33 *Egypt. J. Bot.*, **Vol**. **56**. No. 3 (Part 1) pp. 559 - 571 (2016)

Effect of some Abiotic Factors on Growth, Glycerol and β-carotene Accumulation by *Dunaliella bardawil*

Effat F. Shabana*, Hoda H. Senousy and Hend Y. Mohammad
Botany and Microbiology Department, Faculty of Science, Cairo University.

D*UNALIELLA bardawil* can produce and accumulate large amounts of glycerol and β-carotene in response to stress conditions. The effect of pH, salinity, light intensity and algal inocula as stress factors on growth, β-carotene and glycerol production in *D. bardawil* was assessed in the present study. The growth profile showed linear relationship for chlorophyll a content, optical density measurements and cell count with time up to the end of the experiment (42 days). The mean growth rate, relative growth rate and number of recycling reached their maximum values after 4 days of growth while generation time recorded the least value at the same time. *D. bardawil* can grow in wide pH range (5.5-9.5) with maximum growth (chlorophyll a and cell count) and glycerol content at pH 7.5 whereas the best pH value for β-carotene production was 8.5. Increasing algal inoculum caused significant increase in growth and β-carotene content while non significant increase in glycerol was observed. Regarding salinity stress, 4 M NaCl was the best concentration for glycerol production and 2.5 M NaCl for growth and β-carotene accumulation. High light stress (292.5 µE m^{-2} s^{-1}) enhanced β-carotene production and the ratio of β-carotene to chlorophyll a reached 1.59.

Keywords: *Dunaliella bardawil*, β-carotene, Glycerol, Stress factors, salinity, light intensity, pH, algal inoculum

The chemical industry is moving towards the adaptation of greener technology for better sustainability. There are several advantages of using microalgae, unlike conventional plant biomass, they have high photosynthetic efficiencies and can grow on non arable land. *Dunaliella* can produce and accumulate large amounts of commercially and economically important compounds as glycerol and β-carotene in response to stress conditions as high light intensity, high salt concentration, pH extremes and nutrient stress (Hadi *et al.*, 2008; Lin *et al.*, 2013). Moreover, Ben-Amotz (2007) stated that environmental factors played the most effective rules on the level of carotenoid production by *Dunaliella* spp. The highest production of β-carotene is observed in high salinity, high temperature and high light intensity. Different *Dunaliella* sp. were cultivated for the production of large amounts of β-carotene and glycerol (Phadwal and Singh, 2003).

*Corresponding author E-mail: dr-effatshabana@hotmail.com

The hydrogen ion concentration (pH) of the culture medium is a very important factor that affects the optimal growth of algal cultures. It appeared that the optimum pH for growth differs according to different algal species. The optimum pH for the growth of marine *D. tertiolecta* is about pH 6 (Craigie and McLachlan, 1964) whereas the optimum pH for the halophilic *D. salina* and *D. viridis* is about 9 (Loeblich, 1972). Wegmann and Metzner (1971) found that the optimum pH for photosynthesis is generally lower than that for growth.

The algal culture density and initial cell concentration are important to be taken into consideration in the design and volume of culture systems. The final concentration of non axenic outdoor mass cultures of *Dunaliella* spp. growth for two or three days in f/2 and 2f media were found to be dependent on initial cell concentration since the cultures started with 80 x 10^3 cell/ml gave better yields than those started with 40 x 10^3 cell/ml (Becerra Dorame *et al.,* 2010). Optimization of the algal density in which the maximal biomass and carotenoid content are performed is an important step both in ponds and photobioreactors (Hosseini-Tafreshi and Shariatie, 2006).

Salt stress in one of the most significant abiotic stresses and affects many aspects of plant physiology and metabolism. Among algae, the only eukaryotic and photosynthetic organism able to grow in media containing a very wide range of salt concentration from 0.5% to 35% is *Dunaliella*. It was found by Ben-Amotz and Avron (1980) that *Dunaliella* cells grown at high salinity (4 M NaCl) contain about half of its weight as glycerol whereas under appropriate cultivation more than 10% of dry weight of *D. bardawil* is accounted for β-carotene. Stress metabolites such as proline, glycine betaine, glycerol and total sugar content increased concomitantly with the increase in salt concentration. Tammam *et al.* (2011) stated that under hyposaline conditions a low content of β-carotene was noticed, whereas hypersaline conditions induced an increase in this product, about 1.4 and 1.1 fold more than thier value were recorded at optimum salinities for both *D. salina* and *D. tertiolecta* respectively.

High light and many environmental stress conditions affect the content of β-carotene in *D. bardawil* and overproduced β-carotene was recorded. Carotenoids protect the photosynthetic apparatus against stress conditions (Telfer, 2002). It was reported by Lamers *et al.,* 2010 that β-carotene accumulation in *D. salina* was induced on increasing light intensity from 200 to 1.400 μE m^{-2} s^{-1}. In the present study the effects of salinity, light intensity, pH and algal inocula on growth, β-carotene and glycerol accumulation in *Dunaliella bardawil* were investigated.

Material and Methods

Algal culture and growth conditions

Dunaliella bardawil was kindly supplied from phycology lab. Faculty of Science, Alexandria University, Egypt. It was grown in batch cultures in MH nutrient medium (Loeblich, 1982) with modification of NaCl concentration to 1.5 M; pH 7.5; temperature 25±1˚C and light intensity of 39 μE m^{-2} s^{-1} (2000 Lux) by

white fluorescent lamps. The initial algal inoculum was adjusted to be (1.5 x 10^6 cell /ml, 2.33 µg/ml chlorophyll a) and three replicates were used for each treatment. In optimum growth conditions the growth of *Dunaliella* cells was followed at two days intervals up to 42 days by measuring growth parameters (chlorophyll a content, cell count and optical density).

Experimental conditions

Effect of pH values (5.5, 6.5, 7.5 , 8.5 and 9.5); algal inocula (1.5, 3, 6 and 12, Cell x 10^6 / ml); NaCl concentrations (1.5, 2, 2.5, 3, 3.5 and 4 M) and different light intensities (39, 78, 117, 195 and 292.5 µE m^{-2} s^{-1}) on growth, β-carotene and glycerol production of *Dunaliella* were studied in four separate experiments.

Growth measurements and glycerol estimation

After 8 days incubation, cells are in their exponential phase of growth, algal cells were harvested and the chlorophyll a, β-carotene, glycerol and cell count were measured in each experiment. Cells were counted by sedquick Rafter cell after the addition of 1-2 drops of Lugol's solution. Cell density was measured at 678 nm, mean and relative growth rates were calculated according to Robert 1979, while generation time and number of recycling were calculated using the formula proposed by Fogg (1975). Chlorophyll a was determined by using 80% acetone and estimated according to Arnon (1949) and APHA (2005). B-carotene was quantified photometrically using the molar extinction coefficient ɛ 450 nm 134, 500 $Lmol^{-1}$ cm^{-1} (Rabbani *et al.,* 1998). Glycerol content was determined by the method of Chitlaru and Pick (1991).

Statistical analysis

The data of the present study were expressed as means $\pm$ SD and were analyzed by one way analysis of variance (ANOVA) using spss statistical package version 20. Comparison of the main effects was performed using a significant level of $P<0.05$ by Duncan's test of homogeneity (Dytham, 1999).

Results and Discussion

Dunaliella, a marine, unicellular, halotolerant green alga possesses a remarkable degree of environmental adaptation by producing β-carotene and glycerol in excess to maintain its osmotic balance (Ghoshal *et al.,* 2002 and Hend, 2014). *Dunaliella* can grow in a wide range of salt concentrations ranging from 0.05 M to 5 M (Chen and Jiang, 2009; Ramos *et al.,* 2011). The ability to grow at very high salt concentrations and the high cell content of β-carotene, glycerol or protein have made this microalga an attractive candidate for commercial production of these compeunds.(Spolaore *et al.,* 2006; Hosseini-Tafreshi and Shariatie, 2009).

The obtained results concerning growth curve of *Dunaliella bardawil;* Table 1 showed linear relationship for growth parameters; chlorophyll a, optical density and cell count during the experimental period (42 days). Because total cell counts may include non viable cells, growth rate was also used to assess the viability of algal populations. The mean growth rate (R), relative growth rate (k) and number

of recycling (Nu)reached their maximum values at day 4 of cultivation after which gradual decrease was observed which is in harmony with those reported by Borowitzka *et al.* (1984). There was a drop into negative growth rate starting after day 34 which indicated that the alga was in the stationary phase. In contrary, the generation time (G) reached the least value at the forth day then gradual increase was observed. The high value of generation time indicated that there was an obvious delay in growth rate with time. Our data are in agreement with El-Badawy (2014) who reported that the maximum specific and relative growth rates and number of recycling of *Chlorella vulgaris* were recorded in the 4th day of growth where the generation time was minimum.

TABLE 1. Growth profile of *Dunaliella bardawil* incubated in 1.5 M NaCl.

Parameter → / Time (days) ↓	Chlorophyll a (µg/ml)	Optical density	Cell count (×10^6 cell /ml)	Relative growth rate (K) (count) day^{-1}	Mean growth rate (R) (count) day^{-1}	generation time (count) day	Number of recycling (Nu)
0	0.55±0.02	0.08±0.01	0.37±0.03	0.00	0.00	0.00	0.00
2	0.85±0.02	0.12±0.01	0.52±0.03	0.45	0.25	0.67	2.98
4	1.62±0.03	0.23±0.02	1.18±0.26	0.66	0.59	0.46	4.36
6	3.45±0.40	0.48±0.01	2.26±0.04	0.58	0.47	0.52	3.88
8	3.80±0.30	0.55±0.01	2.60±0.15	0.36	0.10	0.83	2.40
10	4.93±0.49	0.71±0.01	3.24±0.22	0.40	0.16	0.76	2.64
12	7.02±0.27	0.93±0.05	4.67±0.41	0.46	0.26	0.65	3.06
14	8.74±0.27	0.99±0.01	5.51±0.10	0.37	0.12	0.81	2.48
16	9.83±0.23	1.05±0.09	6.42±0.33	0.37	0.11	0.82	2.44
18	10.86±0.64	1.18±0.03	7.16±0.09	0.35	0.08	0.86	2.32
20	12.60±0.03	1.29±0.03	8.27±0.31	0.36	0.10	0.83	2.42
22	13.92±0.45	1.52±0.04	9.30±0.24	0.35	0.08	0.86	2.34
24	15.73±0.21	1.62±0.00	10.19±0.37	0.34	0.07	0.88	2.26
26	18.09±0.32	1.77±0.02	11.15±0.44	0.34	0.06	0.88	2.26
28	19.43±0.33	1.99±0.02	12.40±0.28	0.35	0.08	0.87	2.31
30	20.66±0.56	2.08±0.01	13.41±0.51	0.34	0.06	0.90	2.23
32	22.75±0.67	2.24±0.01	14.73±0.25	0.34	0.07	0.88	2.27
34	23.27±0.54	2.28±0.01	15.44±0.70	0.32	0.03	0.94	2.14
36	23.09±0.82	2.27±0.01	15.32±0.28	0.30	-0.01	1.01	1.98
38	24.10±0.43	2.28±0.01	15.02±0.92	0.29	-0.01	1.03	1.94
40	25.39±0.74	2.28±0.01	14.99±0.60	0.30	0.00	1.00	1.99
42	25.44±1.14	2.28±0.01	14.34±0.71	0.28	-0.03	1.07	1.87

Data are average of three replicates; each value represents the mean ±SD.

The hydrogen ion concentration (pH) of the growth medium affects many processes associated with algal growth and metabolism including the availability of co_2 for photosynthesis and uptake of ions and hence for the biosynthesis of the bioactive products as secondary metabolites (Borowitzka and Borowitzka, 1988). The optimum pH for the growth and glycerol production of *D. bardawil* was pH 7.5 (control culture). At this pH value chlorophyll a, cell count and glycerol attained their maximum values as illustrated in Table 2. Shifting pH to either the acidic or alkaline side significantly decreased the values of these parameters. However, *Dunaliella*, as reported in literatures, and proved in this study can grow in wide range of pH values (5.5-9.5) and so it can withstand a broad range of pH which may add to the advantages of this promising organism. These results went parallel with the findings of Essa (1995) who reported that the algal cell count of the *Dunaliella sp.* was maximam at pH 7.8, changing the pH value to 6 and/or 9 led to a slight effect on cell count of *Dunaliella cells.* Moreover, he reported that pH 7.8 was the optimum for glycerol production in the cells of *D. salina* and *D. parva*. The present data also showed that the optimum value of β-carotene was recorded at pH 8.5 (a significant decrease at lower and higher pH values). This is in harmony with the results obtained by Anon (1983) and Khalil *et al.* (2010) who recorded that the highest value of β-carotene of *D. bardawil* was obtained at pH 9.0. On the other hand Muthukannan *et al.* (2010) revealed that *D. salina* required a slightly alkaline pH (7.5) for the maximum production of the valuable β-carotene pigment.

TABLE 2. Effect of pH on growth, β-carotene and glycerol production of *Dunaliella bardawil* after 8 days incubation in 1.5 M NaCl.

Parameters → / **pH↓**	**Chlorophyll a (µg/ml)**	**Cell count (x10^6 cell /ml)**	**β-carotene (µg/ml)**	**Glycerol (mg/ml)**
5.5	4.78±0.39^a	3.22±0.58^a	2.64±0.20^a	0.11±0.02^b
6.5	7.04±0.63^b	4.52±0.32^b	3.57±0.19^b	0.13±0.02^b
7.5	7.07±0.07^b	5.19±0.95^b	3.61±0.66^b	0.15±0.03^c
8.5	7.05±0.34^b	5.11±0.48^b	4.04±0.13^b	0.09±0.04^a
9.5	4.49±0.01^a	2.85±0.98^a	2.65±0.66^a	0.09±0.01^a
Initials	2.33	1.5	1.85	0.07

Means marked with the same superscript letters are not-significant (P>0.05), whereas others with different superscript letters are significant (P<0.05).
Data are average of three replicates; each value represents the mean ±SD.

Regarding the effect of initial cell counts (algal inocula) on growth and glycerol production of *D. bardawil*, it was found (Table 3) that chlorophyll a, cell count, β-carotene and glycerol production are directly proportional to algal inocula (as increasing inoculum caused significant increase in the above mentioned parameters). While, the increase in glycerol content was not

significantly altered in most algal inocula. At the largest inoculum used (12 x 10^6 cell / ml), glycerol content reached maximum value but still less than the corresponding initial. This agrees with the findings of Kacka and Donmez, 2008 who mentioned that at high initial inoculum concentration (10 x 10^6 cell / ml) of *Dunaliella* strain T_1, maximum glycerol production and shortest lag period were observed. They also reported that initial cell densities affected cell productivity. In contrary, Martinez and Espinosa (1991) reported that the amount of inoculum of *D. tertiolecta* had no significant effect neither on the population growth rate nor on the maximum cell density achieved, but it does affect the length of time needed to reach the maximum biomass. It is worthy to mention that the highest percentage increase in the values of chlorophyll a (225%), β-carotene (279%) and cell count (266%) was recorded at the least algal inoculum used whereas it was 50%, 33% and 21.2% respectively in the highest algal inoculum. In contrast, there was 38% decrease in glycerol production at the largest algal inoculum used. On the other hand, Vonshak and Richmond, 1985 mentioned that in outdoor raceway ponds self shading by the cells was the most probable cause of growth rate decline during the growth phase. With increasing cell concentration, self shading increased leading to an increase in dark fraction devoid of light supply, this resulted in decreasing specific growth rate.

TABLE 3. Effect of different algal inocula on growth, β-carotene and glycerol production of *Dunaliella bardawil* after 8 days incubation in 1.5 M NaCl.

parameter→ / Algal inoculum (cell x 10^6/ml)↓	Cell count (×10^6 cell /ml)	Chlorophyll a (µg/ml)	β-carotene (µg/ml)	Glycerol (mg/ml)
1.5±0.77	5.50±1.18^a	A: 2.52±1.30 B: 8.19±0.03^a	A: 1.35±0.29 B: 5.12±0.27^a	A: 0.07±0.01 B: 0.18±0.05^a
3.0±0.18	7.05±2.43^b	A: 4.39±1.86 B: 11.27±0.22^b	A: 2.88±0.45 B: 6.29±0.57^{ab}	A: 0.19±0.02 B: 0.21±0.04^a
6.0±1.11	9.41±2.70^c	A: 9.32±0.52 B: 14.57±0.26^c	A: 5.14±0.87 B: 8.09±1.07^b	A: 0.31±0.01 B: 0.17±0.02^a
12.0±2.05	14.54±1.58^d	A: 14.87±0.98 B: 22.44±2.88^d	A: 9.41±1.41 B:12.5±1.77^c	A: 0.48±0.04 B: 0.30±0.03^b

A: initial, B: final

Means marked with the same superscript letters are not-significant (P>0.05), whereas others with different superscript letters are significant (P<0.05). Data are average of three replicates; each value represents the mean ±SD.

Furthermore, Richmond (1992) mentioned that since biomass productivity of an algal culture is dependent on the culture density and the algal growth rate, the culture cell density has to be maintained at an optimum level to support the fastest growth rate at any given irradiance. In this connection, there are wide differences in the design and volume of culture systems as well as in the production routines, which could explain the wide range and the highly variable final cell density and biomass yields (López – Elias *et al.*, 2003).

Regarding the effect of salt stress on *D. bardawil*, it is obvious from the data of the present study (Fig. 1) that chlorophyll a, cell count and β-carotene contents increased gradually and significantly with the increase in sodium chloride concentration to reach their maximum values at 2.5 M after which they gradually decreased to their minimum values at 4 M NaCl. Moreover, the present data also cleared that the highest intracellular glycerol content of *Dunaliella* cells was recorded at 4 M NaCl which is supported by the results reported by Tammam *et al.* (2011). Furthermore, Frank and Wegmann (1974) mentioned that, with increasing salt concentrations in the medium, enzymes of *D.* cells become more and more inhibited, and the only process that remained active is glycerol production. Also, Chitlaru and Pick (1991) reported that with changing external salinity the internal glycerol level changes in direct proportional to the salinity concentrations.

The results reported by Mishra *et al.* (2008) are in conformity with ours as they noticed that no significant differences in chlorophyll a & b contents up to 2 M NaCl and there is decline in their values at 5.5 M NaCl. The present results are in agreement with those obtained by Rad *et al.,* 2011, who mentioned that total carotenoid production as well as cell productivity were affected by salinity stress and the highest carotenoid contents per cell were obtained at 2 M NaCl treated *Dunaliella* sp. In contrary to the present data Loeblich (1982) found an increase in β-carotene concentration per cell of *D. salina* with increase in salinity.

The effect of different light intensities on growth and β-carotene production were followed (Table 4). The maximum growth (chlorophyll a & cell count) of *D. bardawil* was observed at 6000 lux (117 µE m^{-2} s^{-1}) after which there was a significant decrease with further increase in light intensity upto 15000 lux (292.5 µE m^{-2} s^{-1}). There was a linear and significant increase in β-carotene content to reach its maximum at 292.5 µE m^{-2} s^{-1} after 8 days incubation (6.52 µg / ml).

Celekli and Donmez (2006) found that β-carotene increased when light intensity increased while the cell number decreased which is in harmony with the present results. Our results also agreed with those obtained by Abu Sara *et al.* (2011) who mentioned that in response to different light intensities, the maximum growth was obtained at 61 µM m^{-2} s^{-1} and the maximum β-carotene production was at 200 µM m^{-2} s^{-1} while the maximum β-carotene to chlorophyll a ratio was recorded in cells grown at 1000 µ M m^{-2} s^{-1}. In contrary, Abu Rezq, *et al.,* 2010 reported that *D. salina* grows at a significantly faster rate with best growth performance at high light intensity than in low one. In the present study, there was an increase in β-carotene to chlorophyll a ratio from 0.68 to 1.59 with gradual increase in light intensity up to 292.5 µE m^{-2} s^{-1}. The results of Ben Amotz and Avron (1983) concerning the higher β-carotene to chlorophyll a ratio at high irradiance are interpreted as indicating a protecting effect of β-carotene against injury by high irradiance. Under conditions of impairment in chlorophyll content per cell, they also reported that β-carotene accumulation was coupled with chlorophyll depletion under high light intensities which supports the obtained results of the present study. Furthermore, Telfer (2002) suggested that β-carotene protect the algae from damage occurred during excessive irradiances by preventing the formation of reactive oxygen species, quenching the triplet state chlorophyll, reacting with singlet oxygen and functioning as a light filter.

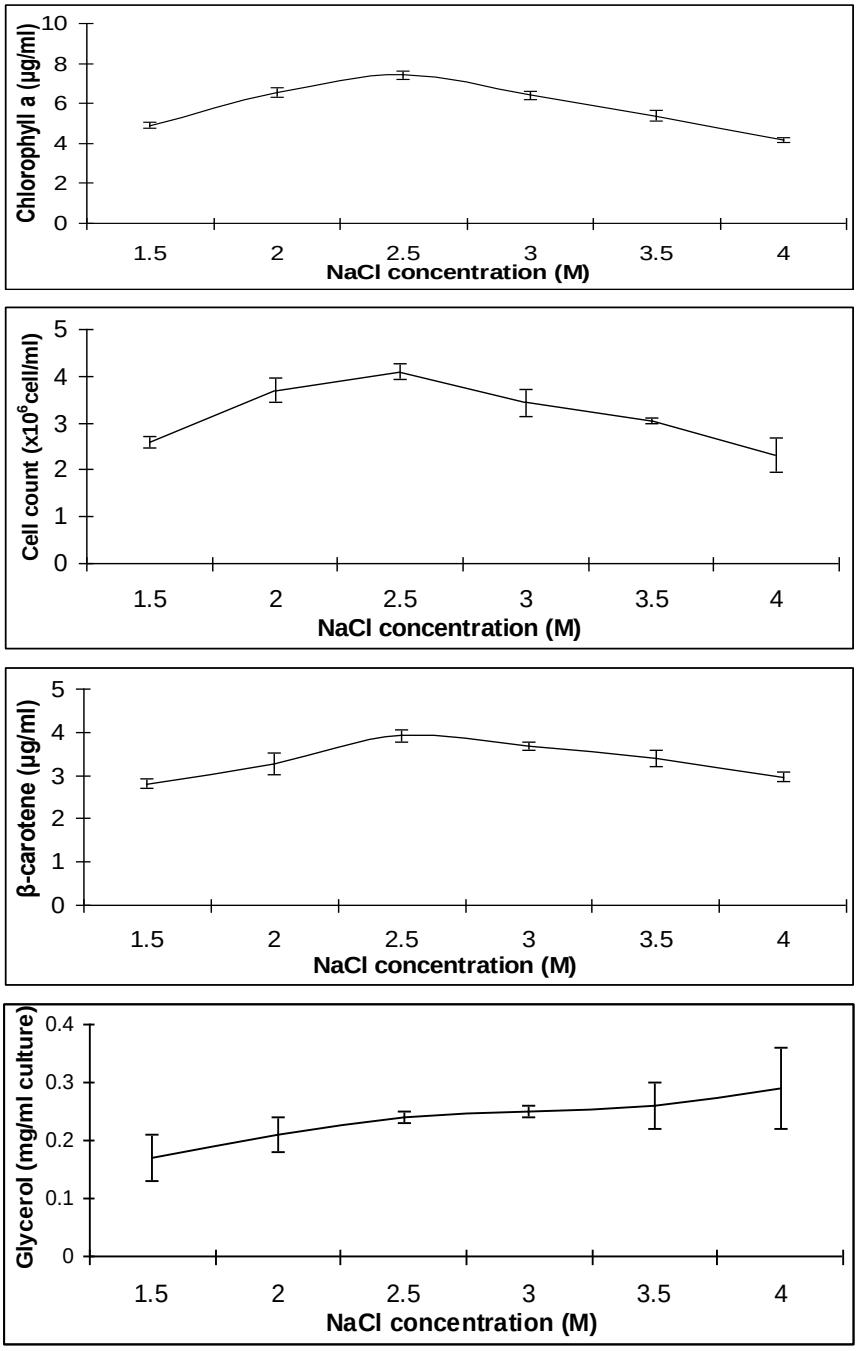

Fig. 1. Effect of different NaCl concentrations on growth, (Chlorophyll a "a", Cell count "b", β-carotene "c" and Glycerol "d") production of *Dunaliella bardawil,* after 8 days incubation.

TABLE 4. Effect of different light intensities on growth, and β-carotene production of *Dunaliella bardawil* after 8 days incubation in 1.5 M NaCl.

Growth parameter→ / light intensity (µE m^{-2} s^{-1}) ↓	Chlorophyll a (µg/ml)	cell count (x 10^6cell/ml)	β-carotene (µg/ml)	β-carotene/ chlorophyll ratio
Initials	2.33	1.5	1.58	0.68
39	5.34±0.03^b	2.49±0.35^a	3.62±0.33^a	0.68
78	6.87±0.07^d	3.56±0.39^b	4.82±0.07^b	0.70
117	7.50±0.20^e	5.89±0.14^d	5.27±0.11^c	0.70
195	5.93±0.03^c	4.14±0.20^c	5.73±0.23^d	0.97
292.5	4.10±0.10^a	3.22±0.02^b	6.52±0.19^e	1.59

Means marked with the same superscript letters are not-significant (P>0.05), whereas others with different superscript letters are significant (P<0.05). Data are average of three replicates; each value represents the mean ± SD.

Conclusions

The present study, provide a base-line information on the optimum growth conditions of the green micro-alga *Dunaliella bardawil* as a model organism for production and accumulations of β-carotene and glycerol. This organism can grow in wide pH range (5.5 – 9.5) with maximum growth and glycerol content at pH 7.5 while the best pH value for β-carotene production was 8.5. Glycerol content was maximum at 4 M NaCl whereas growth and β-carotene production were highest at 2.5 M NaCl. High light stress (292.5 µE m^{-2} s^{-1}) enhanced the production and accumulation of β-carotene in *Dunaliella* cells.

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(*Received* 21/2/2015;
accepted 26/1/2016)

تاثير بعض العوامل الأحيائية على النمو وتراكم الجلسرول والبيتاكاروتين في طحلب *دونااليلا برديل*

عفت فهمي شبانة، هدى حامد سنوسي، هند يوسف محمد
قسم النبات والميكروبيولوجي – كلية العلوم – جامعة القاهرة – الجيزة – مصر .

تم في هذا البحث دراسة تأثير كل من الرقم الهيدروجيني والحقنة الطحلبية والملوحة وشدة الإضاءة كعوامل إجهاد على النمو وتكوين الجلسرول والبيتاكاروتين في طحلب دونااليلا برديل. أوضح منحنى النمو للطحلب الزيادة الخطية التدريجية لقيمة كلورفيل أ وعدد الخلايا والكثافة الضوئية وذلك حتى نهاية التجربة التي استغرقت ٤٢ يوم. سجل كل من معدل النمو النسبي (k) ومعدل النمو (R) وعدد مرات إعادة التدوير (Nu) أعلى القيم في اليوم الرابع للزراعة بينما سجل وقت التضاعف (G) اقل القيم في نفس اليوم.

ينمو طحلب دونااليلابرودويل في مدى واسع للرقم الهيدروجيني (من ٥،٥ إلى ٩،٥) وكان أقصى نمو (كلورفيل أ وعدد الخلايا) وكذلك إنتائج الجلسرول عند رقم هيدروجيني ٧،٥ في حين كان افضل إنتاج البيتاكاروتين عند الرقم الهيدروجيني ٨،٥.

أدت زيادة الحقنة الطحلبية إلى حدوث زيادة معنوية في محتوى الكلوروفيل وعدد الخلايا وصبغ البيتاكاروتين بينما كانت الزيادة في الجلسرول غير معنوية. كما وجد أن أقصى نمو وإنتاج للبيتاكاروتين تم رصدها عند تركيز ٢،٥ مولار كلوريد صوديوم بينما تم الحصول على أكبر قدر من الجلسرول في الخلايا السابق تعريضها لتركيز ٤ مولار كلوريد الصوديوم وذلك بعد ٨ أيام من النمو.

وتم تسجيل أفضل نمو عند شدة إضاءة ($117\ \mu E\ m^{-2}\ s^{-1}$) في حين تم الحصول على أقصى إنتاج للبيتاكاروتين عند ($292.5\ \mu E\ m^{-2}\ s^{-1}$) حيث كانت نسبة البيتاكاروتين للكلوروفيل ١،٥٩. وقد تبين من الدراسة الحالية أن طحلب دونااليلا برديل له قدرة كبيرة على مقاومة وتحمل الإجهادات المختلفة مع القدرة على إنتاج مواد عديدة مثل الجلسرول والبيتاكاروتين.