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Optimum culture conditions required for the locally isolated *Dunaliella salina*

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Key words: salinity, temperature, phosphate, light intensity, pH regimes, algal media.

Abstract

Bloom of *Dunaliella salina* was observed naturally in stagnant puddles of water off Kuwait's Bubiyan Island on 4 July 2004. In order to closely monitor this bloom, alga were collected and maintained as pure culture of the locally isolated *D. salina*. Set of experiments was conducted to study and evaluate the optimum culture conditions required for two samples of *D. salina* (one locally isolated and the other sample brought from Perth, Australia) to achieve the highest growth performance.

The results of the experiments showed that both samples of *D. salina* preferred high salinities (45 psu), low temperatures (20ºC), phosphate concentrations of 30 g m^{-3} d^{-3}, high light intensities ($18x10^3$ lux), and high pH levels of up to 9.18 (without using CO_2 gas), for their optimum growth.

Introduction

Recently, an increasing interest in microalgal biotechnology has emerged due to the variety of biotechnologically interesting compounds that microalgae can synthesize. There is a tremendous potential for microalgae because they can produce anything from foods and feeds to pharmaceutical products. Following the success of *Spirulina* production in the early 1970s, the Cyanotech Corporation was founded in 1983, to produce *Spirulina*, the nutrient-rich dietary supplement, and *Haematococcus*, from which astaxanthin is produced, which is used as a powerful antioxidant with expanding applications as a human nutraceutical, as well as in the aquaculture market and animal feed industries.

The use of *Dunaliella* algal species for aquacultural purposes as a source of *β*-carotene was discussed by Al-Abdul-Elah et al. 1999. They reported, during their technical visit to the Gold Coast Marine Aquaculture hatchery for the black tiger prawn in Australia, that the use of *Dunaliella* would provide the desired pigmentation for farmed prawns, especially two to three days before harvesting. This period is sufficient for the prawn to gain a health red color and better taste, for better marketing and to fetch a higher price.

Dunaliella salina, which is a dinoflagellated green marine algal species (preferred to be called biflagellated by Professor Borowitzka, personal communication) belonging to the class Chlorophyceae (Borowitzka 1990; Avron and Ben-Amotz 1992; Leach et al. 1998) can live in high concentrations of saturated brine (Ahmed et al. 2001; Bhatnagar and Bhatnagar 2005). The *Dunaliella* cell is mainly characterized by a wide basal side with a narrow anterior flagella top. To reproduce, each cell slowly divides lengthwise into two cells. The cell itself consists of one large chloroplast with a single-centered starch surrounded by pyrenoid, a few vacuoles, a nucleus and a nucleolus. Cells of *Dunaliella* are enclosed by a thin elastic plasma membrane covered by a mucous surface coat which permits rapid changes in cell volume 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 *D. salina* is recognized at present as the most salt-tolerant eukaryote known.

Collection and Isolation of pure local sample:

On 4 July 2004, a bloom of *D. salina* was observed in stagnant puddles of water off Kuwait's Bubiyan Island and another bloom with a 1 km x 0.5 km patch size, was also observed in the stagnant land-locked waters of Sulaibikhat Bay (Kuwait Bay) adjacent to the Kuwait Institute for Scientific Research's (KISR)'s head office in Shuwaikh, on 18 and 19 October 2004, and samples were collected. It was observed that there were many flamingoes, other birds, and feeding pelagic fish in the area of the bloom. The samples were submitted to Dr. Faiza Al-Yamani to be identified by her group. They compared Kuwait's *D. salina* with the sample of *D. salina* brought by the Senior author from Professor Borowitzka. Using the published description of this alga (Borowitzka and Siva 2007) and the comparison of samples from the locations, the Kuwait species was confirmed *D. salina*.

The concentration of chlorophyll in the red patch was >120 µg l^{-1}, and the density of cells was >1x10^6 cells l^{-1}. The salinity of the waters was 38.3 psu. The chlorophyll concentration in the first *D. salina* bloom (i.e., 4 July 2004) was 64 µg l^{-1}. When *D. salina* blooms, usually under high light intensity, high salinity and low nitrogen concentration, it produces *β*-carotene in such a large quantity that the water becomes red in color (Dr. Faiza Al-Yamani, personal communication).

Previous local studies conducted:

Previously, Al-Hasan and Sallal (1985) had conducted preliminary studies on the halotolerant alga, *Dunaliella*, from Kuwait's salt marshes in the Khiran area. They observed that a red form of the *Dunaliella* sp. was found under extremely high light intensity (1700 µE m^{-2} s^{-1}, i.e., 150x10^3 lux), temperature (50°C) and salinity (160 psu). Samples of this red alga were transferred to their laboratory, where they were cultured in a 100-rpm shaker incubator, with constant illumination at 61 µE m^{-2} s^{-1}, i.e., 4x10^3 lux, at 30°C), the algal color changed from red to green. Later on, after applying various growth stress, such as increased light intensities, nutrient deficiencies or high salinities, the algal color changed from

green to red due to a decrease in chlorophyll content of the algal cells and an increase in the *β*-carotene content. Al-Hasan et al. (1987) also reported correlative changes in the growth, pigmentation and lipid composition of *D. salina* in response to halostress. Other researchers such as Sallal et al. (1987) worked on the localization of glycollate dehydrogenase in *D. salina*. Nimer et al. (1990) identified the presence of glycollate oxidize and dehydrogenase in *Dunaliella primolecta*.

The objective of this research study was to develop a procedure to mass culture locally isolated *D. salina*, as a step towards enhancing *β*-carotene induction, and then for *β*-carotene extraction. This objective was achieved by evaluating the optimum growth of locally isolated *D. salina* under different salinities, temperatures, light intensities, pH regimes and phosphate concentrations.

At the completion of this research study, new areas of research were developed into plans and developed procedures for *β*-carotene induction and extraction from the locally cultured *D. salina* is in progress.

Materials and Methods

An Innova 4900 Multiple Shaker Environmental Chamber shaker incubator, provided with controlled temperature and light intensity, was used during this period to maintain pure seeds of both strains of *D. salina* (i.e., the locally isolated strain and the strain brought from Australia). 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 provided with sufficient air stones and a standing frame made of stainless steel was also used to carry out all the proposed experiments. Furthermore, glass tubing of different sizes; poly vinyl chloride (PVC) fittings; AFMED algal culture media (Table 1); electrically heated CO_2 regulators (to control culture media's pH level) and natural seawater were also used.

Since that the *D. salina* species tolerance higher salinities, this had facilitated the isolation and purification of the two samples (i.e., Kuwaiti and Australian) of this species. Isolation was carried out using routine dilution methods followed by pipetting under the microscope. The isolated *D. salina* were sampled and maintained as a pure culture at salinity of 120 psu using the procedures performed in the algal growth room at KISR's AFMED, which are used to maintain and mass

produce other marine algal species (Abu-Rezq et al., 1999; James et al., 1988, 1989). In general, the cultures were inoculated under controlled conditions to avoid any possible contamination. After a few days, the cultures were examined under the microscope, and it was observed that the desired species dominated the cultures. The cultures were subcultured again and again using the same process until monospecific cultures were obtained.

Table 1. AFMED Algal Culture Medium Used for D. salina *Seed Maintenance and Production*

Ingredient	Quantity ($g\ m^{-3}\ d^{-3}$)
Urea	10
$CaHPO_4$	5
KNO_4	5
Clewat – 32*	3

*Contents in 1 kg: 3.8 g of iron, 7.7 g of manganese, 0.07 g of copper, 6.3 g of molybdenum, 24.7 g of boron, 0.17 g of cobalt, and 0.17 g of Ethylenediaminetetraacetic acid (EDTA).

The usual technique for microalgal production at KISR's AFMED, has involved a multistep backup system, whereby small-scale cultures are grown and used to inoculate larger scale cultures, which are used as inoculates for even larger ones, with the final culture unit being large, indoor, transparent tubes. The algal production procedures used in this study are described in detail in Abu-Rezq et al. (1999, 2002).

Axenic stock cultures of *D. salina* (Kuwaiti and Australian) were maintained in the seed room, which is equipped with cooling chillers to maintain the temperature at 19 to 21ºC and fluorescent light intensity at $10x10^3$ lux, which is optimal for algal cell production. To maintain and subculture these cultures, nutrients (AFMED algal media, Table 1) and filtered seawater were sterilized at 121ºC using an autoclave. Manufactured by Teikoku Sangyo Co. Ltd. Japan, and supplied by Nippon Trading Co. Ltd., Osaka, Japan.

The salinity of the cultures was maintained at 120 psu, and the algal pH was monitored by chemi-monitors (model 872, Foxboro Inc., US). The controller activated a solenoid valve on a pressurized CO_2-enriched line when a desired pH was exceeded, bubbled gas mixed with compressed air (5% CO_2) then entered the culture through a port at the base of the culture, lowering the pH to the designated.

All of the conducted experiments were on a small scale, in vitro, simulating the actual conditions in the sea. Aliquots from each flask were collected and counted every morning. The algal cell densities were monitored daily using an Improved Neubauer Haemocytometer (Bright-line). Four replicates in 500-ml flasks were utilized for each treatment tested. The algal culture media utilized was the same as applied by James et al. (1986). For all of the flasks, brackish water for the culture media was sterilized by autoclave at 121°C. Prior to the experiment, *D. salina* was adapted to the different salinity regimes required. All of the flasks were exposed 24 hrs to $10x10^3$ lux fluorescent day-light.

The first set of experiments was initiated to study the growth rate of the two samples of *D. salina* (Kuwaiti and Australian) at salinities ranging from 25 to 45 psu at increments of 5 psu. During the experiments to determine the optimum salinity for the highest growth of *D. salina*, the temperature was maintained at 21 to 22°C, which is the algal seed room's normal temperature.

The second set of experiments was carried out to examine the growth rate of *D. salina* (Kuwaiti and Australian) cultured at different temperatures (i.e., 20, 23, 26, 29 and 32ºC).

The third set of experiments studied the growth rate of *D. salina* (Kuwaiti and Australian) cultured at different light intensities (10 and $18x10^3$ lux) providing the

optimum salinity and temperature obtained from the previous experiments.

The fourth set of experiments was aimed to study the growth rate of *D. salina* (Kuwaiti and Australian) cultured at high light intensity ($18x10^3$ lux) with and without injecting CO_2 gas through aeration to the culture media to study the effect of two pH regimes. The pH of the treatments which were exposed to CO_2 gas, was maintained at 6.75-7.25 range using the pH controllers. After the completion of the last four sets of experiments, and getting similar trends of results for salinity, temperature, light intensity and pH level, the following set of experiments was conducted for *D. salina* (Kuwaiti) sample only.

The fifth set of experiments was aimed to study the growth rate of *D. salina* (Kuwaiti and Australian) cultured at high light intensity ($18x10^3$ lux) with two algal culture media (i.e., AFMED algal culture media and the professional Pro-Culture F/2 media (Part A and B, Kent Marine, US, Tables 2 and 3). After the completion of the last five sets of experiments, and getting similar trends of results for salinity, temperature, light intensity, algal culture media and pH level, the following set of experiments was conducted for *D. salina* (Kuwaiti) sample only.

Table 2: Pro-Culture F/2 Media, Part A*

Ingredient	Quantity (%)
Available nitrogen	0.0000
Available phosphate P_2O_5	0.0000
Soluble Potash (K_2O)	0.0000
Iron (minimum)	0.4000
Manganese (minimum)	0.0340
Cobalt (minimum)	0.0020

Zinc (minimum)	0.0037
Copper (minimum)	0.0017
Molybdenum (minimum)	0.0009

* Sources of nutrients are Iron EDTA, manganese EDTA, sodium EDTA, Cobalt chloride, zinc EDTA, copper EDTA, sodium molybdate.

Table 3: Pro-Culture F/2 Media, Part B**

Ingredient	Quantity (%)
Available nitrogen	15.0
Available phosphate (P_2O_5)	2.0
Soluble potash (K_2O)	0.0
Vitamin B_1	0.07
Vitamin B_{12}	0.0002
Biotin	0.0002

** Sources of nutrients are Monosodium phosphate, Thiamine hydrochloride (vitamin B_1), Vitamin B_{12}, Biotin.

The sixth set of experiments examined the effect of different nutritional factors on the growth of *D. salina* (Kuwaiti) providing that the optimum salinity and temperature obtained from the previous sets of experiments were applied. In this set of experiments, the effect of three phosphate concentrations (i.e., 7.5, 15 and 30 g m^{-3} d^{-3} as recommended by James et al., 1986) was studied. Each set of the conducted experiments was terminated after two weeks of growth period.

Results

The results obtained from the first set of experiments regarding the daily cell counts of both samples of *D. salina* (Kuwaiti and Australian) showed that this algae is capable of growing at all of the salinities tested. The growth pattern increased with increasing media salinity, which demonstrate that this alga preferred high salinity (45 psu) rather than low salinity (25 psu). The highest growth was observed at Days 11 to 13 of the experimental period, which may indicate that under these environmental conditions the amount of nutrients added at the beginning of the experiment was able to support the culture for 13 days (Figs. 1 and 2).

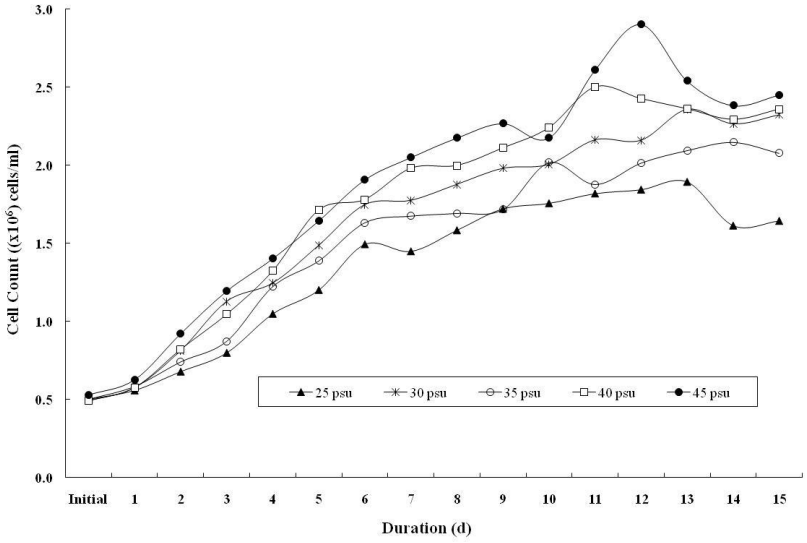


Fig. 1. Performance of locally isolated *Dunaliella salina* (Kuwaiti) at different salinities.

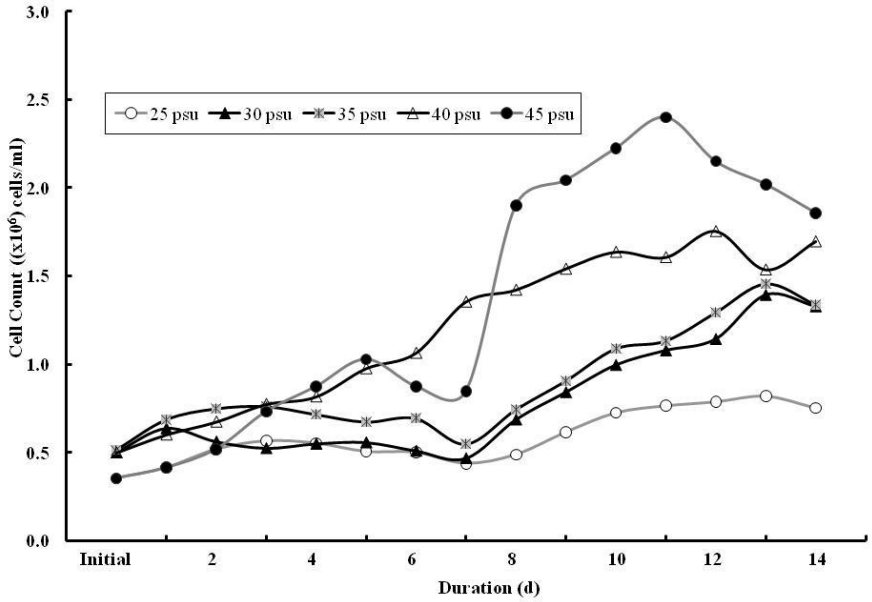


Fig. 2. Performance of *Dunaliella salina* (Australian) cultured at different salinities.

The results obtained from the second set of experiments regarding the growth rate of *D. salina* (Kuwaiti and Australian) cultured at different temperatures (i.e., 20, 23, 26, 29 and 32ºC) showed that the growth pattern in both samples decreased with increasing temperature, an indication that this alga prefers low temperatures (20ºC) rather than high temperatures (32ºC), achieving growth rates of up to $2.90x10^6$ and $2.40x10^6$ cells ml^{-1}, respectively at 20ºC cells ml^{-1} (Figs. 3 and 4).

The results obtained from the third set of experiments regarding the growth rate of *D. salina* (Kuwaiti and Australian) cultured at different light intensities (10 and $18x10^3$ lux) showed that the growth also increased with increasing light intensity, and indicates

that this alga prefers high light intensities rather than low

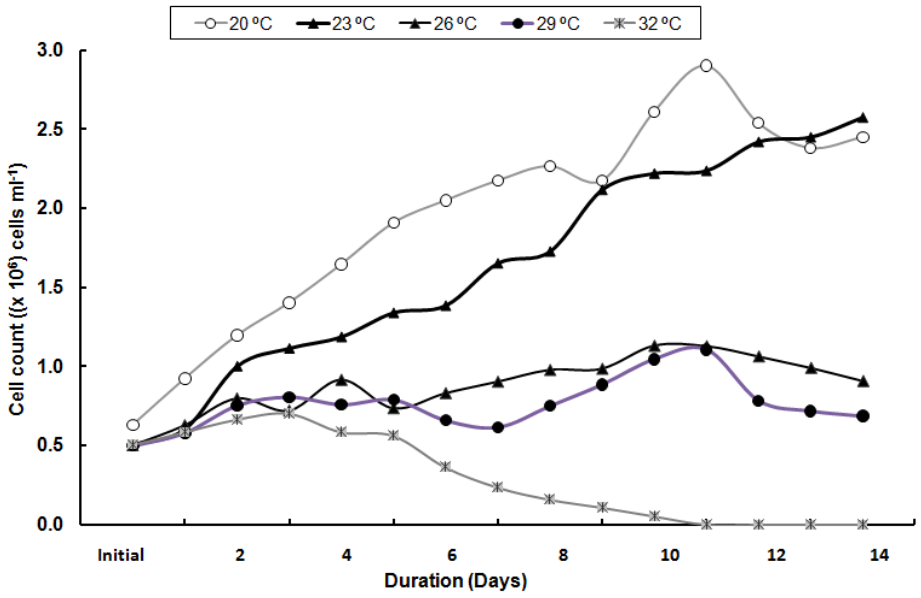


Fig. 3. Performance of *Dunaliella salina* (Kuwaiti) cultured at different temperatures.

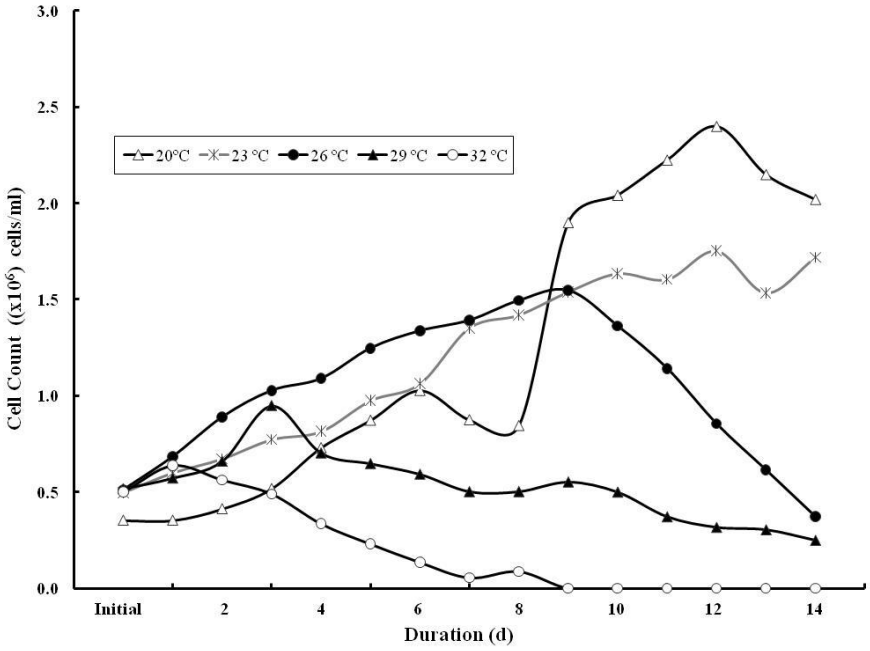


Fig. 4. Performance of *Dunaliella salina* (Australian) cultured at different temperatures.

light intensities. These results indicated that for the Kuwaiti sample, achieving growth rates of up to $4.59x10^6$ cells ml^{-1} at $18x10^3$ lux and only $2.90x10^6$ cells ml^{-1} at $10x10^3$ lux, similarly, for the Australian sample, the highest growth rates up to $3.79x10^6$ cells ml^{-1} at $18x10^3$ lux and only $2.40x10^6$ cells ml^{-1} at $10x10^3$ lux were obtained (Figs. 5 and 6).

The results obtained from the fourth set of experiments regarding the growth rate of *D. salina* (Kuwaiti and Australian) cultured at a high light intensity ($18x10^3$ lux), with and without CO_2 gas (i.e., two pH levels) showed that the growth performance for both samples were higher without CO_2 gas compared to that with CO_2 gas, achieving growth rates of up to $4.59x10^6$ and $4.49x10^6$ cells ml^{-1} at high pHs of up to 9.18 and 9.17, respectively. And up to $2.73x10^6$ and $1.81x10^6$ cells ml^{-1}, respectively at low pHs between 6.75 and 7.25, (Figs. 7 and 8). Furthermore, without CO_2 gas (i.e., at a high pH), the culturing duration

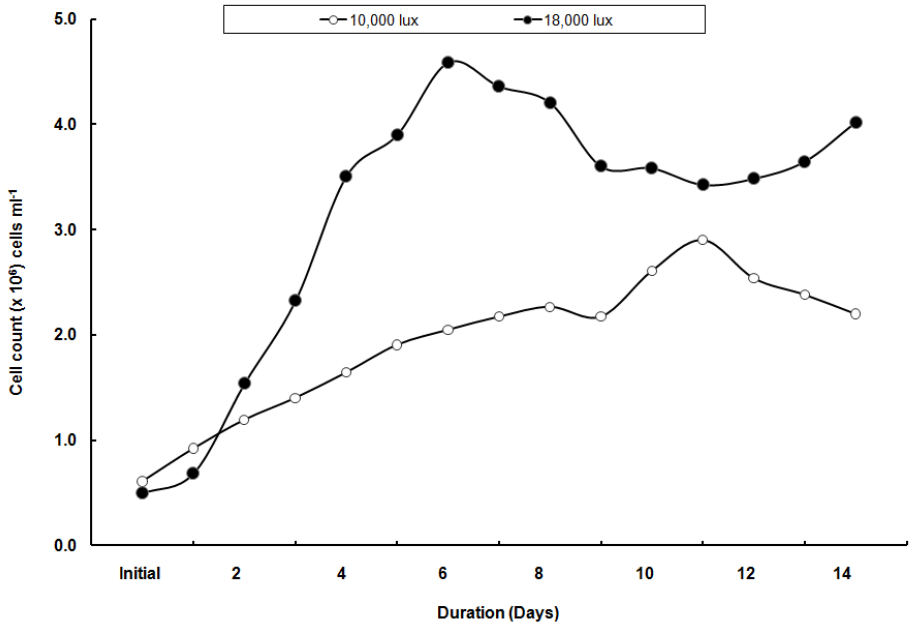


Fig. 5. Performance of *Dunaliella salina* (Kuwaiti) cultured at two different light intensities.

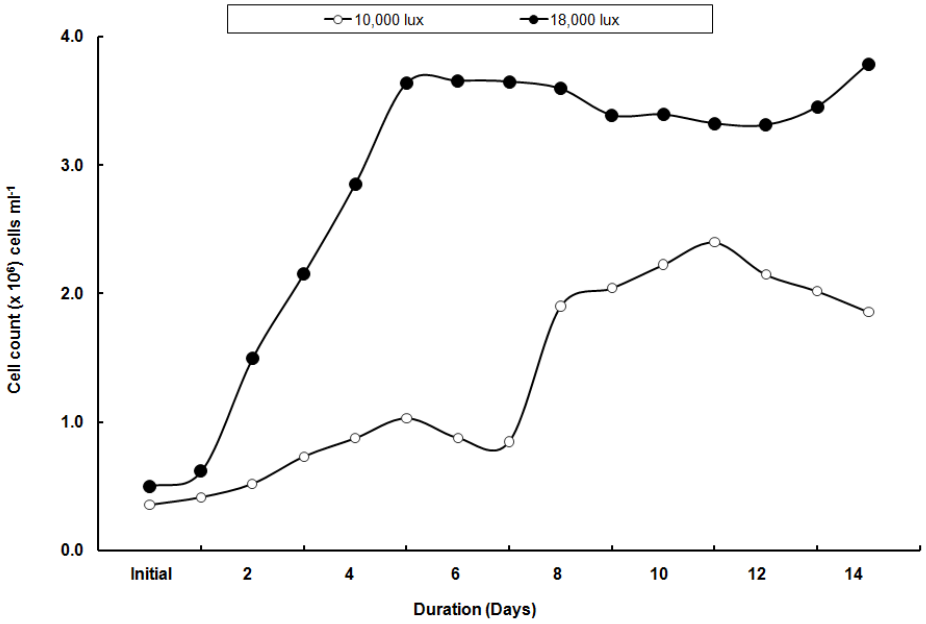


Fig. 6. Performance of *Dunaliella salina* (Australian) cultured at two different light intensities.

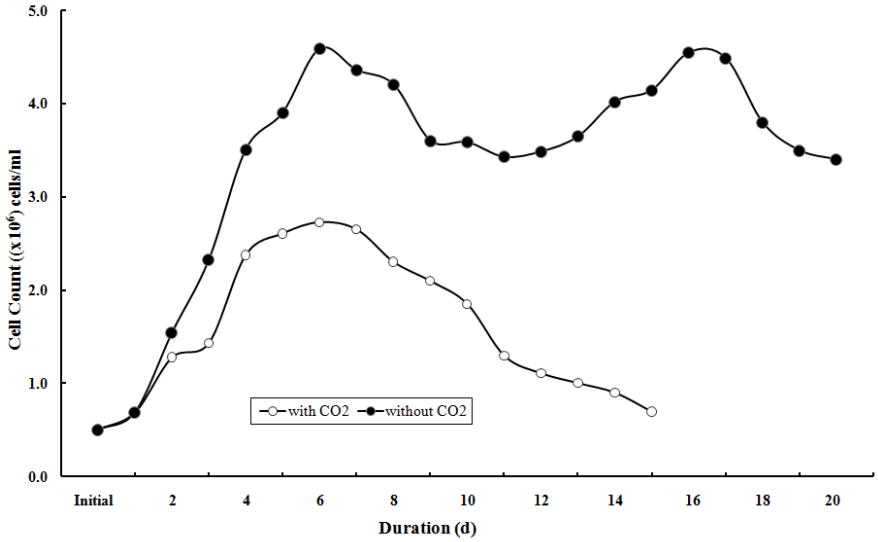


Fig. 7. Performance of *Dunaliella salina* (Kuwaiti) cultured at two pH levels (with and without CO_2 gas).

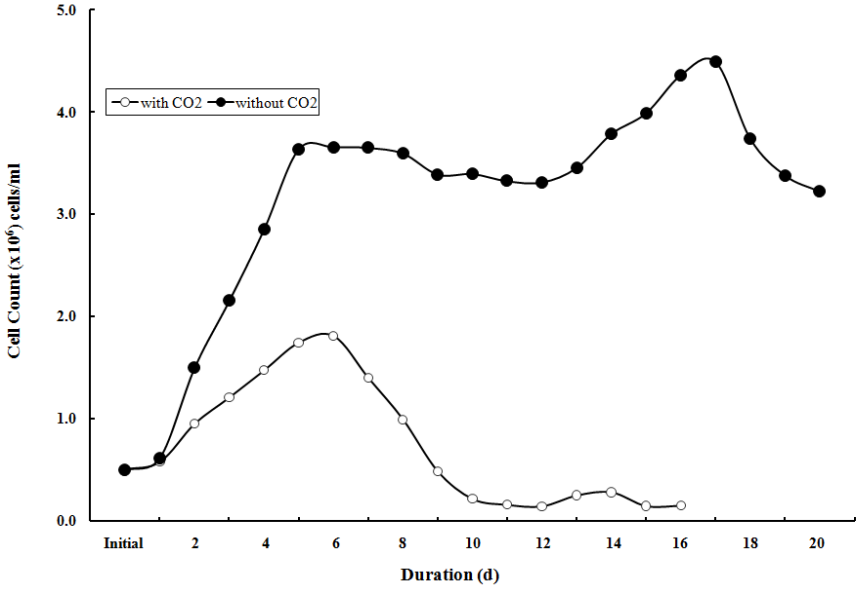
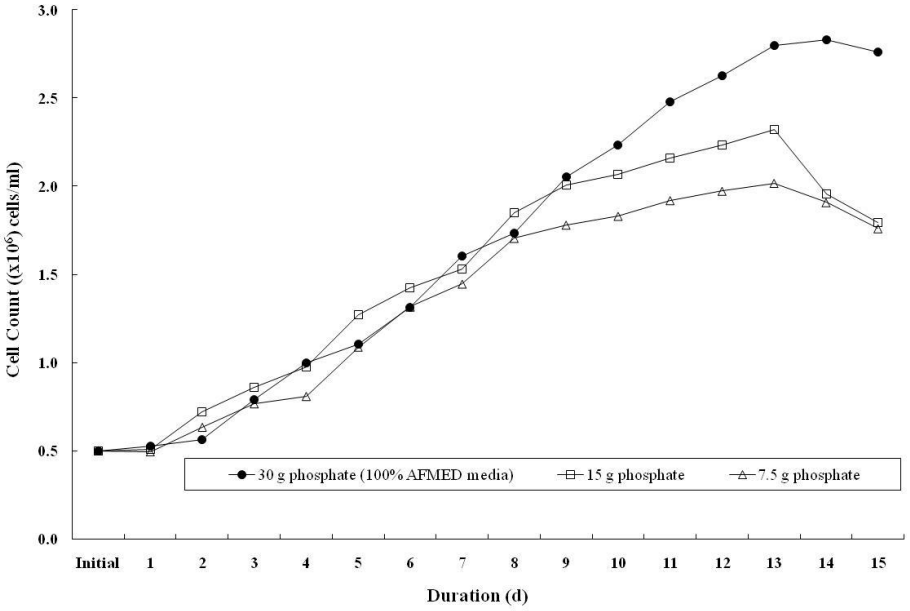


Fig. 8. Performance of *Dunaliella salina* (Australian) cultured at two pH levels (with and without CO_2 gas).

lasting for more than 20 d compared with 15 d and 12 to 16 d, respectively when CO_2 gas was used.

When three different phosphate concentrations were used, *D. salina* (Kuwaiti) preferred a high phosphate concentration (30 g m^{-3} d^{-3}) rather than a low concentration (7.5 g m^{-3} d^{-3}). The highest growth was observed on Days 13 and 14 of the experimental period, which may again indicate that the amount of nutrients added at the beginning of the experiment was able to support the culture for 13 or 14 d (Fig. 9).



AFMED = Aquaculture, Fisheries and Marine Environmental Department

Fig. 9. Performance of locally isolated *Dunaliella salina* at different phosphate concentrations.

The following optimum culturing conditions, i.e., a salinity of >45 psu, temperature of 20ºC, and light intensity of $18x10^3$ lux, without CO_2 gas (i.e., at a high pH), were used to maintain and produce all of the seeds for both *D. salina* samples (from Kuwaiti, and Australian) for further future research studies regarding studying the optimum stress factors required for *β*-carotene induction and possible methods for extraction.

Discussion

Culture Conditions Required for *D. salina*:

The growth pattern increased with increasing salinity (up to 45 psu,) rather than at low salinity (25 psu). These results agreed with that obtained by Ben-Amotz (1987), Dolapsakis et al. (2005), Oren (2005), who found that optimal *Dunaliella* growth could be achieved along the seashore or close to salt lagoons and salt-producing industries due to the ease with

which the desired salt concentration could be obtained by means of natural seasonal.

The results obtained regarding the growth rate of *D. salina* (Australian) cultured at different light intensities (10 and $18x10^3$ lux) showed that growth also increased with increasing light intensity, indicating that this alga prefers high light intensity to low light intensity producing up to $3.79x10^6$ cells ml^{-1} at $18x10^3$ lux and only up to $2.40x10^6$ cells ml^{-1} at $10x10^3$ lux. These results also agreed with the findings of Singh et al. (2000) who reported that *D. salina* grew at a significantly faster rate in high light intensities (400 $\mu Em^{-2}s^{-1}$, i.e., $35x10^3$ lux) than in low light intensities (50 or 200 $\mu Em^{-2}s^{-1}$, i.e., 5,000 or 20 $x10^3$ lux, respectively). Goyal et al. (1998) found that the highest growth for *Dunaliella bardawil*, *D. salina* and *Dunaliella singach* was obtained while using illumination with white fluorescent light at 2 $x10^3$ lux and kept in a shaker operating at a speed of 85 rpm at temperature of 28±0.2ºC. Gomez et al. (1992) concluded that the rate of photosynthesis was significantly higher in the green form at light intensities below 500 $\mu Em^{-2}s^{-1}$, i.e., $50x10^3$ lux. Nevertheless, photosynthetic inhibition by high light was more pronounced in the green form.

The results obtained regarding different phosphate concentrations showed that *Dunaliella* preferred high phosphate concentrations (30 g m^{-3} d^{-3}) rather than low concentrations (7.5 g m^{-3} d^{-3}). These findings were in accordance with those of Singh et al. (2000), who concluded that one of the limiting nutrients for *D. salina* growth was phosphorus, because phosphate-rich cultures grew to levels that were 2.3 times denser than control cultures. Furthermore, *D. salina* grew 1.6 times better in iron rich medium than in the control, and was not inhibited by a lack of CO_2.

Garcia-Gonzalez et al. (2005) discussed the performance of *D. salina* in outdoor cultures in a closed tubular system. They found that maintaining the culture at a temperature of 25ºC with pH of 7.5±0.5, controlled by means of addition of CO_2 gas, a production range of 2 to $4x10^6$ cells ml^{-1} of *D. salina* could be achieved. In the current study, the results showed that growth performance was higher (i.e., up to $4.59x10^6$ cells ml^{-1}) without using CO_2 gas (i.e., at a pH of up to 9.18), compared to that using CO_2 gas (i.e., up to $2.73x10^6$ cells ml^{-1} at pHs of 6.75 to 7.25). Furthermore, without using CO_2 gas (i.e., at high pHs), the culture's duration was extended to more

than 20 d, compared to 15 d when CO_2 gas was used.

It has been well documented by Richmond (1986), Borowitzka (1990), Renaud et al. (1991, 1995) that the chemical composition of several microalgae is influenced by culturing conditions like salinity, temperature, pH, and nutrients. Singh et al. (2000) suggested fixing the water temperature at 30°C while conducting their experiment on limiting nutrients for *β*-carotene induction in *D. salina*. On the other hand, Cifuentes et al. (1992) reported using a temperature of 20±4°C and a photon flux density of 60 µmol m^{-2} s^{-1} equivalent to 5,000 lux under a 12:12 (light and dark phases) photoperiod to test growth and carotenogenesis in eight strains of *D. salina.* The results obtained from the experiments regarding the growth rate of *D. salina* (Kuwaiti) cultured at different temperatures (i.e., 20, 23, 26, 29 and 32ºC) showed that the growth pattern decreased to $2.90x10^6$ cells ml^{-1} with increasing temperature, an indication that this alga prefers low temperatures (20ºC) to high temperature (32ºC).

Leach et al. (1998) concluded that it was possible to obtain a cell concentration of *D. salina* at $0.8x10^6$ cells ml^{-1} when the culture was maintained at a salinity of 18% NaCl w/w and a pH of 8.5. Results also showed that the growth of both examined strains of *D. salina* that both algal culture media used were suitable for culturing them.

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 AFMED laboratories. The results of the experiments conducted showed that this species, from both locations (i.e., Kuwait, and Australia) preferred high salinity (45 psu) to low salinity (25 psu) for optimum growth. Other test results showed that the growth performance for the two strains was best at low temperatures (20ºC). The results also showed that the growth performance for the two strains was best at a high light intensity ($18x10^3$ lux). Furthermore, the results indicated that the growth performance for the two strains was better at high pH levels (i.e., without carbon dioxide gas addition).

With this baseline data it is proposed to workout the stress factors for *β*-carotene extraction.

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