Cultivation technique of *Dunaliellas alina* using partially treated sewage water and discharged desalination water

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Abstract: This study was to examine the feasibility of using alternative low cost technique for *Dunaliella salina* cultivation to be implemented in Saudi Arabia. This work used Dual-phase system to cultivate *D. salina*. The first stage was aimed to maximize *D. salina* biomass and minimize nutrients cost through using different dilution of Johnson's medium (1:0, 1:1, 1:2, and 1:3). The second stage was used two types of water: (partially treated sewage water PTSW) and (discharged desalination water DDW) as a shock to enhances specific biochemical composition product. Protein, carbohydrate, lipid, and beta-carotene of the biomass were measured before and after shock to study the effects of shock on biochemical content. The results showed that the protein and carbohydrate content were higher in the first stage (9.53-13.6 pg/cell;10.3-14.37 pg/cell) respectively, whereas the highest lipid and β-carotene were recorded in DDW shocked cultures (5.66-15.13 pg/cell;16.17-38.93 pg/cell) respectively, compared to lower values in PTSW shocked cultures. This study indicates that the application of discharged desalination water in a Dual-phase culturing system to grow *D. salina* has a great interest if *D. salina* cultivation is targeted for lipids and β-carotene production.

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1. Introduction

Algal culture techniques have been described in several earlier books and articles inthe late 1800s and early 1900s. However, commercial large-scale culture of microalgae only really began to develop in the middle of the last century. The increasing interest in microalgae cultivation has emerged due to the variety of interesting high value products. Microalgae are cultivated as a source of highly valuable molecules such as pigments, lipid and protein that can be incorporated into important industries (Spolaore *et al.,* 2006). There are numerous applications of microalgae range from simple biomass production for food and feed to valuable products for ecological applications. Microalgae are mostly used in the field of human and animal nutrition. They can act as a nutritional supplement, or represent a source of natural food colorants, as well as other commercial uses (Borowitzka, 1999; Soletto *et al*., 2005).

Fewer species of microalgae have economical importance because they are highly productive, easy to maintenance and ideally to culture. Among the various microalgae that have been explored for their suitability for commercial potentials is *Dunaliella salina.* It's proven to be successful due to accumulation of significant amount for valuable chemicals, such as glycerol and carotenoids (Hadi *et al.,* 2008). Many studies have been reported that *Dunaliella salina*is the best commercial source of natural β-carotene (Borowitzka, 1995), which has many applications in nutrition, cosmetic and pharmaceutical products (Cantrell *et al.,* 2003). In recent years, mass cultivation of *Dunaliella salina* for commercial production has being accomplished inseveral countries such as Australia, United States and China (Spolaore *et al.,* 2006). Thus, there are many challenges faced algal cultivation. Algae culture techniques involve high salt consumption to achieve maximum algal production. Salt cost is a major challenge of *D. salina* cultivation. The discharged desalination water can prove beneficial in this felid. Therefore, many companies have attempted to reuse natural brine water or saline waste stream in order to reduce the cultivation cost. Indeed, some of *Dunaliella* production plants have been established close to available sources of salt water, e.g., sea-salt industries, usually in warm and sunny areas where the rate of water evaporation is high and non-agricultural land is abundant (Richmond, 2003).

Hence, it is a worthwhile to develop a new technique to cultivate this highly economically value species with cost effectively method. The reuse of discharged desalination water and seawater as a culture medium for mass production of this microalga could be of great interest since its utilization and further dilution can on the one hand reduce the impact in the environment and on the other hand recycle it into a mean for producing high value bioproducts. With the development of cultivation techniques, microalgal biotechnology can already meet the high

demands of both the food and pharmaceutical industries.

2. Material and methods

Algal source:

A Unialgal culture of *Dunaliella salina* CCAP 19/18 was obtained from CCAP (Culture Collection of Algae and Protozoa, Oban, UK).

Water sampling

Three types of water were used in the experiments: Seawater (Sw), Partially Treated Sewage Water (PTSW) and Discharged Desalination Water (DDW). Seawater was obtained from Offshore Eastern Coast of the Red Sea, (Sharm Obhur, Jeddah). Partially Treated Sewage Water (PTSW) was obtained from BaniMalek Treatment Plant at Jeddah. It was collected from mobile tankers after primary treatment. Discharged Desalination Water (DDW) was taken from out take desalination plant of Saline Water Conversion Corporation (SWCC), Al-Shoaibh. All water samples were stored in reinforced glass bottles at refrigerator for no more than one week. Water samples were filtered gradually through glass microfiber filters (934-AH, Whatman) to remove large particles and suspended solids, (GF/F, Whatman) to remove smaller particulate matter and contaminants, sterilized by autoclaving at 121 ℃ for 27 min. Water parameters (salinity and conductivity) were measured using conductivity meter. Turbidity was measured using turbidity meter. Phosphate, nitrate, sulphate, potassium, calcium and magnesium were determined using ion chromatography whereas copper was determined usingInductivelyCoupledPlasma-Optical Emission Spectrophotometer (ICP-OES).

Media preparation

Two stocks media were prepared with different water sources. The first one was Johnson's medium prepared with distilled water (Johnson *et al.,* 1968) and the second one based on seawater as a solute and source of NaCl. Seawater was enriched with the major elements required for *D. salina* growth according to (Loeblich, 1982), calcium and magnesium salts were reduced to avoid precipitation (Harrison *et al.,* 1980).

Primary experiment

The experiment was designed to allow the selected algae to grow in different water sources through two stages. First step was to compare the effects of different concentrated media (PTSW and DDW) on *D. salina* growth. Both water types were filtered and autoclaved separately, cooled to room temperature then inoculated with 10 ml of stock culture. All cultivations were done in 250 ml Erlenmeyer flasks at controlled room temperature 20 ± 1°C and placed under continuous light regime using day-light fluorescent tubes (PHILIPS TL-D 36 W/34 - 765), with light irradiance of 2000 LUX (28 μmole/m²□sec). Cell concentration of each sample was measured by counting the cell number every three days. Triplicate cultures of each medium were prepared. Since neither PTSW nor DDW enhances *D. salina* growth in the first stage, the PTSW was diluted with seawater to give different concentrations 25%, 50% and 75%. Additional medium was prepared using seawater. The second stage was aimed to determine the optimal and maximum consecration of PTSW for *D. salina* growth. *Dunaliella salina* cells exhibited clumps in seawater media. Therefore, the experiment was designed using PTSW and DDW as a shocking factors in the second stage of dual phase technique as described by (Ben-Amotz, 1995).

Growth rate

Cell number was determined using every two days, using a Model 3 Coulter Multimizer, and associated software V 5.53. The specific growth rate (μ) of *D. salina* was calculated as the slop by plotting the natural log of the cell concentration vs. time in days using equation below (Garcia *et al.,* 2007).

$$\mu = \frac{\ln C1 - \ln C0}{t1 - t0}$$

where*C0*and *C1*(cells per ml) are cell density values at the beginning (*t*0) and the end (*t*1) of a selected time interval between inoculation and maximum cell density, respectively.

Experimental design

The experiments were designed into two stages: the first stage was amid to determine the best dilutions of Johnson's medium for optimum growth of *D. salina*. The second stage amid to use two type of water (PTSW and DDW) as a shocking factor to impose *D. salina* products. The two stages of experiment were conducted in the same time on the same culture conditions.

First stage

In 500 ml Erlenmeyer flasks, 1:0, 1:1, 2:1 and 3:1 dilutions of Johnson's medium versus enriched seawater were used. *Dunaliella salina* was grown in 150 ml of media. Each treatment was done in six replicates divided into two groups to be used in the next stage. Flasks were gently shaken at least once a day. All cultures were incubated in the same culture conditions used for stock cultures. During the experiment time, one ml sample was taken every 48 h to calculate the growth rate for 24 days. Once the growth rate reached the maximum, 10 ml samples were taken for protein, ten ml for carbohydrates, 2 ml for β-carotene and 20 ml lipid determination. Then, all samples were stored in the refrigerator for less than one day.

Second stage

The same cultures that have been previously divided into two groups in the first stage have been shocked. The first group was shocked with 1/3

sterilized DDW. The second group was shocked with 1/2 of the sterilized PTSW that was done due to (Ben-Amotz, 1995) study. The duration was ten days for both groups. The biochemical analysis were done immediately after shock and stored for less than one day.

Biochemical analysis of algal biomass

Beta-carotene content

Beta-carotene was extracted in THF as described by (Hejazi, 2003). A sample of 2 ml was taken from the aqueous phase of each culture medium that had been mixed thoroughly. After 15 min centrifugation at 3500 rpm the upper phase was decanted and 2 ml of tetrahydrofuran (THF) was added to the biomass. Each sample was mixed by vortex for 1-2 min. Then, samples were centrifuged again for 15 min at 3500 rpm for separation of the biomass and the solvent phase. The extracted pigments in the solvent phase were quantified by the spectrophotometric method at wavelength 455 nm. Synthetic all-trans β-carotene (Sigma, type I) was used as stranded.

Carbohydrate content

Total cell carbohydrate was extracted in acidic medium of sulfuric acid according toDubois method (Dubois *et al.,* 1956). Carbohydrate was extracted from samples as following: 10 ml of culture was centrifuged at 3500 rpm for 15 min. In ice bath, one ml of sulfuric acid was added to the pellet overnight. Then, 6 ml of sulfuric acid was added and vortex. To perform carbohydrate assay, about 200 µl of the digested cell walls were mixed with 10 µl of 80% phenol in water and 1.5 ml conc. H_2SO_4. The tubes were left for 5 min. in a boiling water bath followed by 30 min. at room temperature. The absorbance was measured at 485 nm against a blank. The quantity of the sugars was determined from a standard curve of D-glucose.

Total protein content

Protein was quantified using Lowry method (Lowry*et al.,* 1951), the total protein contents were extracted from the pellet in 1 N NaOH placed in 90°C water bath. Protein content was determinate spectrophotometery at 600 nm, using Bovine Serum Albumin (BSA) as standard.

Total lipids content

Total lipids were determined by a modified Bligh and Dyer (1959) method. This method extracts the lipids from harvested cells using a mixture of methanol, chloroform, and water. Firstly, the cells washed three times with 1% NaCl. The supernatant was discarded in order to minimize sample to 2 ml of cell suspension. 7.5 ml of (chloroform/methanol) 1:2 mixture was added with mixing for 10 min. Then, 2.5 ml of chloroform and 2.5 ml of 1% NaCl solution were added with mixing to adjust the ratio of methanol, chloroform and water to 2:2:1 (v/v/v). Tubes were centrifuged for 10 minutes at 3500 rpm. After centrifugation, two layers were formed, a water methanol upper layer and chloroform lower layer which contains the extracted lipids. The upper layer was removed, the chloroform evaporated and the extracts weighed as the total lipid.

3. Results

Chemical characteristic of water samples

In this study, the available phosphate concentration in partially treated sewage water (PTSW) was 1.76 mg/l and it was 0.088 mg/l for seawater, whereas there was no phosphate content detected for desalination water (DDW) (Table 1). Nitrate content was very low (only 9.54 mg/l) in PTSW followed by seawater (1.21 mg/l). No nitrate content was detected in DDW.

The highest calcium content was recorded for DDW (784.41 mg/l), followed by seawater (544.34 mg/l), whereas the lowest content of this element was in PTSW (10.215 mg/l). The maximum magnesium content was recorded for DDW (2493.10 mg/l), followed by sweater (1488.75 mg/l), whereas the minimum content was in PTSW (14.695 mg/l). The highest potassium level was recorded for DDW (680.19 mg/l), followed by seawater (399.66 mg/l), then 1.81 mg/l for PTSW. The highest sulphate was observed in DDW (5469.1 mg/l) secondly seawater (3283.4 mg/l) and the lowest was PTSW (119.1 mg/l). The micronutrient (cupper) was conducted for DDW, whereas was not detected for either seawater or PTSW (Table 1).

The salinity follow conductivity pattern, the highest conductivity and salinity level was observed in PTSW (584 µS/cm), (373 mg/l), respectively followed by value (93.3 mS/cm), (59,700 mg/l) for DDW whereas the lowest conductivityand salinity was recorded for sweater (60.2 mS/cm), (38,500 mg/l). All pH level was almost is the same range for all types of water about 8.

Effect of media dilution on growth parameters (first stage)

In this study, *Dunaliella salina* was capable to grow in a different dilution of Johnson's medium with seawater. Data shows that there was significant difference in growth rate between dilutions. The highest growth rate was obtained in 1:0 with mean slop 0.183±0.00 d^{-1} and the lowest for 1:3 with mean slop 0.143±0.00 d^{-1}. There was no significant difference in the growth rate between 1:0 and 1:1; also between 1:2 and 1:3 (Fig1).

Effect of Dual-phase system on biochemical composition content

Protein

There were variations in the means of protein contents per cell and per liter between dilutions and

stages. In general, protein content in the first stage was higher than the second stage per cell and per liter. In the first stage, maximum protein content was for 1:0 (13.06±0.28 pg/cell) and the lowest was for 1:3 (9.53±0.28 pg/cell). The second stage whether effects DDW or PTSW showed the same pattern (Fig 2). In DDW and PTSW, the dilution 1:0 was highest in the amount of protein content per cell and per liter (6.57 to 8.06 pg/cell). The lowest protein content was for 1:3 (1.9 to 4.53 pg/cell). There was no significant difference between dilutions 1:1, 1:2, 1:2 and 1:3 in protein content per cell. Protein content per liter shows that there was no significant difference between the dilutions 1:1 and 1:2. No significant differences in protein content between shocked with DDW and PTSW per liter, whereas there were significant differences in protein content per cell between all stages (Table 2).

Table 1 Chemical characteristic of water types used in the study.

Parameter	Seawater	Discharged desalination water	Partially treated sewage
Phosphate	0.088	ND	1.76 mg/l
Nitrate	1.21 mg/l	ND	9.54 mg/l
Calcium	544.34 mg/l	784.41 mg/l	10.215 mg/l
Magnesium	1488.75 mg/l	2493.10 mg/l	14.695 mg/l
Potassium	399.66 mg/l	680.19 mg/l	1.81 mg/l
Sulphate	3283.4 mg/l	5469.1 mg/l	119.1 mg/l
Cupper	ND	0.002 mg/l	ND
pH	8.06	8.27	8.90
Conductivity	60.2 mS/cm	93.3 mS/cm	584 µS/cm
Salinity	38,500 mg/l	59,700 mg/l	373 mg/l

Table 2 One-way ANOVA and Tukey HSD test for the effect of Johnson's medium dilutions and stages on biochemical contents. Turkey's test data underscoring indicates where there were no significant differences. Biochemical content of *D. salina* cells expressed as pg per cell.

Parameter	One-way ANOVA (groups and stages)	Turkey's test Between dilutions				Turkey's test Between stages		
Protein (pg cell^{-1})	**	1:0	1:1	1:2	1:3	First	PTSW	DDW
Carbohydrate (pg cell^{-1})	**	1:0	1:1	1:2	1:3	First	PTSW	DDW
Lipid (pg cell^{-1})	**	1:0	1:1	1:2	1:3	First	PTSW	DDW
Beta-carotene (pg cell^{-1})	**	1:0	1:1	1:2	1:3	First	PTSW	DDW

***p*- value ≤ 0.05

Carbohydrate

The highest carbohydrate content was observed in the first stage compared to the second stage shocked by DDW and PTSW (Fig 3).

In the first stage, the highest carbohydrate content was observed for 1:0 (14.37±0.16 pg/cell). In the other hand, the lowest carbohydrate content was recorded for 1:3 (10.3±0.15 pg/cell) (Fig 3 a). In the second stage, generally carbohydrate content was higher in the DDW shocked cultures than shocked by PTSW. Carbohydrate content ranged from 6.8 to 8.23 pg/cell for DDW shocked cultures. Dilution 1:0 in PTSW shocked cultures was highest in carbohydrate content (7.13 ±0.11 pg/cell) (Fig 3). There was no significant difference in carbohydrate content between dilutions 1:0 and 1:1 pg/cell. There were also significant differences in carbohydrate content per cell between all stages (Table 2).

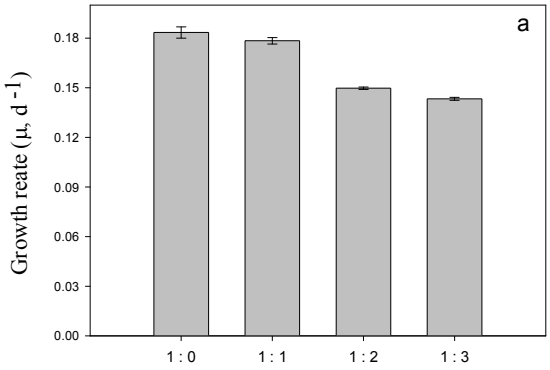

Fig.1 Growth rate of *Dunaliella salina* grown in different dilutions of Johnson's medium during the first stage (µ, day^{-1}), Bars are one standard error ±.

Lipids

In general, lipid content was enhanced by shocking either by DDW or PTSW. In the second stage, the cultures shocked by DDW were achieved

better lipid content (5.66 to 15.13 pg/cell) than cultures shocked by PTSW (3.8 to 8.5 pg/cell) (Fig 4). There were significant differences in the lipid content between all stages per cell and per liter. Also, there were significant differences between all dilutions in lipid content per cell whereas there was no significant difference in lipid content per liter between 1:0 and 1:1 (Table 2).

4.3.4 Beta-carotene

Overall, β-carotene content per cell and per liter were higher in the second stage than first stage. In the first stage, the highest β-carotene content was in dilution of 1:0 (3.65±0.08 pg/cell) and the lowest was in dilution 1:3 (1.13±0.10 pg/cell) (Fig.5a).

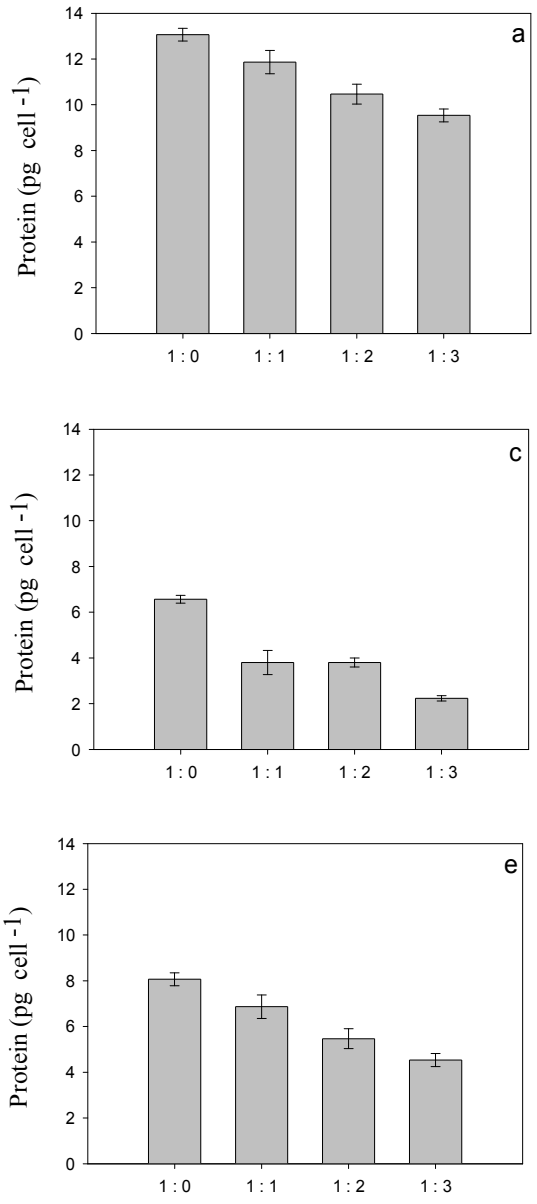

Fig.2 Protein content of *Dunaliella salina*, a. first stage (pg cell^{-1}), b. second stage (shocked with DDW) (pg cell^{-1}), c. second stage (shocked with PTSW) (pg cell^{-1}), of different dilutions of Johnson's medium (1:0, 1:1, 1:1, 1:3). Bars are one standard error ±.

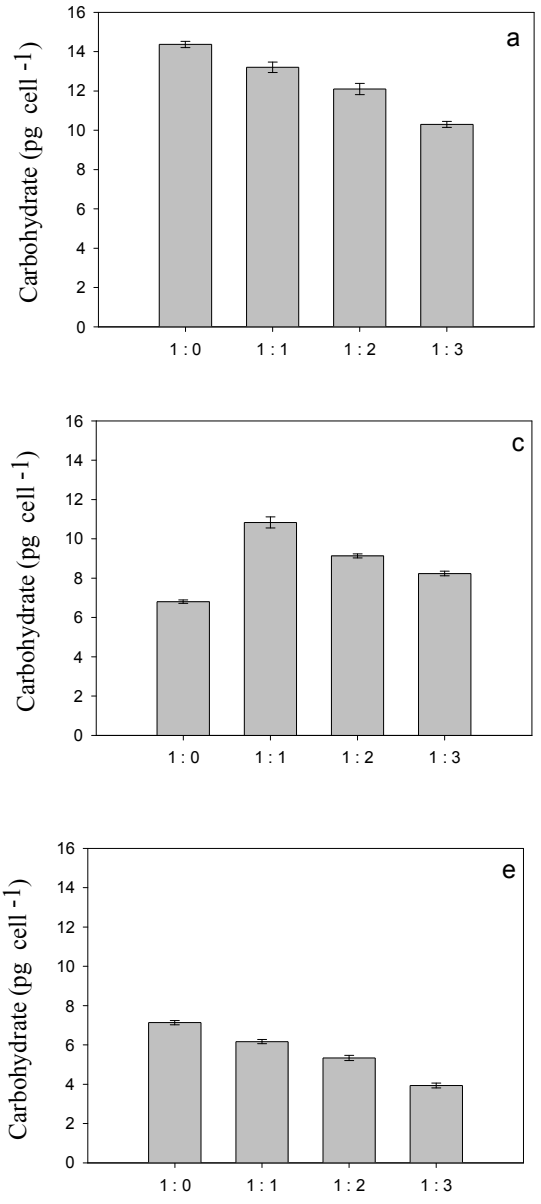

Fig. 3 Carbohydrate content of *Dunaliella salina*, a. first stage (pg cell^{-1}), b. second stage (shocked with DDW) (pg cell^{-1}), c. second stage (shocked with DDW) (pg cell^{-1}), of different dilutions of Johnson's medium (1:0, 1:1, 1:1, 1:3). Bars are one standard error ±.

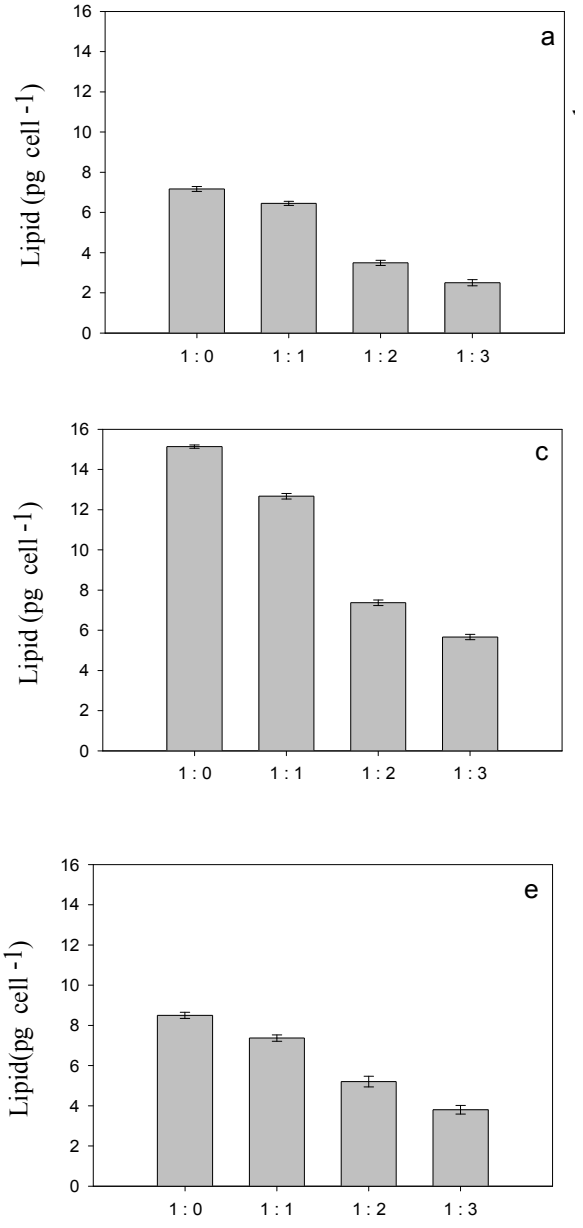

Fig. 4 Lipid content of *Dunaliella salina*, a. first stage (pg cell^{-1}), b. second stage (shocked with DDW) (pg cell^{-1}), c. second stage (shocked with PTSW) (pg cell^{-1}), of different dilutions of Johnson's medium (1:0, 1:1, 1:1, 1:3). Bars are one standard error ±.

The highest β-carotene content per liter wasobtained in second stage shocked by DDW ranged from 16.17 to 38.93 pg /cell compared to PTSW shocked cultures (Fig 5). There was no significant difference in β-carotene between dilutions 1:2 and 1:3 pg/cell (Table 2).

One-way ANOVA showed that there was no significant difference in β-carotene content between dilution 1:2 and 1:3 per cell whereas the rest of dilution has significant differences between them in β-carotene pg/cell (Table3).

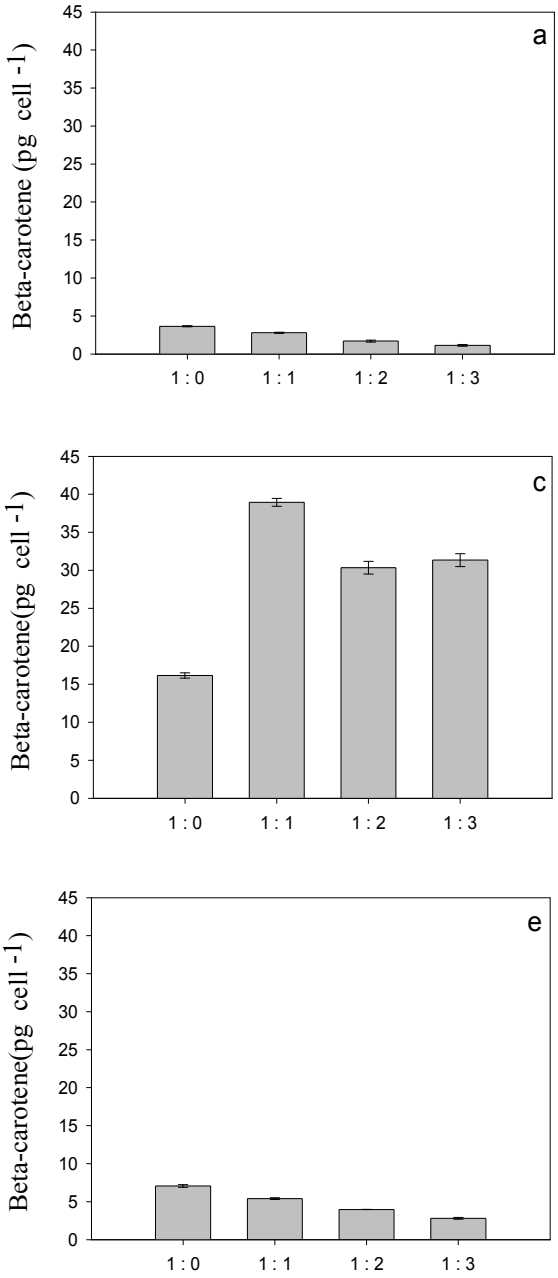

Fig. 5 Beta-carotene content of *Dunaliella salina*, a. first stage (pg cell^{-1}), b. second stage (shocked with DDW) (pg cell^{-1}), e. second stage (shocked with PTSW) (pg cell^{-1}), of different dilutions of Johnson's medium (1:0, 1:1, 1:1, 1:3). Bars are one standard error ±.

Table 3. One-way ANOVA and Tukey HSD test for the effect of DDW shock on beta-carotene content. Turkey's test data underscoring indicates where there were no significant differences.

Parameter	*p*-level	Turkey's test
Beta-carotene (pg cell^{-1})	**	1:0 1:11:2 1:3

***p*- value ≤ 0.05

4. Discussion

Growth parameters reflect the response of microalgae cells to favorite growth conditions (Stein, 1973), however biochemical composition content doesn't follow this trend (Gottumukala, 2010). In this study, Dual-phase system tested the first stage to achieve higher growth performancefor *D. salina* whereas the second stage was designed to boost some specific biochemical composition content.

The maximum growth rates reflect the optimal culture conditions (Ben-Amotz *et al.,* 2009). Mainly, phosphorus and nitrogen are important macronutrients to build up material for microalgae growth (Ben-Amotz, 2003). Therefore, any limitation in these macronutrients could clearly reflected by growth rate value. Although chemical analysis of natural seawater that used in this study show no phosphate content was detected and low nitrate content (see Table 1), nutrients provided by diluted Johnson's medium1:1 (J/Sw) was sufficient enough to support growth of *D. salina.* Whereas further dilutions in seawater were lead to dilute the minimum phosphate and nitrate content (Andersen, 2005).

On the other hand, the biochemical composition content of any microalgal species is clearly related to their growth phase (exponential or stationary) and to culture conditions such as light frequency (Brown *et al.,* 1993), light intensity (Thompson *et al.,* 1993), temperature (Thompson *et al.,* 1992) or culture media (Wikfors *et al.,* 1996). Literature results have been obtain mainly with small experimental volumes under various controlled, but highly variable, conditions (batch/ continuous, L/D cycle, period of harvesting). Gross composition does not always correlated directly with nutritional value owing to possible deficiency in some essential nutrients. However, when specific essential nutrients are in adequate proportion, the gross composition may be important (Stottrup and McEvoy, 2003). Brown *et al.* (1997) reported that overall biochemical of 40 algal species grown under standard condition showed protein as a major organic component (15-52% of dry weight), followed by lipid (2-20%), then carbohydrate (5-12%).

In this study, protein and carbohydrate content in the first stage was higher comparing to the second stage. The first stage present the favorite culture conditions therefore, physiological process of photosynthesis was maximum to enhance carbohydrate content (Wainman and Smith, 1997). In addition, phosphate and nitrate concentration was sufficient to build up protein content in the first stage compared to the second stage (Gottumukala, 2010). In the second stage, the available phosphorus concentration in PTSW was lower than the optimal level required for *D. salina* (Ben-Amtoz *et al.,* 2009) (see Table 1). Nitrogen and phosphorus were limiting macronutrients in DDW (see Table1) that maybe the reason for the lowest protein content that recorded in this study for DDW.

Lipids are known to be a storage product of many algal species (Andersen, 2005). It was found that there are many microalgae species capable of producing high amounts of lipids under nitrogen deficiency (Roessler, 1990). Literature also showed that increasing salinity has significant effect on increasing lipid content (Gautam *et al.,* 1994; Takagi *et al.,* 2006).

This study supported these phenomena as highest lipid content was observed in DDW that has no P and N content recorded (Table 1). That suggesting that DDW could be good addition to *D.salina* cultivation system in Saudi Arabia and other Arab region that has proven to be successful in Spain (Lamela *et al.,* 2010).

Studies, carried out with different *D. salina* strains found variations in β-carotene content depend on stress factor such as: high light intensity, high temperature, high salinity, and nitrogen deficiency (Abu-Rezq*et al.,* 2010). The most highly effective factor is salinity (Ben-Amtoz *et al.,* 2009). Ben-Amtoz (2003) stated that most *Dunaliella* cells turning orange or red in color under the wide range of salt concentrations. High salt content in *Dunaliella* culture media known to induced carotenogensis (Ben-Amtoz, 1995). The data of this work shows that β-carotene content in *D. salin*a shocked with DDW was the maximum recorded content due to high level of salinity. Abd-El-Baky*et al.* (2004) showed similar β-carotene content with similar salinity.

Furthermore, literature shows that the carotenogenesis and growth *Dunaliella* sp. are two separate biological processes (Shaish *et al.,* 1992) that explains the minimum β-carotene content recorded in the first stage of this work.

This study suggests that the dilution of 1:1 is the best to achieve maximum production and quality composition compared to the other dilution. Nevertheless, DDW could be a new addition to be applied in *D. salina* cultivation system. It is known that the amount of flow DDW varies from 20 to 70 % of feed flow of desalination process (UNEP, 1997). Saudi Arabia has the highest desalination capacity worldwide, the use of brine could be a good idea in *D. salina* cultivation to minimize the environmental impacts and benefit from the huge capacity of this

wasted unused water source for lipid and β-carotene production.

Further studies could improve such application in Saudi Arabia. Temperature is known to affect the biochemical of *D. salina* and any other microalgal species. Therefore, it could be selected as the first factor to improve *D. salina* β-carotene production application (Gomez and Gonzalez, 2005).

It is also recommended to search for local strain for any selected cultivation system (Thinh *et al*., 1999). Using locally isolated is better than using international because it would be adapted to local environmental conditions especially in courtiers with high temperature level (Chen *et al*., 2009). Dual-phase technique is suitable for outdoor cultivation system especially with local strain. Furthermore that could be even improve further by genetic modification to fit outdoor cultivation system need and for the specific product targeted (Ben-Amtoz, 1995; Tafreshi and Shariati, 2006).

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