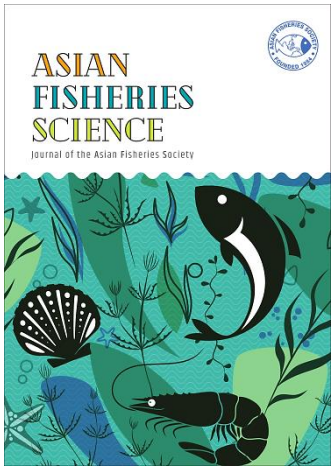

Growth and Carotenoid Production of *Dunaliella salina* (Dunal) Teodoresco, 1905 Cultured at Different Salinities

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Abstract

Microalgae species have been widely used as an alternative source for pigment extraction. The genus *Dunaliella* has been commercially cultured for its carotenoid pigments. Carotenoid is one of the commercially important pigments widely used in various industries. In the present study, different salinities such as 0.3, 0.5, 0.7 and 0.9M were used to culture *Dunaliella salina* (Dunal) Teodoresco, 1905 to determine the ideal salinity for the growth and carotenoid production for 12 days. Growth was monitored daily with respect to cell density. Carotenoids were extracted from samples every alternate day. Results showed that *Dunaliella salina* had highest ($P < 0.05$) cell density when cultured at 0.7M salinity compared to other salinities. Similarly, specific growth rate (0.53 $\mu.day^{-1}$) and carotenoid contents (5.015 $mg.L^{-1}$) were found highest ($P < 0.05$) at 0.7M salinity compared to the other salinities. This study illustrated that *Dunaliella salina* favoured moderately high salinities (0.7M) for optimum cell growth and the production of carotenoid.

Keywords: microalgae, pigment, cell density, salinity, carotenoid

Introduction

Dunaliella salina is a microalga which is the richest sources of natural carotenoids (Xu et al., 2016). *Dunaliella salina*, the major accumulated carotenoid is β-carotene, which is stored in globules of lipid and proline-rich, carotene globule protein in the inter-thylakoid spaces of the chloroplast (βC-plastoglobuli) (Ben-Amotz et al., 1982; Lamers et al., 2012; Davidi et al., 2015).

This natural carotene contains pigments red, yellow to orange used as a colourant in the feed industry (Lamers et al., 2012). *Dunaliella salina* contains almost 5–10 mg β-carotene.g^{-1} dry weight under normal growth which is similar to other green microalgae such as *Chlorella*, *Chlorococcum*, *Scenedesmus, Coelastrum, Desmodesmus* (Del Campo et al., 2007). However, the carotene production is affected by many factors such as salinity, light intensity, nutrient deprivation and temperature. Carotenoid production can increase as much as 10 % of dry weight when this species is cultured in stress conditions such as high salinity, high light intensity, nutrient deprivation and extreme temperatures (Ben-Amotz et al., 1982; Ben-Amotz and Avron, 1983; Borowitzka et al., 1990; Shaish et al., 1993; Ben-Amotz, 1996; Kleinegris et al., 2009).

The genus *Dunaliella* is a unicellular marine green microalga (Prieto et al., 2011) that strives in a multitude of marine habitats such as oceans, brine lakes, salt marshes, salt lagoons and saltwater ditches near the sea. This halo-tolerant and cell wall-lacking microalga adapt to very high salinities. The tolerance is due to the capability of the microalgae in maintaining its energy-yielding processes at high rates (Alyabyev et al., 2011). The immense accumulation of carotenoids by some strains under favourable growth conditions has also guided to fascinating biotechnological applications (Oren, 2005). *Dunaliella* is renown in having the ability to accumulate high levels of carotenoids. Therefore, it has been selected as an important genus for the commercial production of food colouring agents, supplement for food and animal feed and an additive to cosmetics (Edge et al., 1997).

Dunaliella synthesises photosynthetic pigments such as chlorophylls, carotenoids, and phycobilins (Takaichi, 2011; Borowitzka, 2013) in their cells. Carotenoids such as xanthophylls – violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, lutein, loroxanthin, astaxanthin, canthaxanthin, fucoxanthin and β-carotene are commonly found in microalgae (Takaichi and Mochimaru, 2007). The carotenoid accumulation is not only influenced by the salinity but also governed through the culture growth phase and is usually extracted during the exponential phase (Fazeli et al., 2005). The pigment is produced in response to adverse environmental conditions for it has the ability to mob up excessive free radicals formed during these stressful conditions in the cell, thus restoring its physiological balance (Moller et al., 2000; Pisal and Lele, 2005). Carotenoids have a more significant commercial interest (Fazeli et al., 2006) over chlorophylls as they are applied in various fields of production.

Salinity would be the cheapest method of manipulating, controlling, and maintaining the growth environment for higher carotenoid production over temperature and irradiance control, since both involve a higher usage of electricity to maintain the controlled conditions of the culture (Coesel et al., 2008). Therefore, the objectives of this study were to determine the optimum salinity of the local strain of *D. salina* (Dunal) Teodoresco, 1905 for optimum growth and to evaluate the optimum salinity for the highest production and accumulation of total carotenoids.

Materials and Methods

Sample collection, culture and maintenance

Dunaliella salina was isolated from the South China Sea and the stock was preserved at Live Feed Laboratory, Institute of Tropical Aquaculture, Universiti Malaysia Terengganu. Sterilised and filtered seawater (0.5M; 8.0 pH) was used along with Conway medium (Tompkins et al., 1995) to culture *D. salina*. To allow sufficient time for CO_2 equilibration the sterilised medium was kept for 2 days before microalga inoculation. Every 2 weeks sub-culturing was done to maintain pure and healthy stock. The pure and healthy stock culture was used for the experiment.

Media preparation

Conway medium was prepared according to Tompkins et al. (1995). Conway medium consists of macronutrients, trace metal solutions and vitamins (Table 1). One millilitre of macronutrient, 0.5 mL of trace metal, and 0.1 mL of vitamins were added to 1000 mL of filtered and sterilised seawater. Salinity was adjusted at 0.5M before inoculation of pure microalgae culture.

Table 1. Chemical composition of Conway medium.

Conway medium (Tompkins et al., 1995)	
Nitrate	KNO_3 (100 g.L^{-1})
Phosphate	Na_3PO_4 (20 g.L^{-1})
Trace metal	$Na_2H_2EDTA.2H_2O$ (45 g.L^{-1}) $FeCl_3.6H_2O$ (1.3 g.L^{-1}) $ZnCl_2$ (4.2 g.L^{-1}) $MnCl_2.4H_2O$ (0.36 g.L^{-1}) $CoCl_2.6H_2O$ (4.0 g.L^{-1}) $CuSO_4.5H_2O$ (4.0 g.L^{-1}) $(NH_4)_6Mo_7O_{24}.4H_2O$ (1.8 g.L^{-1}) H_3BO_3 (33.4 g.L^{-1})
Vitamin	Thiamin HCl (200 mg.L^{-1}) Cyanocobalamin (10 mg.L^{-1})

Experimental design

Dunaliella salina was grown in sterile 1 L Erlenmeyer flasks containing Conway medium (Tompkins et al., 1995) with increasing sodium chloride (Sigma, USA) concentrations of 0.3M, 0.5M, 0.7M and 0.9M. The pH of the medium was adjusted to 8 by the addition of 1M NaOH (Sigma, St. Louis, MO, USA). The medium was inoculated with 1.5×10^6 cells from the stock culture of the isolates. Cells were grown at 24 ± 1 °C under continuous illumination of 300 µmol m^{-2} s^{-1} (adapted and modified from Fazeli et al., 2006). Light intensities were measured routinely with a quantum light meter (Fieldscout, Spectrum® Technologies, Inc., USA). Three replicates were made for each concentration. The cultures were aerated moderately and sterile cotton plugged at the mouth of the flasks to reduce contamination. Samples of the cultures were examined under the microscope for contamination throughout the experiment. The growth parameters and carotenoid accumulation between these four salinities were observed for 12 days (Prieto et al., 2011).

Cell density and specific growth rate (SGR)

Cell density was determined by cell count daily for each set of salinity and its replicates. Samples were taken from each set of salinity and its replicates every 2 days for carotenoid extraction to determine their pigment concentrations via optical density. Cell density was used to measure the growth of *D. salina*. An aliquot of well-mixed culture suspension was placed on a Neubauer haemocytometer (Assistant, Germany) to count the cell numbers daily for growth monitoring. Cells were tallied in five tiny quadrangles of the central block. Total cell numbers in culture were then estimated by volume and dilution.

The specific growth rate (SGR) of *D. salina* from different treatments were calculated by the following equation (Clesceri et al., 1989):

$SGR\ (\mu.day^{-1}) = \ln X2 - \ln X1 / t2 - t1$

where X1 is the cell concentration at the beginning of the selected time interval, X2 is cell concentration at the end of the selected time interval, t2–t1 is the selected time (11 days) for the determination of cell density of *D. salina*.

Carotenoid determination

A 1 mL aliquot of the algal suspension from each culture was taken at an interval of 2 days and centrifuged at 1000 ×*g* for 5 min. The pellet obtained was extracted with 3 mL of ethanol:hexane 2:1 (v/v). Two millilitres of water and 4 mL hexane (Sigma, USA) was added to the mixture. It was shaken vigorously and centrifuged again at 1000 ×*g* for 5 min. The hexane layer separated and its absorbance (*A*) was determined spectrophotometrically at a wavelength of 450 nm. The amount of extracted carotene from the samples in micrograms was determined by multiplying the absorbance A_{450} with 25.2 (Shaish et al., 1992).

Statistical analysis

The quantitative analysis for cell growth and carotenoid concentration was performed in triplicates by the one-way analysis of variance (ANOVA). Fisher's individual error rate test was applied to resolve the significance between means. The data were expressed as means ± standard error (SE) at a level of $P < 0.05$ which was considered as significant (Fazeli et al., 2005; Hu et al., 2008). Pearson's correlation was used to determine the correlation between cell densities and carotenoid production for each set of salinity.

Results

Cell density

The highest growth of *D. salina* was observed with $2.35 \times 10^6 \pm 0.12$ cells.mL^{-1} at 0.3M salinity and with $2.30 \times 10^6 \pm 0.20$ cells.mL^{-1} at 0.5M salinity both achieved on day 11. Meanwhile, the highest growth for salinities 0.7M and 0.9M were observed on day 7 ($6.95 \times 10^6 \pm 0.00$ cells.mL^{-1}) and day 6 ($1.40 \times 10^6 \pm 0.05$ cells.mL^{-1}), respectively (Fig. 1). In addition, at the end of the experiment, SGR was found to be higher ($P < 0.05$) with *D. salina* cultured at 0.7M salinity (0.53 day^{-1}) followed by 0.9M (0.50 day^{-1}), 0.3M (0.47 day^{-1}), and, 0.5M (0.41 day^{-1}) salinity (Table 2). The salt concentration of 0.7M favours the highest growth of the microalgae with a significant difference in growth ($P < 0.05$) compared to the rest of the treatments.

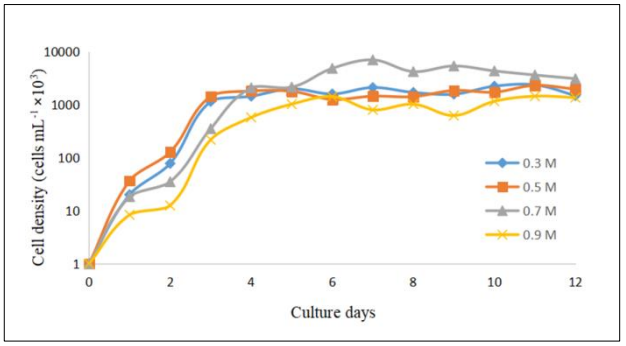

Fig. 1. Cell density (×10^3) cells.mL^{-1} of *Dunaliella salina* cultured at different salinities. Values are mean ± standard error.

Table 2. Specific growth rate of *Dunaliella salina* cultured at different salinities.

Treatments	Specific growth rate (SGR μ.day^{-1})
0.3M	0.476 ± 0.01
0.5M	0.413 ± 0.03
0.7M	0.530 ± 0.02
0.9M	0.504 ± 0.05

Values are mean ± standard error.

Carotenoid accumulation

Total carotenoid content ranged from 0.139 mg.L^{-1} at 0.3M salinity to 5.015 mg.L^{-1} at 0.7M salinity (Fig. 2). The highest carotenoid accumulations were detected at 0.88 ± 0.04 (Day 10), 0.84 ± 0.03 (Day 10), 5.01 ± 0.00 (Day 6) and 1.72 ± 0.13 mg.L^{-1} (Day 2) for salinities 0.3, 0.5, 0.7 and 0.9M, respectively (Fig. 2). The salt concentration of 0.7M favours the highest ($P < 0.05$) carotenoid production of the microalga compared to the rest of the treatments (Fig. 2). A strong positive correlation was discovered between cell density and carotenoid production (Table 3).

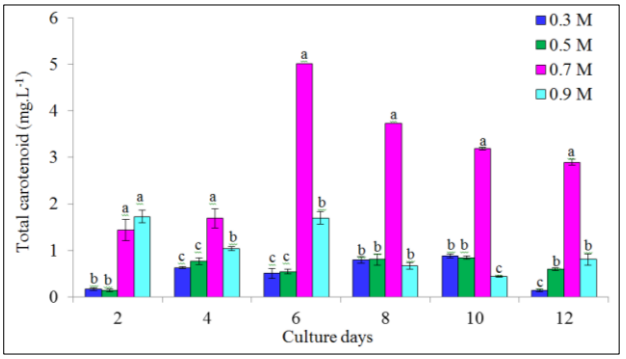

Fig. 2. Carotenoid production (mg.L^{-1}) of *Dunaliella salina* cultured at different salinities. Values are mean ± standard error.

Table 3. Pearson's correlation value for *Dunaliella salina* cultured at different salinities.

	Different treatments			
	0.3M	0.5M	0.7M	0.9M
Correlation	0.736 ± 0.01[b]	0.738 ± 0.01[b]	0.894 ± 0.02[a]	0.783 ± 0.01[b]

Values are mean ± standard error.

Discussion

Dunaliella salina is a type of unicellular and halophilic green biflagellate microalga without a rigid cell wall structure (Ben-Amotz and Avron, 1992). *Dunaliella salina* is renowned in having the ability to accumulate high levels (4.2-5.29 mg.g^{-1}) of carotenoids (Chagas et al., 2015). Therefore, it has been selected as an important species for food colouring agents, a pro-vitamin a supplement for food and animal feed, an additive to food and cosmetics, a health food product (Edge et al., 1997).

Based on the location of the isolated species and culture condition growth may vary for *D. salina* as shown in Table 4.

Table 4. Cell density (cells.mL^{-1}) of *Dunaliella salina* cultured at different salinities.

Salinity (M)	Cell density (cells.mL^{-1})	References
0.4	3.48×10^5	Hamed et al. (2017)
0.6	1.50×10^5	Hamed et al. (2017)
0.7	6.95×10^6	Present study

The optimum salinity for the growth of *D. salina* is 2M (Hadi et al., 2008). However, higher salinities have been reported to retard growth rates but increase the carotenoid levels in them (Fazeli et al., 2005). In the present study, *D. salina* was capable of growing at all the salinities tested. Generally, *D. salina* prefers a moderately high salinity (0.7M) rather than a low salinity (0.3M) or extremely high salinity (0.9M). Therefore, Malaysian *D. salina* demonstrated a higher growth rate at optimum salinity (0.7M). When comparing the results with other findings, there are discrepancies as more factors are involved in affecting the growth rate of the microalgae, including temperature, light, aeration, species, strains, temperature, irradiance, medium used, and culture period. Temperature and light are the two most important parameters that influence microalgal growth and are dependent on each other. The optimal pH for growth of most of the algal species should be between 7.0 and 9.0, although some species that dwell in both acidic and basic environments are also existent (Blinova´ et al., 2015). In addition, the amount of major and minor nutrients in the medium also plays a crucial role in microalgae growth. The importance of the effect of change in pH, salinity, and carbon dioxide on microalgal growth solely depends on the species (freshwater or marine) under microalgae study.

To optimise the microalgae growth, it is essential to control the temperature while culturing microalgae as temperature plays a crucial role in the growth of microalgae (Raven and Geider, 1988). Temperature affects the gross photosynthetic activity of microalgae by undergoing cellular division, which, in turn, affects the biomass productivity of microalgae. *Dunaliella* is able to withstand a temperature range between 0 °C and 45 °C. The ideal growth of *Dunaliella* sp. was determined at 32 °C with a wide growth temperature span ranging between 25 °C and 35 °C (Hosseini Tafreshi and Shariati, 2009). In the present study, the temperature controlled at 26 °C.

Light is vital for microalgae growth and varies with culture density. Light intensity and photoperiod cycles are one of the key factors that may limit or maximise the growth of microalgae cultivation (Parmar et al., 2011). Concentrated microalgae cultured need higher intensity of light to penetrate through the culture (Wahidin et al., 2013). However, if the light intensity is too high, it may inhibit the growth of microalgae or known as photo-inhibition and decrease the photosynthetic rate (Mulders et al., 2014). Most microalgae can tolerate light saturation up to 1700 to 2000 µmol m^{-2} s^{-1} (Griffiths, 2013). When exposed to high light, the *Dunaliella* cells are reported to use the carotenoid synthesis pathway as a protective mechanism against photodamage (Mulders et al., 2014). Different *Dunaliella* strains may vary significantly in their response to light stress and show different sensitivities to the light intensities. However, it is not clear as to whether high or low irradiance is more effective in stimulating the synthesis of 9-cis β-carotene (Orset and Young, 2000).

Majority of microalgal species growth is known to prefer at neutral pH and all strains of microalgae seem to have a limited optimal range of pH (Lutzu, 2012). The effect of photoperiod on the growth of *D. salina* CCAP 19/30 revealed that longer photoperiods led to increased growth of microalgae with higher cell densities (Xu et al., 2016). Growth of microalgae is directly proportional to the uptake rate of the most limiting nutrients. Nitrogen and phosphorous are considered to be the primary nutrient for microalgae growth. In this experiment, the commercial media was used where nitrogen and phosphorous are major nutrients. Nitrogen is considered to be a building

block for proteins and nucleic acids, whereas phosphorus forms parts of phospholipids.

In addition to the higher growth rate, *Dunaliella* preferred 0.7 salinity for the accumulation of carotenoid during the exponential phase (from Day 3 to Day 7). In response to several stress factors or growth-limiting conditions such as salinity, temperature, light, and nutrient deficiencies (Ben-Amotz and Shaish, 1992; Fazeli et al., 2005; Mojaat et al., 2008), *D. salina* synthesises and accumulates β-carotene in lipid globules in the stroma of chloroplasts. The extent of carotenoids accumulation in oil globules within the inter-thylakoid spaces of their chloroplast is directly proportional to the integral amount of light to which *D. salina* cells are exposed during a division cycle (Ben-Amotz and Avron, 1983). The pigment has the ability to mob up excessive free radicals formed during these stressful conditions in the cell, thus restoring its physiological balance (Pisal and Lele, 2005). Accumulation is enhanced under several harmful conditions such as high irradiance, stress temperatures, high salt concentration and/or nutrient deficiency (Ben-Amotz and Shaish, 1992). Under these conditions, up to 10 % of the algae dry weight is β-carotene. *Dunaliella* β-carotene occurs as a number of isomers, two of which, 9-cis and all-trans, make up approximately 80 % of the total isomers (Prieto et al., 2011).

A study done by Fazeli et al. (2005) showed that low salinities of 0.1M and 0.5M NaCl favoured both total carotenoids and chlorophyll-a production by *D. tertiolecta* when compared on a volume basis. However, productivity on a cellular basis (per gram of total carotenoids and chlorophyll-a per cell) was significantly higher at extreme salt concentration (3M NaCl). Although high salinity favoured total carotenoid production by *D. tertiolecta* on a cellular basis, it negatively affected on a per-volume basis because cell growth was repressed at elevated salt concentrations. In another study by Marin et al. (1998), they reported that the adjustment of light in combination with salinity is the best methods to achieve optimal carotene production in commercial cultures of *D. salina*.

In the present study, the highest carotenoid concentration of 5.015 $mg.L^{-1}$ (Day 6) was found at 0.7M. In a separate study, Hadi et al. (2008) have reported that 1.66 $mg.L^{-1}$ of total carotenoid was extracted from *D. viridis* at 1.0M salinity. However, the findings of the present study showed that the Malaysian *D. salina* is capable of accumulating 1.730 $mg.L^{-1}$ (Day 2) carotenoid at only 0.7M salinity, using different media and irradiance compared to other studies. The local *D. salina* may, therefore, have the potential to accumulate higher amounts of carotenoids if cultured at higher salinities.

Conclusion

The microalgae had the maximum growth rate and highest total carotenoid accumulation at 0.7M salinity. Therefore, the local strain *Dunaliella salina* can be regarded to have an optimum salinity for growth and total carotenoid accumulation at 0.7M salinity as compared to other salinities (0.3, 0.5 and 0.9M). The results of this study can be applied in the production of carotenoid from the local strain of *D. salina* for application in the food industry, pharmaceutical, cosmeceutical and also aquaculture. Further studies are required to do mass cultivation of *D. salina* at the optimum salinity (0.7M) for open outdoor cultures (such as open tanks) and closed cultures (such as tubular reactors) to utilise the biomass in different industries.

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