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The Effect of Certain Environmental Factors on Growth and β-Carotene Production by *Dunaliella sp.* Isolated from the Dead Sea

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Abstract

Microalga *Dunaliella sp.* was isolated and identified from ponds of the south-east shores of the Dead Sea. The most suitable media for the isolated *Dunaliella sp.* was found to be M1 (modified BG-11) which gave the highest growth and β-carotene production. The effects of different physical (temperature and light intensity) and chemical (different nitrogenous and sulfate compounds) factors were tested. The best salinity for *Dunaliella* growth was 2.5 % NaCl, while the maximum β-carotene to chlorophyll *a* (Chl. *a*) ratio was found in high salinities: Dead Sea water-M1 (DSw-M1) dilution (1:1), 10% NaCl and DSw-M1 (3:1). By using $NaNO_3$ at 40 mg N l^{-1} concentration as a nitrogen source and $MgSO_4$ at 25 mg l^{-1} concentration as a sulfate source, the maximum growth and β-carotene production was obtained. In response to different light intensities, the maximum growth was obtained at 61 µmol s^{-1} m^{-2}, and the maximum β-carotene production was at 200 µmol s^{-1} m^{-2}, while the maximum β-carotene to chlorophyll *a* ratio was recorded in cells grown at 1000 µmol s^{-1} m^{-2}.

الملخص

تم في هذه الدراسة عزل الطحالب الدقيقة المحبة للملوحة "دونياليلا" من السبخات المائية المالحة والمتناثرة في الجزء الجنوبي من البحر الميت. حيث كان أفضل وسط غذائي من المواد اللاعضوية لهذه الطحالب هو M1 (BG-11). وكان أفضل نمو للطحالب عند درجة حرارة 20 C م ، بينما ماتت معظم الخلايا عند درجة حرارة 40 و 50 C م . وقد تم دراسة تأثير عوامل وظروف فيزيائية وكيميائية مختلفة على نمو هذه الطحالب وانتاجها لمادتي البيتاكروتين والكلوروفيل . وقد سجل أعلى نمو لهذه الطحالب عند استخدام كلوريد الصوديوم بنسبة 2.5 % ، بينما كانت أعلى نسبة بيتاكاروتين/كلوروفيل أ عند تراكيز الملوحة العالية DSw- , 10 % M1 (1:1), DSw-M1 (3:1) . تم الحصول على أعلى نمو ونسبة انتاج البيتاكاروتين عند استعمال نترات الصوديوم (كمصدر للنيتروجين) بتركيز 40 ملغ نيتروجين/لتر ، وكبريتات المغنيسيوم (كمصدر للكبريت) بتركيز 25 ملغ/ل. وبعد دراسة تأثير شدة الضوء وجد أن أعلى نمو تم تسجيله عند شدة اضاءة 61 (مايكرومول/ث.م2) وأعلى انتاج للبيتاكاروتين كان عند شدة اضاءة 200 (مايكرومول/ث.م2) بينما كانت أعلى نسبة بيتاكاروتين/كلوروفيل أ عند شدة اضاءة 1000 (مايكرومول/ث.م2).

Keywords: Dunaliella, β - Carotene, Dead Sea, Halophiles

1. Introduction

The Dead Sea is the lowest exposed surface on earth (416 m below sea level) and is one of the world's saltiest lakes, with a total dissolved salt concentration of 340 gl^{-1}. It is considered sterile and, therefore, unsuitable for fishery (Gavrieli *et al*., 1999).

The eukaryotic algae *Dunaliella* was first described by Teodoresco in 1905, and first reported to be present in the Dead Sea by Elazari-Volcani in 1940 (Oren, 1999). *Dunaliella* is a unicellular, motile, green microalgae which lacks a rigid cell wall and has a single large cup-shaped chloroplast that fills the posterior part of the cell (Butcher, 1959; Javor, 1989).

Great interest in *Dunaliella* has arisen because of its ability to withstand various environment stresses, especially those associated with hypersaline conditions. Its halotolerance is predominantly mediated by an accumulation of glycerol as an osmoregulator (AL-Hasan *et al*., 1987; Ginzburg, 1987).

A number of studies have revealed that growth (Ben-Amotz and Avron, 1983; Ginzburg, 1987) and pigment compositions (Ben-Amotz *et al*., 1989) of this algae are affected by halostress conditions. It was found that the β-carotene to chlorophyll *a* ratio gradually increased with an increase in NaCl concentration, and, as a result, the algae changed its appearance from green to deep orange (Ben-Amotz and Avron, 1983; AL-Hasan *et al*., 1987).

Overproduction of β-carotene is induced by high light intensity (Kleinegris *et al.*, 2010) and by other environmental factors such as nutrient deprivation, or high salt concentration (Ben-Amotz and Avron, 1983; Raja *et al.*, 2007; Macias-Sa'nchez *et al*., 2009). β-Carotene was found to be accumulated in oil globules in the interthylakoid space of the chloroplast and it is composed of two major stereoisomers: all-*trans* and 9-*cis*

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β-carotene (Ben-Amotz *et al*., 1989; Macias-Sa'nchez *et al*., 2009; Kleinegris *et al*., 2010).

From the other side of the Dead Sea facing Jordan, *Dunaliella sp.* was isolated. So, the effect of certain environmental conditions on growth and β-carotene is reported.

2. Material and methods

2.1. Sampling and growth conditions

Sampling was carried out during spring season (April-May, 2003) from ponds present at the east-south basin of the Dead Sea. All collected samples were examined microscopically for the presence of a green algae *Dunaliella* and a preliminary cultivation was carried out using inorganic medium (AL-Hasan *et al*., 1987). Identification of the isolated and cultivated *Dunaliella* was carried out morphologically according to Butcher (1959).

Dunaliella cells were harvested by centrifugation at 3500 rpm for 15 min and washed several times with sterile 10% (w/v) NaCl solution to minimize bacterial contamination. Cells were then cultivated in an inorganic medium containing 5 mM $MgSO_4$, 0.3 mM $CaCl_2$, 5 mM KNO_3, 0.2 KH_2PO_4, 1.5 µM $FeCl_3$, 50 mM $NaHCO_3$, 30 µM EDTA, 5% NaCl, 300 U polymyxin B. ml^{-1}, 150 U penicillin G. ml^{-1}, 1000 U streptomycin. ml^{-1}, pH 8 (AL-Hasan *et al*., 1987). The antibiotics were sterilized separately and added to eliminate residual bacteria. Cultures were incubated at room temperature 25±2 C˚ with continuous slow stirring using a magnetic stirrer. Cultures were sparkled with sterile air. A constant illumination of 61 µmol m^{-2} s^{-1} was provided at the surface of the vessels using cool white fluorescent lamps.

Five different types of media were tested for their effects on the growth of *Dunaliella sp.*; these are: **M1** (BG-11 medium, Stanier *et al*. (1971), **M2** (f2 medium, Jeffrey and LeRoi (1997), **M3** (f/2 medium, Guillard (1962), **M4** medium of Ben-Amotz *et al*. (1989), and **M5** of Sallal *et al*. (1987).

2.2. Growth parameters and pigments

Cell number was determined using a haemocytometer (Jeffrey and LeRoi, 1997). Chlorophyll *a* and β-carotene were extracted from algal pellet with 80% (v/v) acetone according to (Ben-Amotz and Avron, 1983). $\boldsymbol{E}^{1\%}$ of 87.67 at 664 nm and $\boldsymbol{E}^{1\%}$ of 2273 at 480 nm have been used to calculate chlorophyll *a* and β-carotene concentrations, respectively (Ben-Amotz and Avron, 1983 and Jeffrey *et al*., 1997).

2.3. Cultivation of Dunailella cells in different nutrient media

M1 medium with different NaCl concentrations, 1.25%, 2.5%, 5%, 10%, 20%, and 30%, was used to grow *Dunailella sp.* Different dilutions of Dead Sea water and M1 medium were also used (DSw : M1) (1:1) and (3:1). Cultures were incubated at room temperature 25±2 C˚ under constant light illumination of 61 µmol m^{-2} s^{-1}.

Different nitrogenous sources such as $NaNO_3$, NH_4Cl, $Ca(NO_3)_2$, and NH_4NO_3 were prepared with the following concentrations: 10, 20, 30, 40, 50 mg Nl^{-1} in 250 ml conical flasks containing M1 nitrogen-free medium. Various concentrations of $MgSO_4$: 0, 25, 50, 75 mg/l were also prepared in M1 sulfate-free media. All cultures had 2.5% NaCl concentration, and were incubated at room temperature 25±2 C˚ and constant light illumination of 61 µmol m^{-2} s^{-1}.

Dunaliella culture grown in M1 medium were also illuminated at different light intensities: 61, 200, and 1000 µmol m^{-2} s^{-1} using cool white fluorescent lamps and Halogen lamp (Phoenix electric, China). Light intensity was measured using a photometer (LI-COR model LI-189, USA).

2.4. Pigment analysis

TLC was carried out for the extracted pigments as described by Stahl (1965). Two developing solvents were applied separately on TLC aluminum sheets of silica gel: (1) *n*-hexane : acetone : *iso*-propanol (69:30:1 v/v/v) to resolve individual pigments, (2) petroleum ether : acetone (97: 3 v/v) to resolve β,β-carotene (Repeta and Bjornland, 1997).

2.5. β-Carotene crystallization

β-Carotene crystallization was performed as described by Repeta and Bjornland (1997). 2 ml extract was dissolved in 10 ml benzene and cooled to a solid phase at - 20 C˚. 30 ml of pre-cooled methanol was added on the top of the solid benzene layer and the biphasic system was left at – 20 ˚C, for 1 to 3 days. The epiphasic methanol layer will slowly dissolve the solid benzene and the β-carotene will get crystallized at the interface between the layers.

3. Results

Collected samples from brine ponds at the east-south basin of the Dead Sea were examined microscopically for the presence of a green algae *Dunaliella* which was isolated and cultivated in M1 medium. *Dunaliella* cells were identified as *Dunaliella sp.* according to the description given by Butcher (1959).

In TLC, two structurally related β,β-carotene (β-carotene) and β,ε-carotene (α-carotene) were resolved using the developing solvent 1 (Fig. 1a) in addition to four other spots with different colors (blue-green, green, faint green, and faint yellow) and different $\boldsymbol{R_f}$ values (Fig. 1a). Using developing solvent 2, a yellow-orange β,β-carotene spot with a $\boldsymbol{R_f}$ value= 0.82, was also resolved (Fig. 1b).

Dunaliella cells were cultivated in 5 different types of media (M1, M2, M3, M4 and M5) under constant illumination and at room temperature 25±2 ˚C. Through 14 days, the maximum growth and β-carotene production were obtained with M1 media as shown in Fig. 2. Chlorophyll *a* was 7.5 mgl^{-1} while β-carotene was 5.2 mgl^{-1} (Fig.2). M5 media gave the maximum β-carotene to chlorophyll *a* ratio 0.8 while M4 gave the lowest ratio 0.6 as shown in Table1 and Fig2.

Table 1 shows the average number of *Dunaliella* / ml grown in five different media. The maximum number of *Dunaliella* cells was found in M1 medium, which agrees with the result obtained in Fig. 2. M5 medium gave $4.8x10^6$ cell/ml in comparison with $6.5x10^6$ cells/ml in

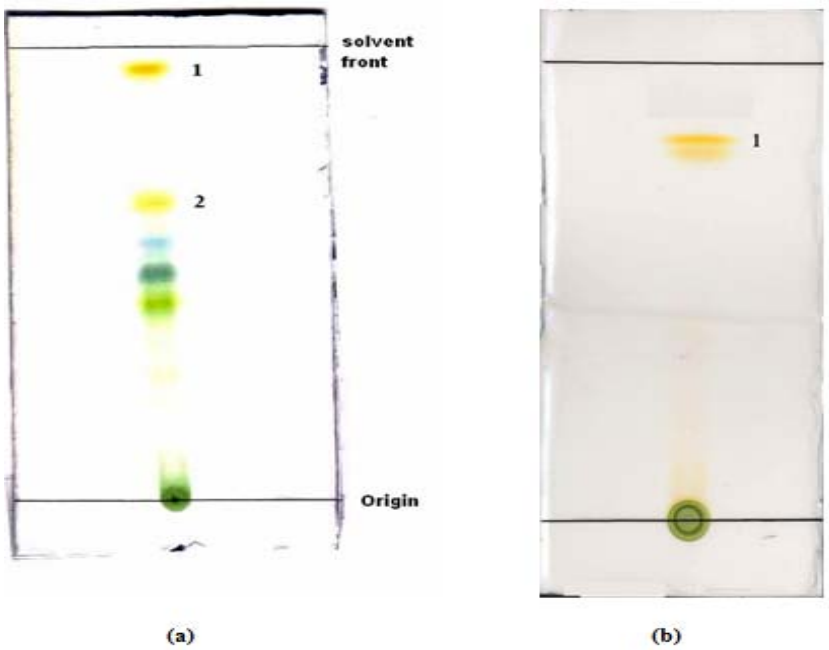

Figure 1. Thin layer chromatography (TLC) plates showing:

(a) The structurally related β,β- and β,ε-carotenes (1,2 respectively). Stationary phase: silica G, Mobile phase: *n*-hexane : acetone: *i*-propanol. (b)β,β-carotene (1) with R_f=0.82. Stationary phase: as in (a). Mobile phase: petroleum ether : acetone.

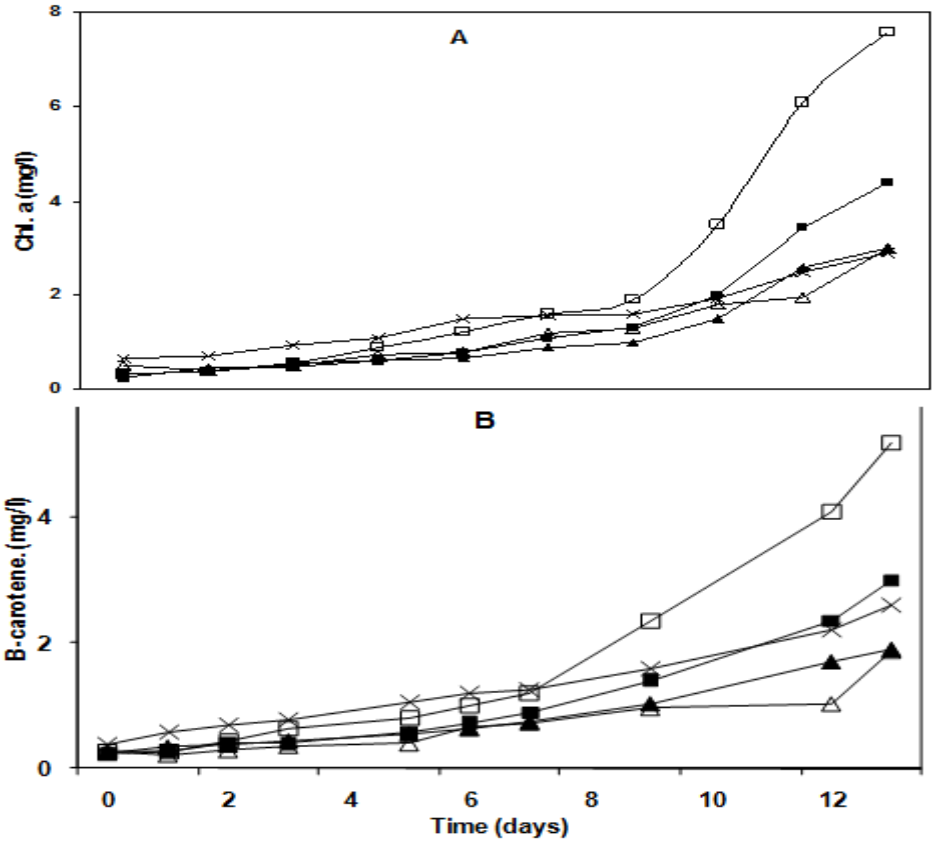

Figure 2. Growth (A) and β-carotene (B) of *Dunaliella sp.* in five different types of media: M1 □, M2 ■, M3 ▲ , M4 △, and M5 ✕ .

medium M1, while other types of media gave less numbers than these two types of media after 14 days of growth (Table 1).

Table 1. The average number of *Dunaliella sp*. cells grown in different types of media. ($x10^6$/ml) .

Days / Media	0	2	5	7	9	12	14
M1	0.5	1.7	1.5	3.9	4.1	6.4	6.5
M2	0.5	1.2	1.6	1.7	2.3	2.6	2.9
M3	0.45	1.6	2.0	2.1	2.2	2.4	2.4
M4	0.6	1.6	1.7	2.2	3.0	3.8	4.4
M5	0.5	1.2	1.4	1.9	2.7	3.1	4.8

Maximum growth was obtained at 20 ˚C where chlorophyll *a* and β-carotene concentrations were 3.4 and 2.1 mgl^{-1}, respectively, after 10 days. A slight growth was observed at 30 C˚, while no growth observed at 40 C˚ and 50 C˚.

The highest growth of *Dunaliella* cells was found toat 40 $mgNl^{-1}$. However $NaNO_3$ enhanced the highest growth and β-carotene production compared to other nitrogenous compounds used (Fig. 3). The different concentrations presented in this figure were the highest for each nitrogenous compound.

Sodium nitrate ($NaNO_3$) at a concentration of 40 $mgNl^{-1}$ gave 5.17 mgl^{-1} and 4 mgl^{-1} for chlorophyll *a* and β-carotene, respectively . However, the maximum β-carotene/chlorophyll *a* ratio was found to be 0.82 at 20 $mgNl^{-1}$ (Fig. 3).

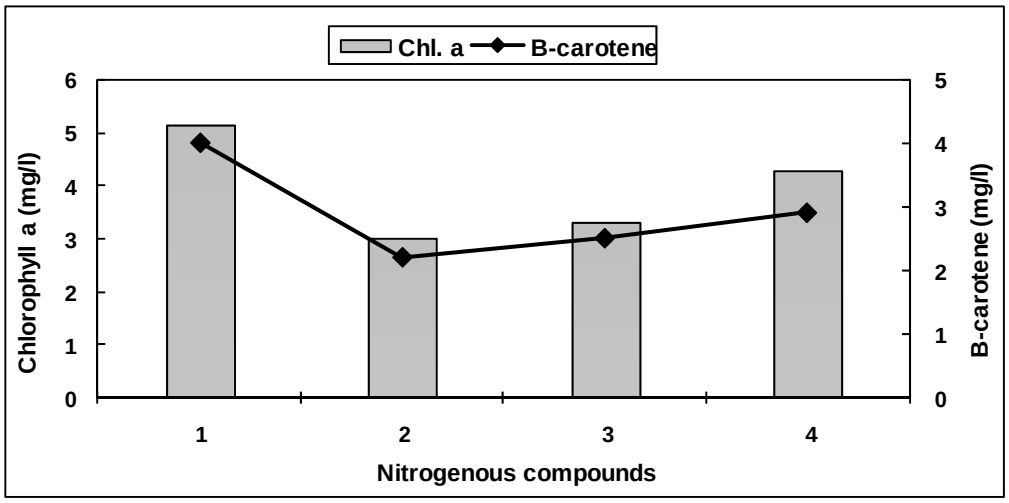

Figure 3. Growth and β-carotene production after 15 days of growth using M1 medium supplemented with different nitrogenous compounds: 1, 40 mg NL^{-1} $NaNO_3$; 2, 40 mg NL^{-1} $Ca(NO_3)_2$; 3, 40 mg NL^{-1} NH_4NO_3; and 4, 50 mg NL^{-1} NH_4Cl.

The effect of different concentrations of magnesium sulphate on *Dunaliella sp.* was studied. *Dunaliella* growth and β-carotene production were found to be the highest at 25 mgl^{-1} $MgSO_4$. So, chlorophyll *a* and β-carotene concentration were 3.4 mgl^{-1} and 2.4 mgl^{-1}, respectively (data not shown).

The maximum chlorophyll *a* and β-carotene production were obtained at 2.5% NaCl with 5 mg/l and 4.2 mg/l chlorophyll *a* and β-carotene respectively (Fig. 4). However, the decrease in chlorophyll *a* under laboratory conditions was noticed in 30% and DSw-M1 (3:1)(0.2mg/l) (Fig. 4), an increase in β-carotene production was 2.5mg/l noticed in *Dunaliella* grown in DSw-M1 (1:1) as compared to DSw-M1 (3:1). The best ratio of β-carotene/chlorophyll *a* was recorded in culture grown in DSw-M1 (1:1) which was 1.1.

Effects of different light intensities on *Dunaliella* growth are shown in Fig. (5). Chlorophyll *a* contents increase in *Dunaliella* with 4 and 2.6 mgl^{-1}, respectively, at both 61 and 200 µmol $s^{-1}m^{-2}$ after 12 days culture old, but *Dunaliella* chlorophyll *a* content decreased to 0.14 mgl^{-1} under 1000 µmol $s^{-1}m^{-2}$ light intensity (Fig. 5). *Dunaliella* grown under 1000 µmol $s^{-1}m^{-2}$ gave the maximum β-carotene/chlorophyll *a* ratio (1.25) at day 8, while cells grown under 61 µmol $s^{-1}m^{-2}$ gave 0.66 ratio.

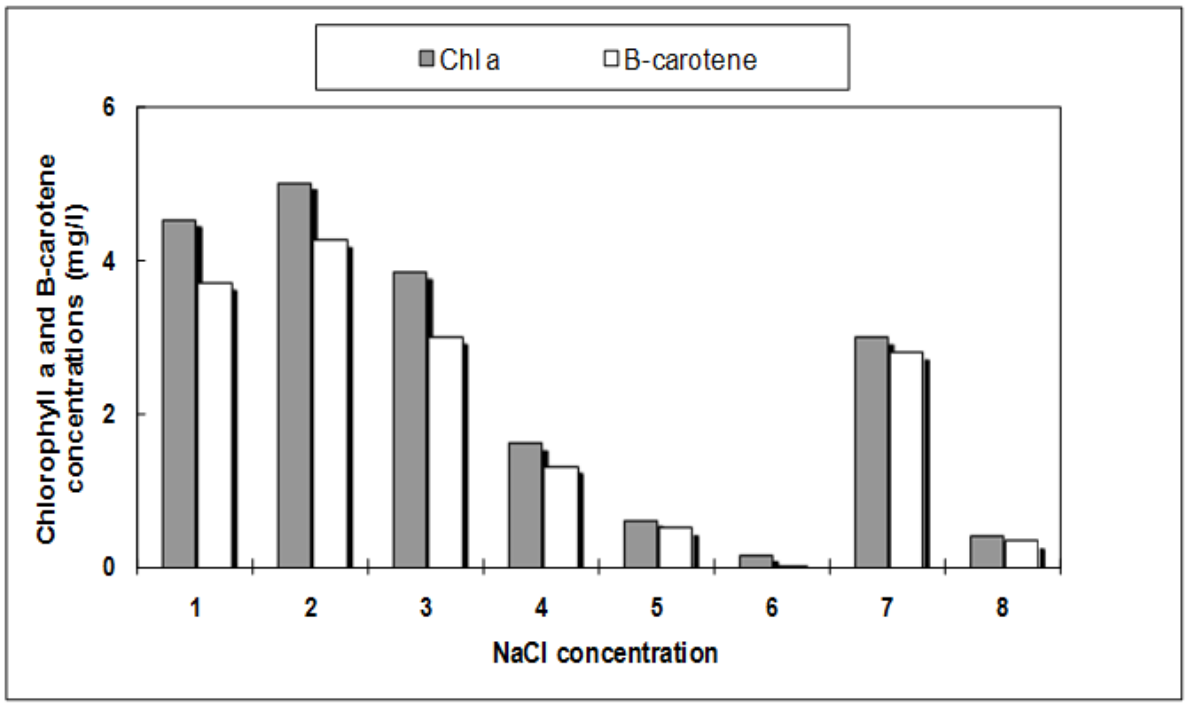

Figure 4. Effect of different NaCl concentrations: 1, 1.25%; 2, 2.5%; 3, 5%; 4, 10%; 5, 20%; 6, 30%; 7, 1:1 (DSw:M1); 8, 3:1 (DSw:M1) on the growth and β-carotene production of *Dunaliella sp.*

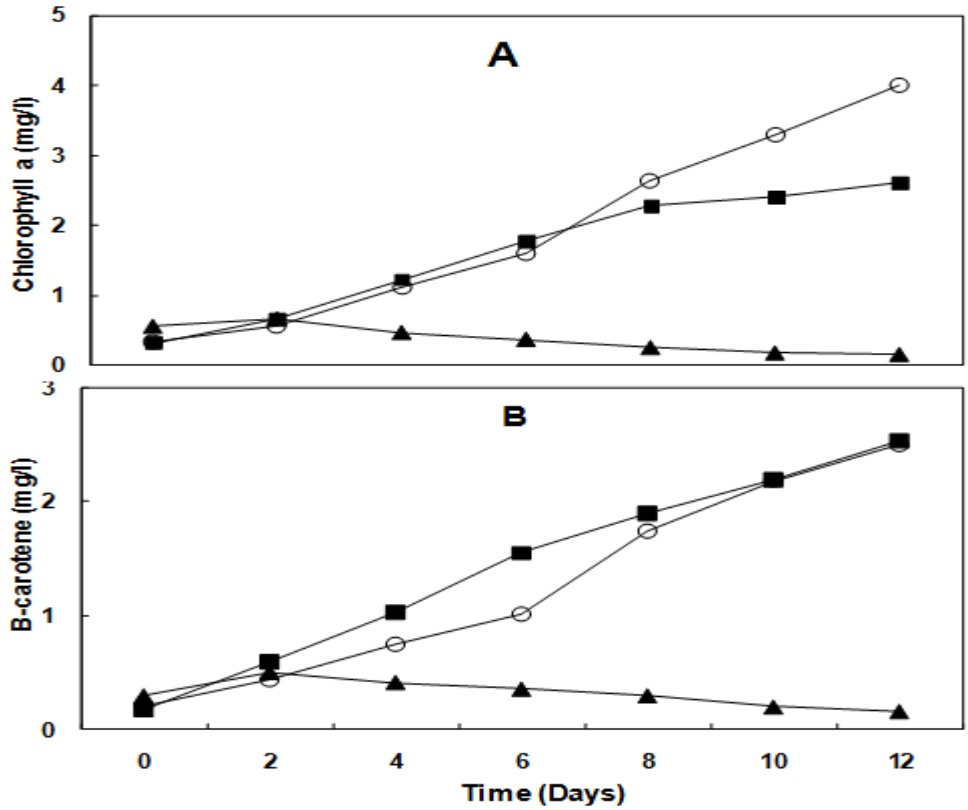

Figure 5. Effect of different light intensities on *Dunaliella sp.* growth (A) and β-carotene production (B). ¤ 61 µmol s^{-1} m^{-2}, ■ 200 µmol s^{-1} m^{-2}, ▲ 1000 µmol s^{-1} m^{-2}.

4. Discussion

Dunaliella sp. culture in this study remained green in color at all tested conditions. According to Sammy (1993) *D. viridis* remained green at all salinities, but he reported "a red flowering" growth of *Dunaliella salina*. Al-Hasan *et al.* (1987) recorded a red *Dunaliella* forms in Kuwait salt marshes during summer, while in winter *Dunaliella* cells turned green and became smaller in size.

The different species of *Dunaliella* have different optimal growth temperatures. *Dunaliella salina* was found to grow optimally at 30˚C, *D. viridis* at 37˚C, and *D. tertiolecta* at 20˚C (Brown and Browitzka, 1979). Other *Dunaliella* strain (AL-Hasan *et al*., 1987) remained viable at 55˚C during summer and at 12˚C during winter. In this study, *Dunaliella* cells were found to grow optimally at 20˚C.

Dunaliella sp. were found to grow better in $NaNO_3$ and NH_4Cl than in NH_4NO_3 (AL-Hasan and Sallal, 1985), which agrees with the results in this study as presented in Fig. 4. Gibor (1956) found that both *D. salina* and *D. viridis* grow much better with NO_3^--N than with NH_4^+-N; however, *D. salina* from the Great Salt Lake preferred NH_4^+ - N over NO_3 - N (Post, 1977).

The depletion of sulfate in the medium causes *Dunaliella* cells to stop dividing and start accumulating β-carotene inside the cells (Lers *et al*., 1990; Phadwal and Singh, 2003), and, as a consequence, an increase in β-carotene/chlorophyll a ratio (Ben-Amotz and Avron, 1983). These results are in agreement with this study where β-carotene production and β-carotene/chlorophyll *a* ratio increased at low sulfate concentration 25 mgl^{-1} $MgSO_4$.

In this study, *Dunaliella* cells grow optimally at 2.5 % salinity and tolerated up to 20% salinity (Fig. 4). In many studies, the major species of *Dunaliella* were found to have different salt concentrations. *D. viridis* grow optimally in 5.8 – 8.9% salinity and tolerate up to 23.2% salinity (Browitzka *et al*., 1977), while *Dunaliella salina* isolated from Kuwait marshes had an optimum growth at 2.5 – 5% (w/v) NaCl and the growth continued up to 30% NaCl (AL-Hasan *et al*., 1987). The strain of *Dunaliella tertiolecta* tolerated 0.5 – 34% NaCl (Wegmann, 1981).

The β-carotene/chlorophyll *a* ratio in this study was the highest at high salinities DSw-M1 (1:1), 10 % and DSw-M1 (3:1); this agrees with many studies that reported that β-carotene to chlorophyll *a* ratio increased in high NaCl concentration (Ben-Amotz and Avron, 1983). Javor (1989) reported that carotenoid content continued to increase in cells with respect to salinity in medium with >10% NaCl. Other study found that the highest β-carotene production per cell was obtained at 2 M NaCl in *D. salina* and *D. bardawil* in comparison with 1 and 3 M NaCl concentrations (Gomez *et al*., 2003).

Optimal light conditions for both growth and β-carotene production have been reported for several strains of *Dunaliella* (Van Auken and McNulty, 1973; AL-Hasan and Sallal, 1985; Javor, 1989). Light intensity is the major induced factor for β-carotene production, which is highly effective in protecting *Dunaliella* cells against photoinhibition due to the ability of β-carotene to quench damaging singlet oxygen and hydroxyl radicals (Ben-Amotz *et al*., 1989; Prescott *et al*., 2005). This is in accordance with the results of this study: β-carotene production increased at high light intensity (200 $\mu mol\ m^{-2}\ s^{-1}$) (Fig. 5), and the highest β-carotene/chlorophyll *a* ratio was obtained at 1000 $\mu mol\ m^{-2}\ s^{-1}$. Hejazi and Wijffels (2003) also reported that β-carotene content of the *Dunaliella salina* cells increased by increasing the light intensity.

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