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Induction and extraction of *β*-carotene from the locally isolated *Dunaliella salina*

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Abstract

Two samples of *Dunaliella salina* (one locally isolated from Kuwait's puddle stagnant waters off Bubiyan Island and the other sample brought from Perth – Australia) were maintained and pure cultures were produced. Set of experiments was conducted to study and evaluate the stress environmental factors required for *β*-carotene induction. Results showed that the two samples of *D. salina* tended to change color from green to yellow or brown under the following culturing conditions: high salinity (200 to 250 psu), high temperature (38.5ºC), high light intensity (55x10^3 lux) and low culture media concentrations, i.e., at 25% of either the AFMED or F/2 algal culture media. Another set of trials was conducted to carry out *β*-carotene extraction using the Pressured Fluid Extraction method. The results obtained showed that both of the *D. salina* samples contained relatively good amounts of *β*-carotene 33.8- 96.5 pg cell^{-1}. Due to the success and encouraging results obtained in this study, it is recommended that a pilot scale production of *D. salina* culture using outdoor shallow raceways to be conducted in the near future.

Key words: salinity, temperature, light intensity, chromatography, carotenoids.

Introduction

Microalgae are currently cultivated commercially for human nutritional products around the world in small to medium scale production systems, with total world commercial algal biomass production estimated at about 10,000 tons per year. The main algae currently cultivated

photosynthetically (e.g. with light energy) for various nutritional products are *Spirulina, Chlorella, Dunaliella* and *Haematococcus* (Campo *et al.*, 2007; Benemann, 2008).

Al-Abdul-Elah *et al.*,(1999) discussed the possible use of *Dunaliella* algal species for aquacultural purposes as a source of *β*-carotene. They reported, during their technical visit to the Gold Coast Marine Aquaculture hatchery in Australia, that the use of *Dunaliella* two to three days before harvesting would be sufficient for the farmed black tiger prawn to gain a health red color and better taste. These two good preferred characters would be promising better marketing and fetch a higher price.

Dunaliella salina which is a biflagellated green marine algal species belonging to the class Chlorophyceae (Avron and Ben-Amotz, 1992; Leach *et al.*, 1998; Prof. Borowitzka, personal communication) can live in high concentrations of saturated brine (Ahmed *et al.*, 2001). Cells of *Dunaliella* are enclosed by a thin elastic plasma membrane covered by a mucous surface coat which permits rapid cell volume changes in response to extracellular changes.

Ben-Amotz *et al.*,(1982); Loeblich (1982); Garcia-Gonzalez *et al.*,(2003); Gomez *et al.*,(2003); Gomez and Gonzalez (2005) reported that *Dunaliella*, under certain stress conditions (i.e., high salinity, high solar radiation and low nitrogen), have been shown to change their colors from green to orange or red and accumulate large amounts of *β*-carotene. They also stated that *D. salina* is recognized at present as the most salt-tolerant eukaryote known.

Commercial Importance of β-Carotene

D. salina is a unique species of alga that has a high content of natural carotenoids, especially *β*-carotene, antioxidants and essential vitamins. This algal species has served and still as the major source for the extraction of *β*-carotene for commercial applications (Bosma and Wijffels, 2003; Leon *et al.*, 2003; Ye *et al.*, 2008). Carotenoids act as secondary pigments in photosynthetic organisms, and as antioxidants and provitamin factors for non-photosynthetic organisms. Their antioxidant and colorant properties make them useful for the therapeutic, dietetic and industrial applications of carotenoids (Leon *et al.*, 2003).

Dunaliella natural *β*-carotene productions are widely distributed in many different markets under three different categories: *β*-carotene extracts, *Dunaliella* powder for human use and dried *Dunaliella* for feed use (Ben-Amotz, 2004). Today, the price of extracted and purified natural *β*-carotene is much higher than that of synthetic *β*-carotene ($1,000 to $2,000 kilogram^{-1} for natural versus $400 to $800 kilogram^{-1} for synthetic). The price difference reflects that the consumers prefer the natural products from the synthetic *β*-carotene (Caswell and Zilberman, 2000).

Commercial production of *D. salina* as a source of *β*-carotene became the fourth major microalgal industry when culture and production facilities were established by Western Biotechnology Ltd. and Betatene Ltd. in Australia in 1986 (Borowitzka, 1999). These were soon followed by other commercial plants in China, Israel, United States (US), as well as other plants in Chile, Iran and Japan (Borowitzka, 1999; Ben-Amotz, 2004; Gomez and Gonzalez, 2004).

In Kuwait, the cost of 100 tablets (500 mg each contain carotenes in addition to other mixtures of antioxidant vitamins) ranges between KD 6.500 and 13.500 (US$ 20 and 40), depending on the brand, source and ingredients of each. Thus, it is worthwhile to develop a technique by which to extract pure, natural *β*-carotene from locally available marine *D. salina* and make it available for public consumption in Kuwait's market. Such pure, natural *β*-carotene extracted from *D. salina* will cost less than the existing commercial carotenoid products available in Kuwait.

Due to the success in maintaining and finding out the optimum indoor culture conditions required for the highest growth rate of *D. salina* (Abu-Rezq *et al.,* 2010), this research work was conducted. The two pre-produced samples of *D. salina* (i.e., Kuwait and Australia) were used at the Aquaculture, Fisheries and Marine Sciences Department (AFMED), Kuwait Institute for Scientific Research's (KISR) laboratories. The main objective was to develop a procedure to culture locally isolated *D. salina,* as a step towards enhancing *β*-carotene induction, and then testing and adopting methods for *β*-carotene extraction.

Materials and Methods

β-Carotene Induction

Innova 4900 Multiple Shaker Environmental Chamber shaker

incubator, provided with controlled temperature and light intensity, was used during this study to maintain pure seeds of both samples of *D. salina*. A water bath tank made from reinforced/tempered glass having the following dimensions: 200-cm length, 65-cm width and 25-cm height with a 10-mm glass thickness was used for the set of experiments carried out.

Set of experiments were conducted on a small scale, in vitro, simulating the actual conditions in the sea. Aliquots from each 1000-ml flask were collected and counted every morning using an Improved Neubauer Haemocytometer (Bright-line). Each experiment was terminated after two weeks of growth, unless stated otherwise.

The locally isolated *D. salina* in this study is referred as *D. salina* (Kuwait) while the *D. salina* brought from Australia is referred as *D. salina* (Australia). The algal seed maintenance and production procedures were as described in Abu-Rezq *et al.*,(1999, 2002 and 2009).

The culture media salinity was manipulated by the addition of natural sea salt to the culture media to increase the salinity to the desired concentration as recommended by Professor Borowitzka (Personal communication). Prior to the experiment, both *D. salina* (i.e., from Kuwait and from Australia) were acclimated to the different salinity regimes, as required.

The first set of experiments studied the growth rate of *D. salina* (Kuwaiti) cultured at light intinsity $10x10^3$ lux, salinity at 140 and 190 psu, and 25 and 100% of AFMED algal culture media concentrations, without using carbon dioxide (CO_2) gas. The temperature was maintained at 21 to 22ºC. The second set of experiments studied the growth rate of *D. salina* (Kuwaiti) cultured at $10x10^3$ lux at different high salinities (50, 100, 150 and 200 psu) under the same culturing conditions as the first set of experiments. The third and fourth sets of experiments studied the growth rate of *D. salina* (Kuwaiti and Australian) cultured at different high salinities (i.e., 50, 100, 150, 200 and 250 psu) and at higher light intensity (i.e., $18x10^3$ lux) and temperature was also maintained at 21 to 22ºC. These two experiments were terminated after 35 days of observation.

The fifth and sixth sets of experiments studied the effect of different high salinities (100, 150 and 200 psu) on the induction of *β*-carotene in both samples of *D. salina* (Kuwaiti and Australian), at 30ºC using a

temperature-controlled water-bath provided with immersable heaters and with fluorescent daylight to achieve a light intensity of $18x10^3$ lux. The seventh and eighth sets of experiments studied the effect of different salinities (i.e., 100, 150 and 200 psu) on the induction of *β*-carotene in both samples of *D. salina* (Kuwaiti and Australian) at 35ºC. The ninth and tenth sets of experiments studied the effect of different salinities (i.e., 100, 150 and 200 psu) on the induction of *β*-carotene in both samples of *D. salina* (Kuwaiti and Australian) at 38.5ºC. The eleventh, twelfth and thirteenth sets of experiments were designed to compare the effect of three high light intensities (i.e., 18, 25 and $55x10^3$ lux) on the time required to start the *β*-carotene induction in both samples of *D. salina* (i.e., Kuwaiti and Australian). The other stress culture factors applied for these three sets of experiments were as follows: initial culturing salinity of 200 psu; no CO_2 gas; low culturing media concentrations, i.e., at 25% AFMED algal media and the professional Pro-Culture F/2 media (Abu-Rezq *et al.*, 2009); and water bath temperature of 38.5ºC.

β-Carotene Extraction

After reviewing several of the methods that are used for *β*-carotene extraction and given the facilities available at KISR's laboratories, the Pressurized Fluid Extraction (PFE: Dionex® ASE 300) method (Denery *et al.*, 2004) were chosen for this study to extract the total carotene from the prepared *D. salina* samples. The choice of acetone as the solvent for the extraction of *β*-carotene has several advantages, such as easier handling, and lack of contamination in equipment used for PFE. The pure acetone (99.8% pesticide residue analysis grade) used for this task was filtered through a 0.45-µm membrane filter prior to use. All of the glassware and accessories for the ASE 300 were washed with acetone prior to extraction.

Several samples of *D. salina* samples (Kuwaiti and Australian) were produced as a result of the experiments conducted earlier in this study. As the samples were sensitive to light, they were handled under low light conditions using an ice box.

Moisture Analysis: The moisture content was determined on all samples in triplicate according to the American Association of Cereal Chemists method of analysis (AACC, 1990,

Abu-Rezq *et al.*,2009). It was calculated using Equation 1:

$$Moisture\ (\%) = \frac{(Initial\ Weight\ of\ Sample - Dry\ Weight\ of\ Sample)}{Initial\ Weight\ of\ Sample} \times 100$$

(1)

Freeze-Drying: Brown *D. salina* samples (500-ml) were transferred into labeled aluminum trays, covered properlyand kept overnight at -20ºC for freezing. The freeze-dryer (a Vitris Unitop 800L with temperature rise at 5°C min^{-1}) was stabilized and made ready for loading the frozen samples at -40°C for 72 to 96 h.

The alga samples were lyophilized and nitrogen-packed to prevent oxidation.

Spectrophotometric Analysis: A UV-Visible spectrophotometer (i.e., a Shimadzu UV Pharma Spec 1700 connected to a printer) was used to measure the total carotenoids in the acetone extract from the alga samples.

The absorbance at the 452-nm wavelength of the respective samples was used in Equation 2 to determine the total carotenoids in the extract (Handout, Professor Borowitzka, personal communication; Rodriguez-Amaya and M. Kimura, 2004; Abu-Rezq, 2007). $C = A_{452} \times 3.86 \times V_e / V_t$ (2)

where, C = Total carotenoids (mg l^{-1}), A_{452} = absorbance at 452 nm, V_e = volume of extract (ml) and V_t = volume of culture sample (ml).

Qualitative determination of β-carotene by HPLC:

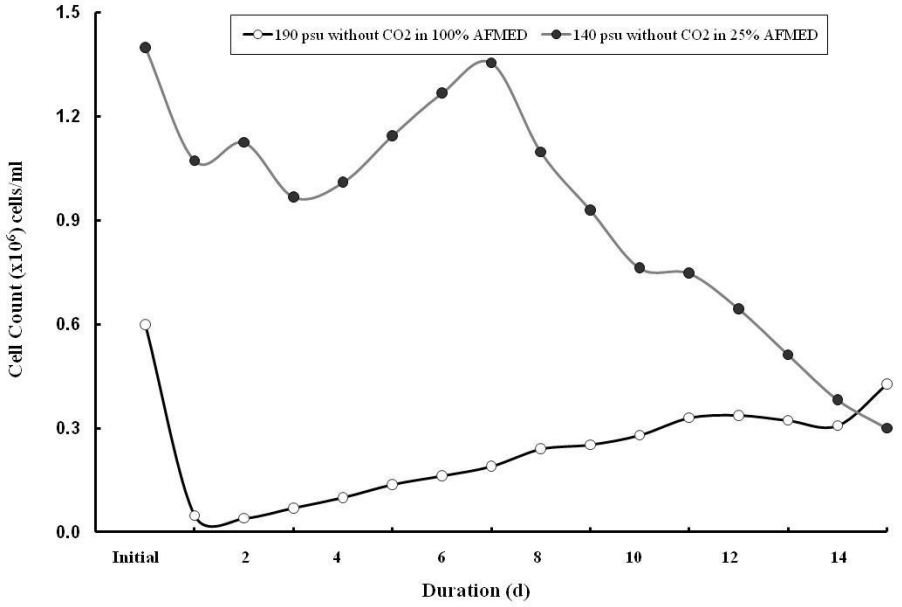
In order to calculate the percentage of *β*-carotene among the total carotenoids obtained from each produced *D. salina* sample, the High Performance Liquid Chromatography (HPLC) was used. HPLC is a form of column chromatography that is used frequently in biochemistry and analytical chemistry to separate, identify and quantify compounds. In HPLC, each sample were run through the HPLC column under specific conditions and the retention time of the peak obtained was compared with that of standard *β*-carotene run under similar conditions. The area under the peaks hence obtained was used to calculate the concentration of *β*-carotene in the sample. Thereby, the percentage of *β*-carotene in each sample was calculated using the results of both UV-Visible spectrophotometry and HPLC.

Results

β-Carotene Induction

The results obtained from the first set of experiments, when *D. salina*

(Kuwaiti) was cultured at $10x10^3$ lux, 140 and 190 psu, different culture media concentrations (25 and 100% AFMED media) and without CO_2 gas, showed that growth performance was not enhanced even with 100% AFMED media. This may indicate that salinity has a stronger effect on the growth performance than does the media concentration (Fig. 1). The results obtained from the second set of experiments, when *D. salina* (Kuwaiti) was cultured at $10x10^3$ lux and different high salinities (50 to 200 psu), showed that the growth performance was better at the lowest salinity of 50 psu than at higher salinity of 200 psu. Meanwhile, changes in the color (from green to yellow and brown) were observed in the *D. salina* (Kuwaiti) cultured at higher salinities. This may indicate that at high salinity, *β*-carotene induction was initiated (Fig. 2). The results obtained from the third set of experiments, in which *D. salina* (Kuwaiti) was cultured at a high light intensity ($18x10^3$ lux) and different salinities (50 to 250 psu), showed that growth performance was better at the lowest salinity (i.e., 50 psu) with cell densities of up to $4.59x10^6$ cells/ml, than that at the highest salinity (250 psu) with cell densities of up to $1.14x10^6$ cells/ml. In general, the growth performance of *D. salina* (Kuwaiti) at this light intensity (i.e., $18x10^3$ lux) under the different salinity regimes was higher than those at the lower light intensity (i.e., $10x10^3$ lux) used in the second set of experiments. Meanwhile, as in the second set of experiments, the color of the *D. salina* cultures changed from green to yellow and brown at higher salinities



AFMED = Aquaculture, Fisheries and Marine Environmental Department

Fig. 1. Performance of *Dunaliella salina* (Kuwaiti) cultured at different high salinities, with different culture media concentrations, and without CO_2 gas.

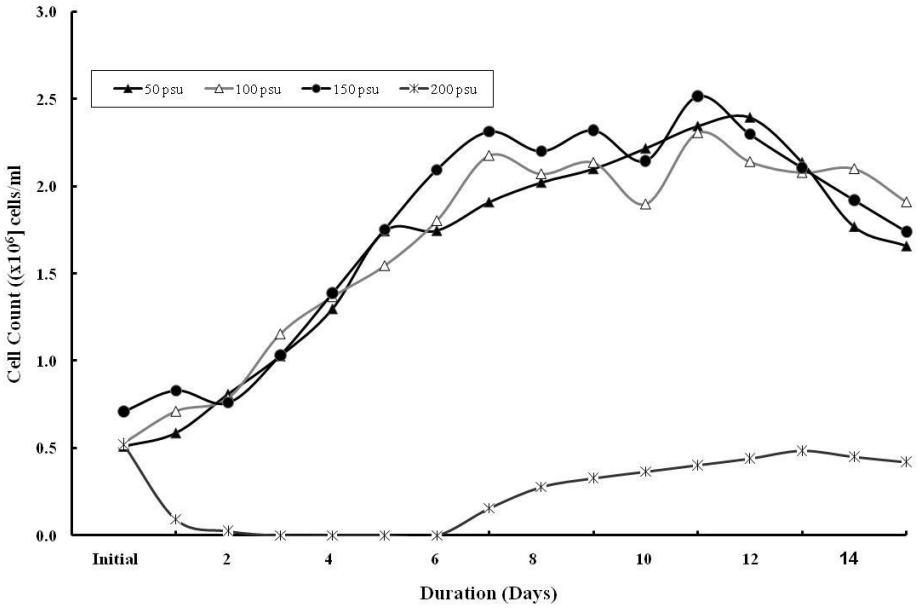


Fig. 2. Performance of *Dunaliella salina* (Kuwaiti) cultured at $10x10^3$ lux and at different high salinities (50 to 200 psu).

(i.e., at 200 and 250 psu). This may indicate that at higher salinity, *β*-carotene induction was initiated (Fig. 3). A further investigation for this

change in color was examined later in this study during *β*-carotene extraction procedures.

The results obtained from the fourth set of experiments, in which *D. salina* (Australian) was cultured at a high light intensity ($18x10^3$ lux) and at different salinities salinity of 50 psu with cell densities of up to $4.49x10^6$ cells ml^{-1}, compared with that at the highest salinity of 250 psu, with cell densities of $0.63x10^6$ cells ml^{-1}. Again, changes in color (to yellow and brown) were observed for cultures subjected to higher salinities as obtained in the third set of experiment (Fig. 4).

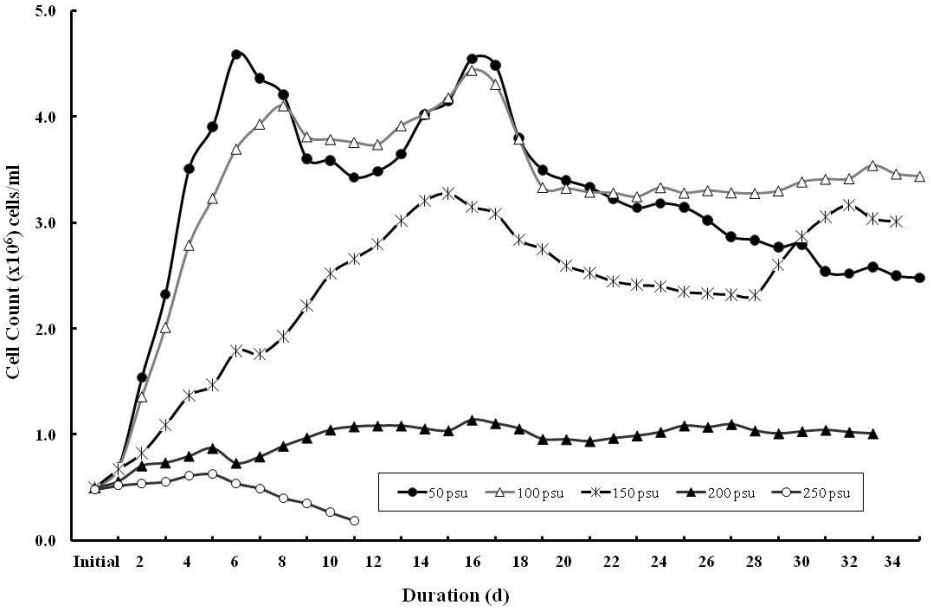


Fig. 3. Performance of *Dunaliella salina* (Kuwaiti) cultured at $18x10^3$ lux and at different high salinities (50 to 250 psu).

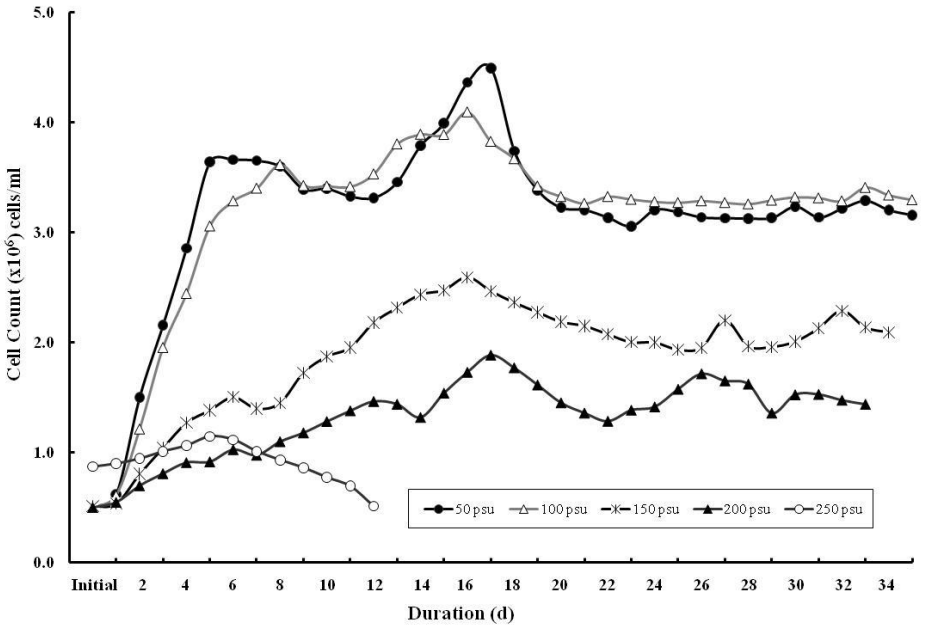


Fig. 4. Performance of *Dunaliella salina* (Australian) cultured at 18x10^3 lux and at different high salinities (50 to 250 psu).

The results obtained from the fifth and sixth sets of experiments regarding the effect of different salinities (i.e., 100, 150 and 200 psu) on the induction of *β*-carotene in *D. salina* (Kuwaiti and Australian) at 30ºC showed that the two samples of this species tended to change cell color from green to brown after 24 to 25 d of culturing when cultured at a higher salinity starting from 200 psu. This change in color may indicate that *β*-carotene induction had started (Figs. 5 and 6).

The results obtained from the seventh and eighth sets of experiments regarding the effect of different salinities (100, 150 and 200 psu) on the induction of *β*-carotene in *D. salina* (Kuwaiti and Australian) at 35ºC showed that the two samples of this species tended to change cell color from green to brown after 17 to 18 d of culturing when they were also cultured at the higher salinity starting from 200 psu (Figs. 7 and 8).

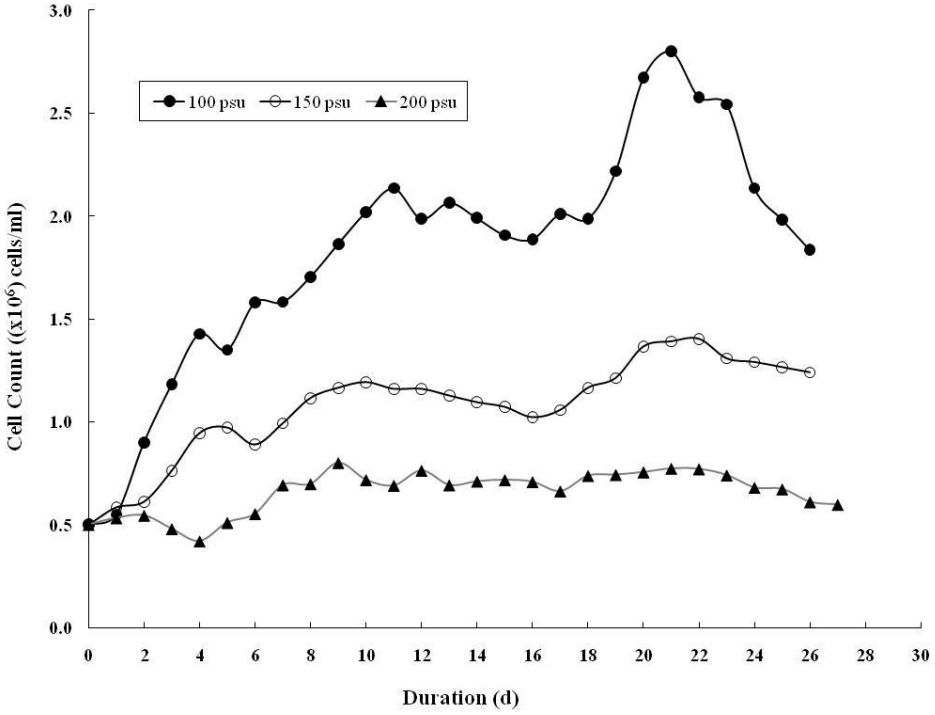


Fig 5. Performance of *Dunaliella salina* (Kuwaiti) cultured at different salinities at 30°C.

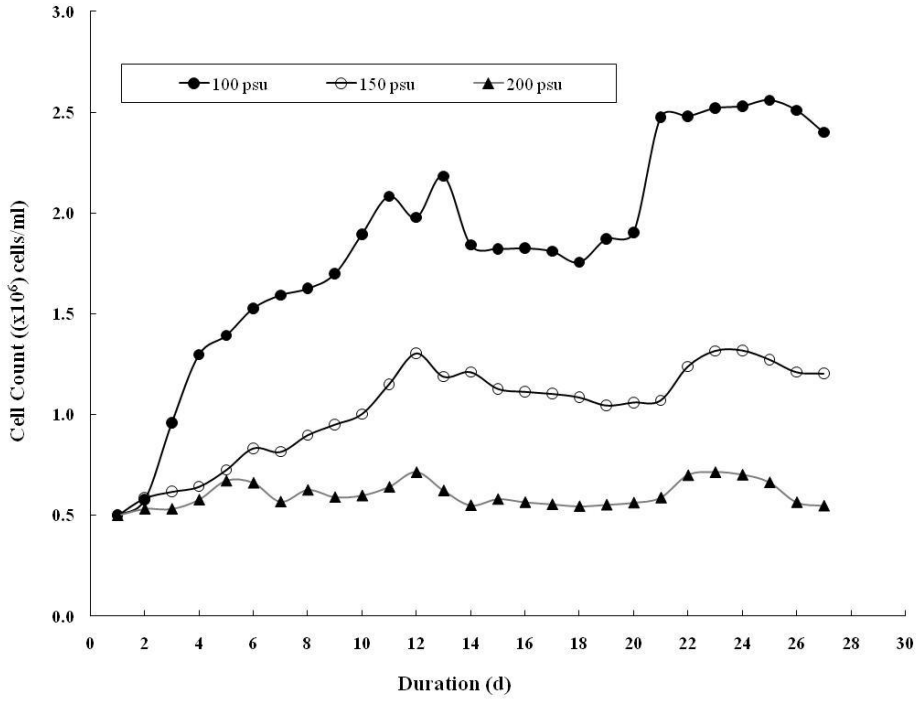


Fig 6. Performance of *Dunaliella salina* (Australian) cultured at different salinities at 30°C.

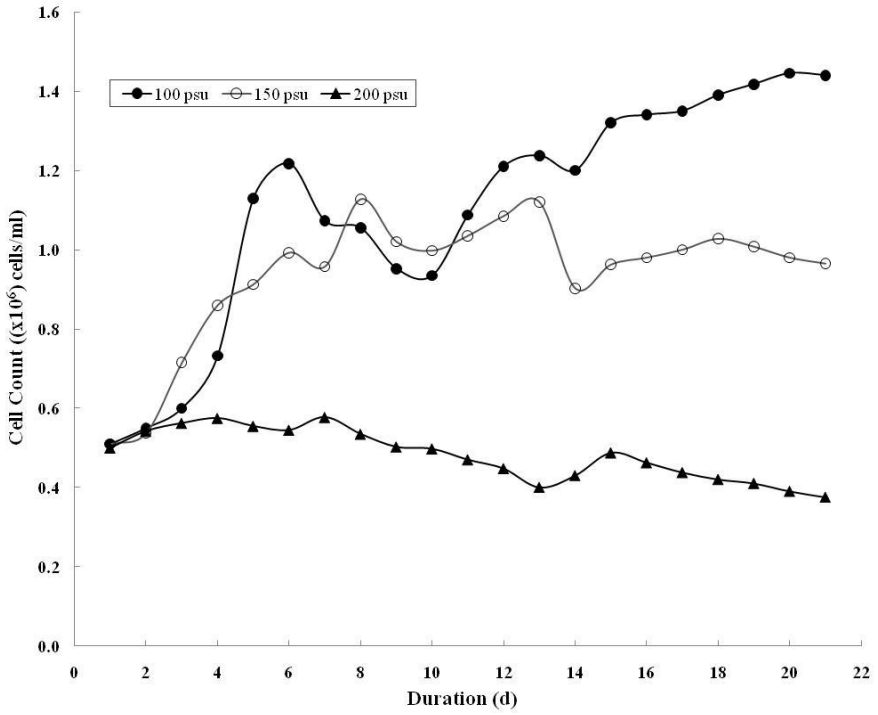


Fig. 7. Performance of *Dunaliella salina* (Kuwaiti) cultured at different salinities at 35°C.

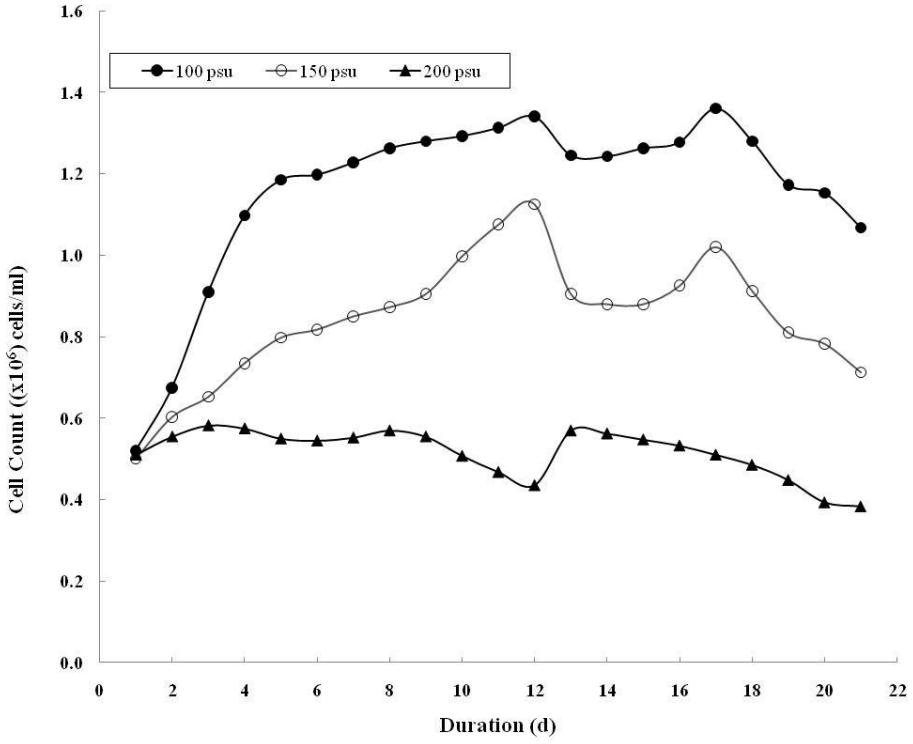


Fig. 8. Performance of *Dunaliella salina* (Australian) cultured at different salinities at 35°C.

Similarly, the results obtained from the ninth and tenth sets of experiments on the induction of *β*-carotene in *D. salina* (Kuwaiti and Australian, respectively) at 38.5ºC showed that the two samples of this species tended to change cell color from green to brown after 14 to 15 d of culture when they were also cultured at high salinity starting from 200 psu (Figs. 9 and 10). Samples of the brown *D. salina* were sent to KISR's laboratories to prepare for the *β*-carotene extraction trials.

The eleventh, twelfth and thirteenth sets of experiments, which studied the effect of three high light intensities and two culture media (18, 25 and 55x10^3 lux, and AFMED and F/2) on the time required to start the *β*-carotene induction in both *D. salina* samples showed that regardless of the culture media used, at 18x10^3 lux, the brown color (indicating *β*-carotene induction) started after 15 to 16 d of culturing. This period was reduced to 10 or 11 d when culturing was conducting at a higher light

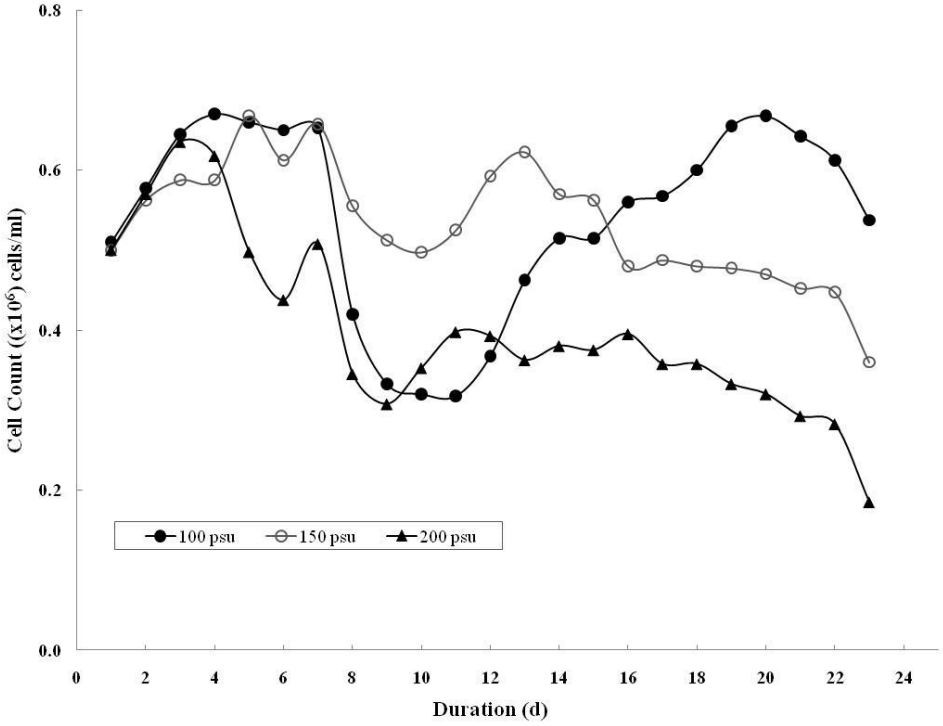


Fig. 9. Performance of *Dunaliella salina* (Kuwaiti) cultured at different salinities at 38.5ºC.

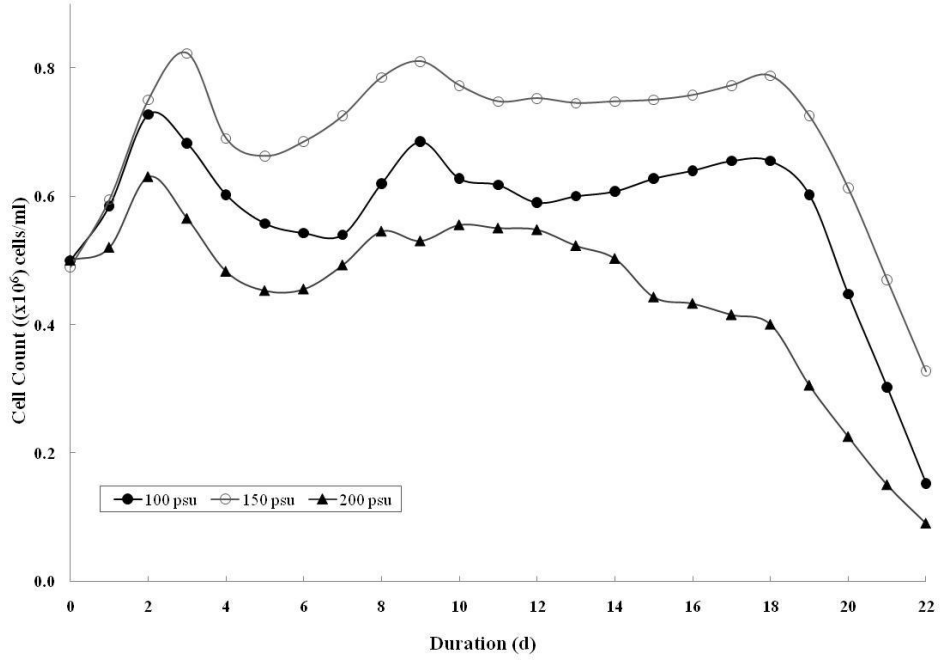
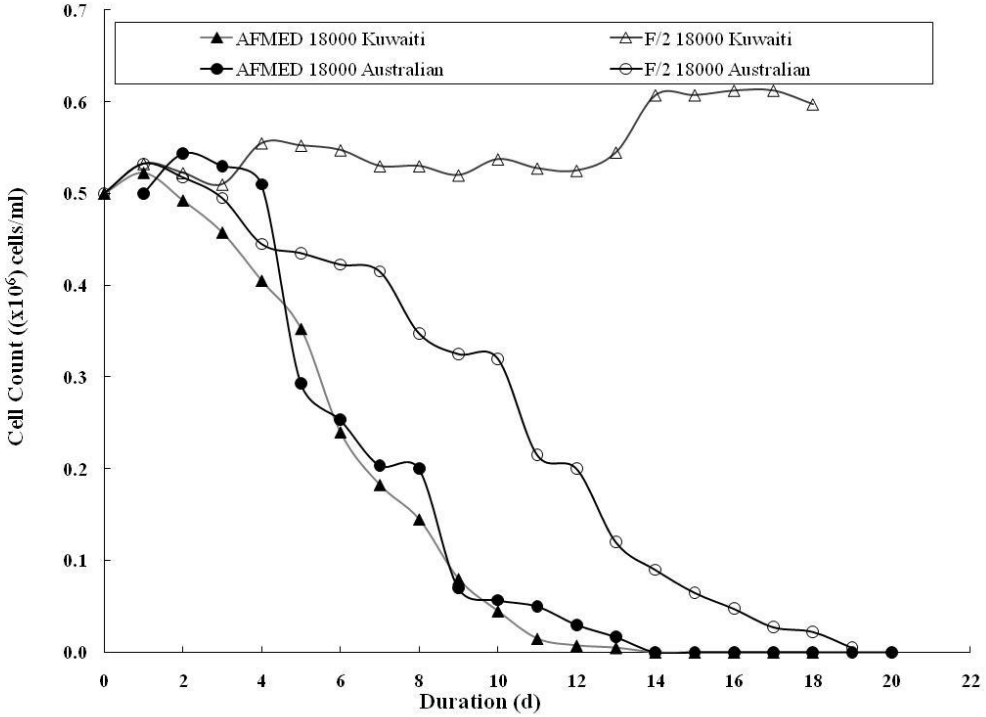


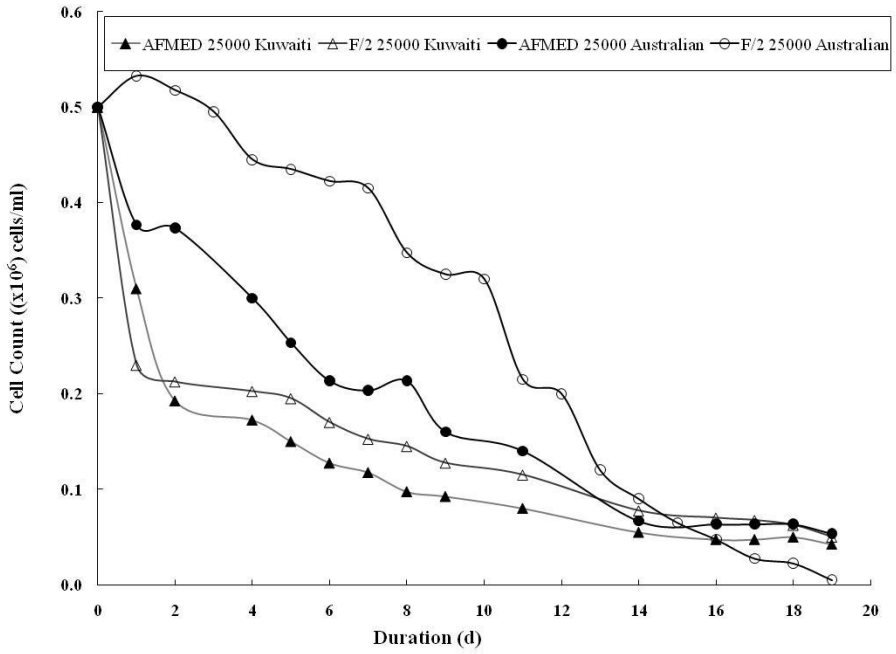
Fig. 10. Performance of *Dunaliella salina* (Australian) cultured at different salinities at 38.5°C.

intensity ($25x10^3$ lux), and it was further reduced to 5 or 6 d, when culturing was conducted at $55x10^3$ lux, i.e., when the salinity was >250 psu (Figs. 11 through 13). These results showed that both culture media (AFMED and F/2 media) can be used for *β*-carotene induction.



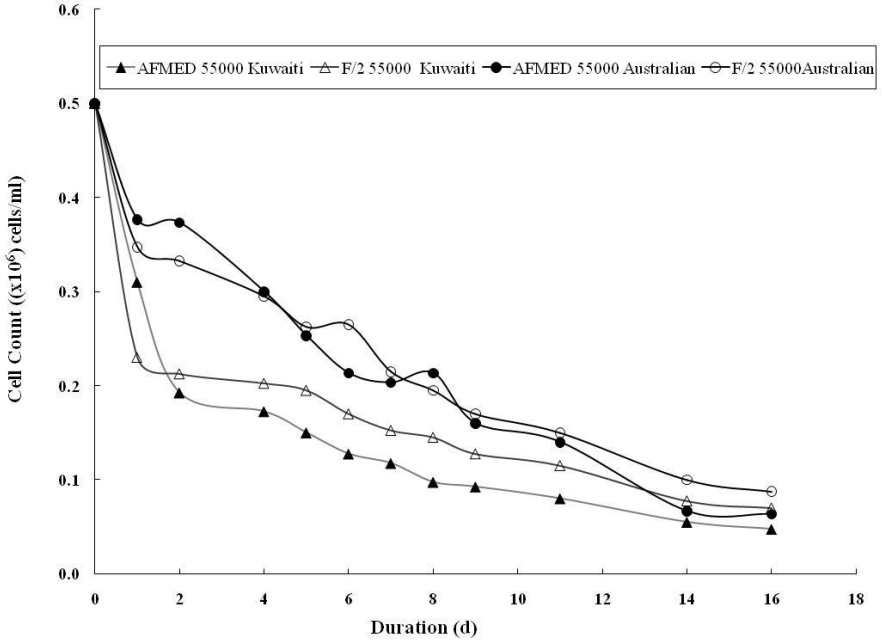
AFMED = Aquaculture, Fisheries and Marine Environmental Department

Fig. 11. Performance of *Dunaliella salina* (Kuwaiti and Australian) at 18x10^3 lux.



AFMED = Aquaculture, Fisheries and Marine Environmental Department

Fig. 12. Performance of *Dunaliella salina* (Kuwaiti and Australian) at 25x10^{3}lux.



AFMED = Aquaculture, Fisheries and Marine Environmental Department

Fig. 13. Performance of *Dunaliella salina* (Kuwaiti and Australian) at 55x10^3 lux.

β-Carotene Extraction

The moisture analysis done showed that the moisture content of both samples were almost equal ($p>0.05$), i.e., for the *D. salina* (Kuwaiti) sample, it was 72.14±0.17%, and for the *D. salina* (Australian) sample, it was 72.55±0.34%. Furthermore, the dry weight for the two tested samples, obtained from 550 ml from the original culture, after being dried in the freeze-dryer with no significant difference between them ($p>0.05$). They were as follows: for the *D. salina* (Kuwaiti) sample, the dry weight was 182.22 g and for the *D. salina* (Australian) sample, the dry weight was 179.50 g.

The results from the UV-Visible spectrophotometric analysis showed that the maximum absorbance for samples of both Kuwaiti and Australian samples was in at a wavelength of 452 nm, which is characteristic of *β*-carotene expressed as total carotenoids (Fig. 14).

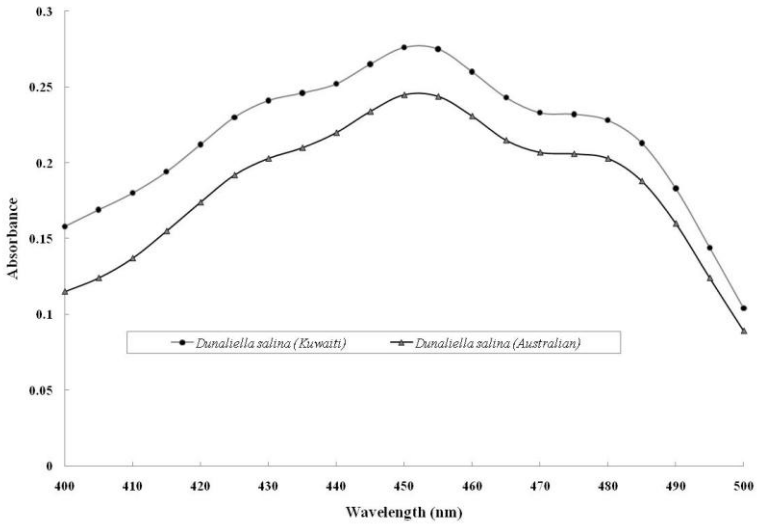


Fig. 14. Wavelength absorbance curve as obtained from the spectrophotometer.

Using Equation 2 (mentioned before), the total carotenoid contents were calculated for each sample. The total carotenoids extracted from the *D. salina* (Kuwaiti) sample was 4.71 mg l^{-1}. Considering that the cell density of the sample tested was $0.13x10^6$ cells ml^{-1}, the concentration of total carotenoids per cell of *D. salina* (Kuwaiti) can be expressed as $(4.71x10^{-3}$ g $l^{-1}) \div (0.13x10^9$ cells $l^{-1})$, which

$= (4.71x10^{-3}$ g $l^{-1}) / (130x10^6$ cells $l^{-1})$

$= 0.03619x10^{-9}$ g $cell^{-1}$

$= 36.19x10^{-12}$ g $cell^{-1}$

$= 36.19$ pg $cell^{-1}$.

Similarly, the concentration of total carotenoids per cell of *D. salina* (Australian) can be expressed as $(5.06x10^{-3}$ g $l^{-1}) / (0.15x10^9$ cells $l^{-1})$, which is

$= (5.06x10^{-3}$ g $l^{-1}) / (150x10^6$ cells $l^{-1})$

$= 0.03375x10^{-9}$ g $cell^{-1}$

$= 33.75x10^{-12}$ g $cell^{-1}$

$= 33.75$ pg $cell^{-1}$.

These amounts of total carotenoids obtained from the samples of *D. salina* (Kuwaiti and Australian) cultured, induced, prepared and extracted at KISR, concur with the results recorded by several other researchers who reported that *β*-carotene is accumulated in oil droplets inside the cells of *D. salina* under stress conditions up to the concentration of 10 to 12% of the sample's dry weight. The total carotenoid contents of other sets of

samples sent to KISR's laboratories, for *β*-carotene extraction demonstrated that the total carotenoid contents in the green samples (in the growth phase) for both samples of *D. salina* (Kuwaiti and Australian) were very low, i.e., 2.24-5.48 pg cell^{-1}, and 3.75-5.0 pg cell^{-1}, respectively ($p>0.05$). At the same time, the total carotenoid contents of the red, i.e., stressed, samples ranged between 47.20-84.59 pg cell^{-1} and 38.3-96.47 pg cell^{-1}, respectively, regardless of the algal culture media used.

Qualitative determination of β-carotene by HPLC

The HPLC calculation performed was to express the concentration of *β*-carotene in the acetone-extracted sample. For example, the *β*-carotene calculation for the red *D. salina* (Kuwaiti) sample cultured with F/2 algal media was conducted as follows. The dry extract was dissolved in 2 ml of the mobile phase used for HPLC before injection. Since the concentration of *β*-Carotene = 36.7 mg l^{-1}, it is equivalent to express it as 36.7x10^{-3} mg ml^{-1}. Since the sample had been dissolved in 2 ml of the mobile phase, the sample contained (36.7x10^{-3} x2) mg of *β*-carotene i.e., 73.4x10^{-3} mg of *β*-carotene.

Since the weight of the freeze-dried sample taken for extraction was 2.02 g, the amount of *β*-carotene in 2.02 g of freeze-dried sample was, therefore, 73.4x10^{-3} mg. In addition, the amount of *β*-carotene in 1 g, as well as in 1 kg of freeze-dried sample was determined mathematically.

$$\frac{73.4X\,10^{-3}\ \text{mg}\ \beta-\text{Carotene}}{2.0151\ \text{g of Freeze}-\text{Dried Sample}} = \frac{36.42\ X\ 10^{-3}\ \text{mg}\ \beta-\text{Carotene}}{\text{g of Freeze}-\text{Dried Sample}} \quad (3)$$

Therefore, 1 g of freeze-dried red *D. salina* (Kuwaiti) sample cultured with F/2 media contained 36.42x10^{-3} mg of *β*-carotene. So 1 kg of freeze-dried sample contained 36.42 mg of *β*-carotene. Therefore, the percentage of *β*-carotene in this sample calculated by Equation 4.

$$\%\ \beta\text{-carotene} = \frac{\beta-\text{carotene mg/kg}}{\text{Total Carotenoids mg/kg}}\ x100 \quad (4)$$

$$= \frac{36.42}{40.57}\ x100$$

$$= 89.77\ \%$$

Similarly, calculations showed that 1 kg of freeze-dried *D. salina* (Australian) cultured with AFMED media contained 52.596 mg of *β*-carotene and the percentage of *β*-carotene was found to be 65.66 %.

Discussion

β-Carotene Induction and Extraction

The encouraging results obtained from this study showed that, it was possible to maintain pure cultures of both samples (the locally isolated *D. salina* and the *D. salina* sample brought from Australia) at KISR laboratories. Results also indicated that the two samples of this species tended to change their cell color from green to yellow, or brown under certain indoor controlled culture condition. This change in color indicated that *β*-carotene induction was initiated. The optimum environment condition for *β*-carotene induction under laboratory conditions for the two samples was while using high salinity (200-250 psu); high temperature (38.5ºC); high light intensity ($55x10^3$ lux) and at low culture media concentrations (25% of either the AFMED or F/2 media).

The results obtained from the experiments regarding the effect of different salinities (100, 150 and 200 psu) on the induction of *β*-carotene in *D. salina* (Kuwaiti and Australian) at 30ºC showed that the two samples of this species tended to change cell color from green to yellow or brown after 24 to 25 d of culture, when they were cultured at the high salinity range of 200 to 250 psu. Moreover, the time required for this changes in colour became shorter when the culture temperature was increased (i.e., at 38.5ºC, the required time was 5-6 days). This change in color may have indicated that *β*-carotene induction had started.

The results obtained on the effect of the three high light intensities and two culture media (18, 25 and $55x10^3$ lux, and at low AFMED and F/2 media concentrations) on the time required for induction of *β*-carotene to begin in both *D. salina* samples (Kuwaiti and Australian) showed an inverse relationship between light intensity and the time required for *β*-carotene induction to be initiated, regardless of the culture media used. These results demonstrated that both culture media (AFMED and F/2) can be used for *β*-carotene induction under the experimental culturing conditions used in this study.

The obtained results agreed with that concept which is generally accepted that *D. salina* synthesizes large quantities of *β*-carotene when subjected to certain stress factors, which is evidenced by the orange color acquired by these algae (Leon *et al.*, 2003). Ben-Amotz (1987); Borowitzka *et al.*,(1990); Gomez-Pinchetti *et*

al.,(1992); Hejazi and Wijffels (2003); Bosma and Wijffels (2003), Leon *et al.*,(2003); and Ye *et al.*,(2008) reported that these factors (i.e., high light intensity, nutrient limitation, high salinity, high temperature and high pH) can be applied separately or in combinations of two to induce *β*-carotene formation. Sarmad *et al.*,(2006) and Hosseini and Shariati (2006), who conducted their experiments on four and three samples of *D. salina*, respectively, reported that the effect of salinity on *β*-carotene accumulation per cell was sample dependent.

The results obtained from the UV-Visible spectrophotometric analysis showed that the maximum absorbance for both samples (Kuwaiti and Australian) was at a wavelength of 452 nm, which is characteristic of *β*-carotene expressed as total carotenoids.

During this study a Dionex® ASE 300 available at KISR's laboratories was utilized for the PFE with acetone of total carotenoids from prepared, dry *D. salina* samples. Similarly, Denery *et al.*,(2004) conducted experiments on carotenoid extraction from *Haematococcus pluvialis* and *D. salina* using PFE. Based on the results, they concluded that PFE produced comparable extraction efficiencies to traditional extraction techniques. On the other hand, Hejazi and Wijffels (2004), discussed milking of microalgae, i.e., a new method developed for milking *β*-carotene from *D. salina* grown in a two-phase bioreactor. In their technique, cells were grown first under normal growth conditions then stressed by excess light to produce *β*-carotene. The second step was started by adding biocompatible organic phase to extract *β*-carotene via continuous re-circulation of biocompatible organic solvent. Therefore, the cells were continuously reused and do not need to be grown again. But, the use of milking technique requires understanding the mechanisms involved in at a cellular level.

β-carotene can be also extracted from sources other than *D. salina*. Choo *et al.*,(1991) reported different method for *β*-carotene extraction from palm oil. It involves selective adsorption of carotenoids from alkyl esters in an open column. High recovery of carotenoids (> 90 %) could be obtained, and the column could be used over 50 times without any loss of activity. At the same time, for *β*-carotene extraction from palm oil Choo *et al.*,(1992) and Ooi *et al.*,(1991) reported different methods that involve

distillation for successful production of carotenoids at a concentration of >80,000 ppm from their pilot study. A higher carotene-enriched concentration (>90%) was also discussed by further subjecting the carotenoid concentrate, through column chromatography (Ooi *et al.,* 1994). Mendes *et al.,*(2003) reported another method, supercritical carbon dioxide, for *β*-carotene extraction from different algal species (*Botryococcus braunii*, *Chlorella vulgaris*, *D. salina* and *Arthrospira maxima*). Their method was carried out in a flow apparatus at temperatures between 313.1 and 333.1 K and pressures up to 35.0 MPa.

Results also indicated that the pressurized fluid extraction (PFE) method for total carotenoids extraction and the percentage of the *β*-carotene in that total carotenoids extracted using the High performance liquid chromatography (HPLC) for small scale *β*-carotene extraction was effective and suitable under laboratory conditions. These results showed that both the *D. salina* samples (locally isolated and from Australia) contained relatively good amount of total carotene contents (36.19 - 84.59 pg $cell^{-1}$ and 33.75 - 96.47 pg $cell^{-1}$, respectively, Abu-Rezq et al, 2009).

The obtained results were higher compared with that of Cifuentes et al (1992), they reported that low carotenoids content (13.7 mg l^{-1}, 8.1 pg $cell^{-1}$) could be increased under enriched sea water to 49 pg $cell^{-1}$ (A.S. Cifuentes, unpublished results). Ben-Amotz, 2004 recorded that the total harvest of 3-9 kg *β*-carotene can be produced from shallow water pond of 20 cm on an area of 300 m^2 (600 m^3).

Hejazi *et al.,* (2002) concluded that *β*-carotene of high purity can be produced from green active *D. salina* by using biocompatible solvents. Recently, Hejazi *et al.,*(2003); Hejazi and Wijffels (2004); Hejazi *et al.,*(2004) developed a new method for milking *β*-carotene from *D. salina* efficiently and continuously in a two-phase bioreactor.

Conclusions

Under the conditions of this study, it was possible to establish and maintain pure cultures of *D. salina* from both Kuwait and Australia at KISR's laboratories. The results of the experiments conducted related to stress factors required for *β*-carotene induction, showed that both the samples of this species tended to change color from green to yellow or brown under certain culturing conditions such as: high salinity (200

to 250 psu), high temperature (38.5ºC), high light intensity ($55x10^3$ lux) and low culture media concentrations, i.e., at 25% of either the AFMED or F/2 media.

Positive results in the trials performed related to *β*-carotene extraction indicated clearly that this change in color marked the initiation of *β*-carotene induction. In addition, the PFE of carotenoids used in this research was suitable for extracting *β*-carotene under laboratory conditions. The results also showed that both of the *D. salina* samples (Kuwaiti and Australian) contained relatively good amounts of *β*-carotene.

Due to the encouraging results obtained in this study, it is recommended that a future pilot scale production of *D. salina* culture using outdoor raceways to be conducted as soon as possible.

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