3) NS = Non-significant.

Table 4: GC/MS analysis of n-hexane extract of isolate EG-Bb01 of *Botryococcus braunii* cultured on modified Chu 13 medium and incubated at 48.6 ± 2.7 µ mol photons m^{-2} s^{-1}

Identification (IUPAC nomenclature)	Formula	Area %			
		EG-Bb01		EG-Bb04	
		Control	Treated	Control	Treated
2-Hexanone	$C_6H_{12}O$	-	16.59	-	16.40
3-Methylcyclopentanol	$C_6H_{12}O$	-	3.378	-	3.100
4-Methyl-2-hexanone	$C_7H_{14}O$	-	5.912	-	3.358
2,2-Dimethylpentanal	$C_7H_{14}O$	-		-	1.143
3-Hexen-2,5-diol	$C_6H_{12}O_2$	-	4.264	-	2.616
5-methyl-2-hexanone	$C_7H_{14}O$	-	2.719	-	1.813
Octane	C_8H_{18}	-	2.142	-	1.658
2-Nonanone	$C_9H_{18}O$	-	0.793	-	0.618
2-Methyloctane	C_9H_20	-	0.917	-	1.133
2,7-Dimethyloctane	$C_{10}H_{22}$	-	0.587	-	0.515
Decane	$C_{10}H_{22}$	-	2.760	-	2.390
Hendecane	$C_{11}H_{24}$	-	0.948	-	1.030
2-methyldecane	$C_{11}H_{24}$	-	2.657	-	3.893
Dodecane	$C_{12}H_{26}$	-	0.639	-	0.515
Tetradecane	$C_{14}H_{30}$	-	5.068	-	3.512
Pentadecane	$C_{15}H_{32}$	-	3.111	-	2.462
Hexadecene	$C_{16}H_{32}$	0.586	-	4.964	-
Hexadecane	$C_{16}H_{34}$	0.594	1.174	5.554	2.585
Heptadecane	$C_{17}H_{36}$	-	0.999	-	1.030
Octadecene	$C_{18}H_{36}$	0.745	0.948	11.90	0.587
Octadecane	$C_{18}H_{38}$	0.364	-	5.948	-
6,10,14-trimethylpentadecan-2-one(1)	$C_{18}H_{36}O$	0.216	26.53	13.86	40.38
Nonadecane	$C_{19}H_{40}$	-	1.452	3.047	1.257
Icosene	$C_{20}H_{40}$	0.741	-	11.45	-
Icosane	$C_{20}H_{42}$	0.256	0.361	4.571	0.515
[(2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen^{-1}-ol](2)	$C_{20}H_{40}O$	0.956	-	1.966	-
Docosa-3,6,9-triene	$C_{22}H_{40}$	-	-	10.37	-
Docosene	$C_{22}H_{44}$	0.654	-	4.571	0.773
Docosane	$C_{22}H_{46}$	0.221	0.206	4.424	-
Docosanol	$C_{22}H_{46}O$	-	1.277	-	0.886
Tetracosene	$C_{24}H_{48}$	0.478	5.500	5.456	-
Tetracosane	$C_{24}H_{50}$	-	0.567	-	-
Pentacosadiene	$C_{25}H_{48}$	0.902	2.627	-	-
Hexacosene	$C_{26}H_{52}$	0.596	-	2.31	-
Hexacosane	$C_{26}H_{54}$	-	-	-	-
Heptacosadiene	$C_{27}H_{52}$	11.04	1.040	-	0.278
Heptacosene	$C_{27}H_{54}$	1.505	-	0.934	-
Octacosadiene	$C_{28}H_{54}$	0.737	-	1.229	-
Octacosene	$C_{28}H_{56}$	1.117	-	2.217	-
Nonacosadiene	$C_{29}H_{56}$	54.06	-	1.475	-
Nonacosene	$C_{29}H_{58}$	2.812	0.299	-	0.268
Nonacosatriene	$C_{29}H_{54}$	1.076	-	-	-
Hentriacontadiene	$C_{31}H_{60}$	18.059	0.144	0.541	-

(1) Hexahydrofarnesyl acetone 2) Transphytol

Table 5: GC/MS analyses of *n*-hexane extract of the test isolates of *Botryococcus braunii* cultured on modified Chu 13 medium supplemented with 17mM NaCl

Identification	Formula	Area %			
		EG-Bb01		EG-Bb04	
		Control	Treated	Control	Treated
2-Hexanone	$C_6H_{12}O$	-	14.43	-	12.04
3-Methylcyclopentanol	$C_6H_{12}O$	-	3.718	-	2.76
4-Methyl-2-hexanone	$C_7H_{14}O$	-	5.521	-	3.049
2,2-Dimethylpentanal	$C_7H_{14}O$	-	1.720	-	0.979
3-Hexen-2,5-diol	$C_6H_{12}O_2$	-	4.923	-	1.772
5-methyl-2-hexanone	$C_7H_{14}O$	-	2.843	-	1.782
Octane	C_8H_{18}	-	2.760	-	-
2-Nonanone	$C_9H_{18}O$	-	0.731	-	1.195
2-Methyloctane	C_9H_{20}	-	2.173	-	1.082
2,7-Dimethyloctane	$C_{10}H_{22}$	-	0.999	-	-
Decane	$C_{10}H_{22}$	-	4.285	-	2.06
Hendecane	$C_{11}H_{24}$	-	2.276	-	0.577
2-methyldecane	$C_{11}H_{24}$	-	3.214	-	3.214
Dodecane	$C_{12}H_{26}$	-	1.123	-	-
Tetradecane	$C_{14}H_{30}$	-	6.221	-	2.76
Pentadecane	$C_{15}H_{32}$	-	2.668	-	2.019
Hexadecene	$C_{16}H_{32}$	0.586	-	4.964	-
Hexadecane	$C_{16}H_{34}$	0.594	1.009	5.554	2.462
Octadecene	$C_{18}H_{36}$	0.745	1.236	11.90	0.422
Octadecane	$C_{18}H_{38}$	0.364	-	5.948	-
6,10,14-trimethylpentadecan-2-one(1)	$C_{18}H_{36}O$	0.216	22.16	13.86	49.87
Nonadecane	$C_{19}H_{40}$	-	1.391	3.047	1.318
Icosene	$C_{20}H_{40}$	0.741	-	11.45	-
Icosane	$C_{20}H_{42}$	0.256	0.402	4.571	-
[(2E,7R,11R)-3,7,11,15-tetramethyl-2-hexadecen^{-1}-ol](2)	$C_{20}H_{40}O$	0.956	-	1.966	0.979
Docosa-3,6,9-triene	$C_{22}H_{40}$	-	-	10.37	-
Docosene	$C_{22}H_{44}$	0.654	0.247	4.571	1.277
Docosane	$C_{22}H_{46}$	0.221	-	4.424	-
Docosanol	$C_{22}H_{46}O$	-	1.627	-	1.082
Tetracosene	$C_{24}H_{48}$	0.478	3.770	5.456	1.854
Pentacosadiene	$C_{25}H_{48}$	0.902	2.194	-	-
Hexacosene	$C_{26}H_{52}$	0.596	-	2.31	-
Hexacosane	$C_{26}H_{54}$	-	-	-	0.052
Heptacosadiene	$C_{27}H_{52}$	11.04	1.267	-	-
Heptacosene	$C_{27}H_{54}$	1.505	-	0.934	-
Octacosadiene	$C_{28}H_{54}$	0.737	-	1.229	-
Octacosene	$C_{28}H_{56}$	1.117	-	2.217	-
Nonacosadiene	$C_{29}H_{56}$	54.06	-	1.475	-
Nonacosene	$C_{29}H_{58}$	2.812	0.834	-	0.546
Nonacosatriene	$C_{29}H_{54}$	1.076	-	-	-
Hentriacontadiene	$C_{31}H_{60}$	18.059	-	0.541	-

(1)Hexahydrofarnesyl acetone (2) Transphytol

contribution to the lipid content of EG-Bb01 with wt. % of 1.12, 1.51, and 2.81, respectively (Table 4). The triene compound nonacosatriene (C29H54) was only identified among the n-hexane extract of the isolate EG-Bb01. Three dienes namely heptacosadiene (C27H52), hentriacontadiene (C31H60), and nonacosadiene (C29H56) contributed heavily to the lipid content of the isolate EG-Bb01 with 11.04%, 18.06%, and 54.06% wt. %, respectively (Table 4).

The n-hexane extract of the isolate EG-Bb04 includes 9 monoenes, 4 alkanes, 1 ketone, 1 diterpenoid, 3 diene and 1 triene (Table 13). Based on wt. %, the compound octadecene C18H36 (11.9%), hexahydrofarnesylacetone, C18H38O (13.86%), icosene, C20H40 (11.45%) and docosatrine (C22H40) (10.37%) were the major constituents. Compared to other isolates, the monoenes (C19H38) and the triene docosatrine (C22H40) were only identified in lipid fraction of the isolate EG-Bb04. The odd numbered hydrocarbons, heptacosene (C27H54) (wt. % 0.93), nonacosadiene (C29H56) (wt. % 1.47) and hentriacontadiene (C31H60) (wt. % 0.54) exhibited minor contribution to the hydrocarbon content of EG-Bb04 (Table 13).

It is evident that the isolate EG-Bb01 produces C25-C31 odd numbered hydrocarbons (wt. % 89.45) including two monoenes (C27 heptacosene and C29 nonacosene) four dienes (C25 pentacosadiene, C27 heptacosadiene, C29 nonacosadiene, and C31 hentriacontadiene) and a single triene (C29 nonacosatriene) while the isolate EG-Bb04 produces C25-C31 odd numbered hydrocarbons (wt. % 2.95) including only one monoenes (C27 heptacosene), two dienes (C29 nonacosadiene, and C31 hentriacontadiene) and a single triene (C29 nonacosatriene) (Table 4). This finding strongly indicates that the Egyptian isolates belong to the chemical race A of *B. braunii* [21, 25,30, 45].

GC/MS analysis of n-hexane extract of the test isolates of *Botryococcus braunii* cultured on modified Chu 13 medium under 3.6 ± 0.2 klux light intensity

Detailed qualitative and quantitative compositions of the n-hexane extract of isolate EG-Bb01and isolate EG-Bb04 are shown in Table 4. A total of 29 C6-C31 and 27 C6-C29 different compounds were identified in n-hexane extract of isolates EG-Bb01 and EG-Bb04, respectively (Table 4). The short C6-C15 chain compounds were only detected in lipid fraction of the treated cultures. These short chain compounds contributed by 52.48% and 46.15% to the total lipid fraction of the isolates EG-Bb01and EG-Bb04, respectively (Table 4). The wt. % of the C18 ketone, hexahydrofarnesylacetone, contributed significantly to wt.% of lipid fraction of isolates EG-Bb01 (26.53%) and EG-Bb04 (40.38%) (Table 4).

Compared to control cultures, a relatively low wt. % of the odd numbered hydrocarbons were produced by the test isolates cultures under 48.6 ± 2.7 µmol photons m^{-2} s^{-1} light intensity (Table4). The odd numbered hydrocarbons produced by the isolate EG-Bb01 (4.11 wt. %) were represented by C25 Pentacosadiene (2.627%), C27 Heptacosadiene (1.04%), C29 Nonacosene (0.299%) and C31 Hentriacontadiene (0.14%) (Table 4). The odd numbered hydrocarbons produced by the isolate EG-Bb04 (0.546 wt. %) were C27 Heptacosadiene (0.278%) and C29 Nonacosene (0.268%) (Table 4). Lacking any previous published information and data about the effect of light intensity on lipid composition of *B. braunii*, makes it difficult to explain the production of short chain hydrocarbons by *B. braunii* at high light intensity.

GC/MS analysis of n-hexane extract of the test isolates of *Botryococcus braunii* cultured on modified Chu 13 medium supplemented with 17mM NaCl

Detailed qualitative and quantitative composition of the n-hexane extract of isolates EG-Bb01and EG-Bb04 cultured on control medium supplemented with 17mM NaCl are shown in Table 5. A total of different 27 C6-C29 and 23 C6-C29 different compounds were identified in n-hexane extract of the isolates EG-Bb01 and EG-Bb04, respectively. Considerable numbers of short C6-C15 chain compounds -mostly hydrocarbon- were detected in biomass of cultures grown in Chu13 supplemented with 17mM NaCl (Tables 5). These short chain compounds contributed by 59.61% and 35.29% (w/w %) to the lipid content of isolates EG-Bb01 and EG-Bb04, respectively. The wt. % of the C18 ketone, hexahydrofarnesylacetone, contributed heavily to lipid fractions of both isolates EG-Bb01 (22.16%) and EG-Bb04 (49.87%). The transphytol (C20H40O) was detected on lipid fraction of isolate EG-Bb04 in relatively low wt % (0.979%) (Table 5).

A relatively low wt. % contribution of the odd numbered hydrocarbons was reported in n-hexane extract of the two test isolates cultured on medium supplemented with 17mM NaCl compared to control cultures (Table 5). The odd numbered hydrocarbons produced by the isolate EG-Bb01 (4.29 wt. %) were represented by C25 pentacosadiene (2.194 wt. %), C27 heptacosadiene (1.267 wt. %) and C29 Nonacosene (0.834 wt. %). For isolate EG-Bb04, a single odd numbered hydrocarbon, C29 Nonacosene (0.546 wt. %) was only detected (Table 5).

References

1. Neenan B, Feinberg D, Hill A, McIntosh R, Terry K (1986) Fuels from microalgae: Technology status, potential, and research requirements. Publ. No. SERI/SP-231-2550, Solar Energy Research Institute, Golden, CO pp149.
2. Liu Z. Y, Wang GC, Zhou B C (2008) Effect of iron on growth and lipid accumulation in Chlorella vulgaris. Bioresource Technology 99: 4717-4722.
3. Qin J (2005) Bio-hydrocarbons from algae-impacts of temperature, light and salinity on algae growth. Rural Industries Research and Development Corporation. Barton, Australia.
4. Piorreck M, Baasch KH, Pohl P (1984) Biomass production, total protein, chlorophylls, lipids and fatty acids of freshwater green and blue-green algae under different nitrogen regimes. Phytochemistry 23: 207-216.
5. Sawayama S, Minowa T, Dote Y, Yokoyama S (1992) Growth of the hydrocarbon rich microalga Botryococcus braunii in secondarily treated sewage. Applied Microbiology and Biotechnology 38: 135-138.
6. Singh Y, Kumar HD (1992) Lipid and hydrocarbon production by Botryococcus sp. under nitrogen limitation and anaerobiosis. World Journal of Microbiology and Biotechnology 8: 121-124.
7. Illman AM, Scragg AH, Shales SW (2000) Increase in Chlorella strains calorific values when grown in low nitrogen medium. Enzyme and Microbial Technology 27: 631- 635.
8. Lynn SG, Kilham SS, Kreeger DA, Interlandi SJ (2000) Effect of nutrient availability on the biochemical and elemental stoichiometry in freshwater diatom Stephanodiscus minutulus bacillariophyceae. Journal of Phycology 36: 510-522.
9. Reitan KI, Rainuzzo JR, Olsen Y (1994) Effect of nutrient limitation on fatty acid and lipid content of marine microalgae. Journal of Phycology 30: 972-979.
10. Vázquez-Duhalt R, Arredondo-Vega BQ (1991) Oil production from microalgae under saline stress. Biomass for

energy and industry 5th E. C conference. Policy, Environment, Production and Harvesting 1: 547-551.

11. Derenne S, Metzger P, Largeau C, Van Bergen PF, Gatellier JP, et al. (1992) Similar morphological and chemical variations of in Ordovician sediments and cultured Botryococcus braunii as a response to changes in salinity. Organic geochemistry. Oxford etc 19: 299-313.
12. Takagi, M Karseno, Yoshida T (2006) Effect of salt concentration on intracellular accumulation of lipids and Triacylglyceride in marine Microalgae Dunaliella cells. Journal of Bioscience and Bioengineering 101: 223-226.
13. Rao RA, Dayananda C, Sarada R, Shamala TR, Ravishankar GA (2007) Effect of salinity on growth of green alga Botryococcus braunii and its constituents. Bioresoure Technology 98: 560-564.
14. Brenckmann F, Largeau C, Casadevall E, Corre B, Berkaloff C (1985) Influence of light intensity on hydrocarbon and total biomass production of Botryococcus braunii. Relations with photosynthetic characteristics. In: Paiz W, Coombs J, Hall DO (Eds.): Energy from Biomass, Elsevier Applied Science Publication, London pp722-726.
15. Kojima E, Zhang K (1999) Growth and hydrocarbon production of microalga Botryococcus braunii in Bubble column photobioreactors. Journal of Bioscience and Bioengineering 87: 811-815.
16. Lupi F M, Fernandes HML, Sa-Correia I, Novais JM (1991) Temperature profiles of cellular growth and exopolysaccharide synthesis by Botryococcus braunii Kütz. UC 58. Journal of Applied Phycology 3: 35-42.
17. Renaud SM, Thinh LV, Lambrinidis G, Parry DL (2002) Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. Aquaculture 211: 195-214.
18. De Castro AS, Garcia VMT (2005) Growth and biochemical composition of the diatom Chaetoceros cf. wighamii Brightwell under different temperature, salinity and carbon dioxide levels. I. Protein, carbohydrates and lipids. Aquaculture 246: 405-412.
19. Senousy HH, Beakes GW, Hack E (2004) Phylogenetic placement of Botryococcus braunii (Trebouxiophyceae) and Botryococcus sudeticus isolate UTEX 2629 (Chlorophyceae). Journal of Phycology 40: 412-423.
20. Komárek J, Marvan P (1992) Morphological differences in natural populations of the genus Botryococcus (Chlorophyceae). Arch. Protistenkd 141: 65-100.
21. Metzger P, Largeau C (1999) Chemicals of Botryococcus braunii. In: Cohen Z (ed) Chemicals from microalgae. Taylor & Francis, London pp 205-260.
22. Metzger P, Largeau C (2005) Botryococcus braunii: a rich source for hydrocarbons and related ether lipids. Applied Microbiology and Biotechnology 66: 486-496.
23. Metzger P, Villarreal-Rosales E, Casadevall E (1991) Methyl branched fatty aldehydes and fatty acids in Botryococcus braunii. Phytochemistry 30: 185-191.
24. Metzger P, Allard B, Casadevall E, Berkaloff C, Coute A (1990) Structure and chemistry of a new chemical race of Botryococcus braunii (chlorophyceae) that produces lycopadiene, a tetraterpenoid hydrocarbon. Journal of Phycology 26: 258.
25. Banerjee A, Sharma R, Yusuf C, Banerjee UC (2002) Botryococcus braunii: a renewable source of hydrocarbons and other chemicals. Critical Reviews in Biotechnology 22: 245-279.
26. Andersen RA, Kawachi M (2005) Traditional Microalgae Isolation Techniques. In: Andersen RA, ed. Algal culturing techniques. Phycological Socity of America pp 94-11.
27. Prescott G (1951) Algae of the Western Great Lakes Area. Cranbrook Bull. Inst. Sci. No. 31. WC Brown, Co., Dubuque, Iowa pp977.
28. Bourrelly P (1966) Les Algues d'Eau Douce. Initiation à la systématique. Tome I : Les algues vertes. Éditions N. Boubée et Cie, Paris pp511.
29. Bourrelly P (1990) Les Algues d'Eau Douce. Tome I. Les Algues Vertes. Second Édition. N Boubée et Cie, Paris pp576.
30. Largeau C, Casadevall E, Berkaloff C, Dhamelincourt P (1980a) Sites of accumulation and composition of hydrocarbons in Botryococcus braunii. Phytochemistry 19: 1043-1051.
31. Dayananda C, Sarada R, Kumar V, Ravishankar GA (2007) Isolation and characterization of hydrocarbon producing green alga Botryococcus braunii from Indian freshwater bodies. Electronic Journal of Biotechnology 10: 79-91.
32. Samorì C, Torri C, Samorì G, Fabbri D, Galletti P, et al. (2010) Extraction of hydrocarbons from microalga Botryococcus braunii with switchable solvents. Bioresource Technology 101: 3274 -3279.
33. Dayananda C, Sarada R, Bhattacharya S, Ravishankar G A (2005) Effect of media and culture conditions on growth and hydrocarbon production by Botryococcus braunii. Process Biochemistry 40: 3125-3131.
34. Sadasivam S, Manickam A (1996) Biochemical methods. 2nd ed. New Age International (p) Limited, Tamil Nadu Agricultural University ISBN: 81-224-0976-8.
35. Rafael VD, Bertha OAV (1991) Haloadaptation of the green alga Botyococcus braunii (Race A). Phytochemistry 30: 2919-2925.
36. Casadevall E, Dif D, Largeau C, Gudin D, Chaumont D, et al. (1985) Studies on batch and continuous cultures of Botryoeoeeus braunii: Hydrocarbon production in relation to physiological state, cell ultrastructure and phosphate nutrition. Biotechnology and Bio-engineering 27: 286-295.
37. Fernandes HL, Tomè MM, Lupi FM, Fialho AM, Sá-Correia I, et al. (1989) Biosynthesis of high concentrations of an exoploysaccharide during the cultivation of the microalga Botryococcus braunii. Biotechnology Letters 2: 433-436.
38. Scott SA, Davey MP, Dennis JS, Horst I, Howe CJ, et al. (2010): Biodiesel from algae: challenges and prospects. Current Opinion in Biotechnology 21: 277-286.
39. Yeesang C. and Cheirsilp B. (2011): Effect of nitrogen, salt, and iron content in the growth medium and light intensity on lipid production by microalgae isolated from freshwater sources in Thailand. Bioresource Technology, 102: 3034-3040.
40. Ruangsomboon S (2012) Effect of light, nutrient, cultivation time and salinity on lipid production of newly isolated strain of the green microalga, Botryococcus braunii KMITL 2. Bioresource Technology 109: 261-265.
41. Cepák V, Lukavský J (1994) The effect of high irradiances on growth, biosynthetic activities and the ultrastructure of the green alga Botryococcus braunii (strain Droop 1950/807-l). Archiv für Hydrobiologie 102: 115-131.
42. Niyogi KK (1999) Photoprotection revisited: Genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology 50: 333-359.
43. Solovchenko AE, Khozin-Goldberg I, Didi-Cohen S, Cohen Z, Merzlyak MN (2008) Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga Parietochloris incisa. Journal of Applied Phycology 20: 245-251.
44. Ben-Amotz A, Tomabene TG, Thomas WH (1985) Chemical

profile of selected species of microalgae with emphasis on lipids. Journal of Phycology 21: 72-81.

45. Dayananda C, Sarada R, Srinivas P, Shamala TR, Ravishankar GA (2006) Presence of methyl branched fatty acids and saturated hydrocarbons in botryococcene producing strain of Botryococcus braunii. Acta Physiologiae Plantarum 28: 251-256.