

## Monitoring of growth and biochemical composition of *Dunaliella salina* and *Dunaliella polymorpha* in different photobioreactors

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### Cite this article as:

Demirel, Z. (2022). Monitoring of growth and biochemical composition of *Dunaliella salina* and *Dunaliella polymorpha* in different photobioreactors. *Aquatic Research*, 5(2), 136-145. <https://doi.org/10.3153/AR22013>

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Submitted: 09.12.2021

Revision requested: 01.02.2021

Last revision received: 03.03.2022

Accepted: 03.03.2022

Published online: 08.03.2022

### ABSTRACT

In this study, the isolation of green algae were collected from two different stations of Aegean Sea and Seyfe Lake. The molecular identification of *Dunaliella* species using their 18S ribosomal DNA genes were sequenced and investigated with the BLAST program in the NCBI database. After the morphological and molecular identification, two different *Dunaliella* species were deposited in Ege University Microalgae Culture Collection. *D. salina* and *D. polymorpha* cells were firstly produce in both bubble column to monitor the growth profiles and then the species were cultivated in bubble column and stirred column photobioreactors (PBRs) under both high light intensity and different mixing conditions to investigate the total protein, carbohydrate, lipids and carotenoid concentrations. Moreover, this study aims to evaluate the production of  $\beta$ -carotene using two different PBRs. As a result of this study, *D. salina* in stirred PBR obtained the highest lipid ( $334.79 \pm 0.02$  mg/L), total carotenoid ( $96.7 \pm 0.02$  mg/L), and  $\beta$ -carotene content ( $21.18 \pm 0.03$   $\mu$ g/mL), while the maximum dry cell mass of 0.906 g/L was reached by *D. polymorpha* in bubble column PBR. The aim of this study was to investigate the nutritional values and  $\beta$ -carotene content of *Dunaliella salina* and *D. polymorpha* isolated from Turkey.

**Keywords:** *Dunaliella salina*, *Dunaliella polymorpha*, Isolation, Molecular identification, Carbohydrate content,  $\beta$ -carotene, Lipid content

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## Introduction

Microalgae have been long known for utilized of raw material for food products and feed animals. The three most important phyla of microalgae are to be in *Bacillariophyta* (diatoms), *Chlorophyta* (green algae), and *Chrysophyta* (golden algae). There are many trade applications of green microalgae products acquiring for carotenoids, lipids, proteins as used in various industries such as energy, cosmetics, pharmaceutical, bakery, and aquaculture. For instance, *Haematococcus pluviialis* is essential carotenoid as a source of astaxanthin, *Chlorella vulgaris* as a supplementary food product or food ingredient and the saline species of *Dunaliella* as a  $\beta$ -carotene resource. The  $\beta$ -carotene has a lipophilic terpenoid pigment, which can commercialize as food additives and provitamin A (Jesus and Filho, 2010). *Dunaliella* cells have a wide range of advantages in the production of chemicals such as carotenoids and xanthophylls with antioxidant, anticancer and anti-inflammatory activity, in bioremediation techniques, and in the production of biofuels used for *D. tertiolecta* biomass (da Silva et al., 2021). *D. salina* can grow up extensive open systems using raceway ponds in Australia, China, India, Chile, U.S., and Israel as a pigmenting agent ( $\beta$ -carotene) (Carvalho et al., 2006). Human bodies are not able to synthesize carotenoids; people must obtain enough amount from foods as a source of dietary supplements. More recent articles are determined the benefits of carotenoids to provide human healthcare such as the lower risk of inflammation, cardiovascular disease, neurodegenerative disease and diabetes, cancer prevention, improved ophthalmological diseases (Maoka, 2020). Although we provide carotenoids from nutrients of fruits and vegetables, a good alternative may be to use carotenoids from microalgae.

*Dunaliella* sp. is unicellular flagella green algae significantly found in halophilic environments world. *Dunaliella* is the richest resource of the carotenoid  $\beta$ -carotene and producing value compounds as high concentrations of glycerol and fatty acids (Elleuch et al., 2019). Halotolerant algae of *D. salina* has to accumulate large amounts of carotenoids (nearly 10-14% of the algal dry weight) and unicellular green microalgae of *D. salina* cells changes from red color under high light intensity, high salinity, limiting nutrient supplies (Zarandi-Miandoab et al., 2019). The morphologic taxonomy of *Dunaliella* has not openly defined under the different environmental conditions because *Dunaliella* do not own an apparent cell wall (Borowitzka and Silva 2007). However, the taxonomy on morphological of the genus *Dunaliella* has significant differences in environmental conditions such as brackish lake and marine species. Anatolia, accommodating extensive saline areas, is the findable region as salt and brackish water lakes (Tuz Lake, Seyfe Lake and Sultansazlıđı Lake) in Turkey.

Sandy-clay-loam textured soils of the region were found to be light and strong alkaline, too salty, very calcareous and low organic matter content (Abaci-Bayar et al., 2020).

The aim of this study is to investigate the growth and biochemical composition of isolated and identified *D. salina* and *D. polymorpha* in both bubble column and stirred column photobioreactors (PBRs) under light intensity of 300  $\mu\text{mol photons/m}^2\text{s}$  condition. Moreover, this study aims to evaluate the production of carotene especially  $\beta$ -carotene by indigenous *Dunaliella* species cultivated in two different PBRs.

## Material and Methods

### Isolation

Benthic samples were collected from the different stations of Aegean Sea/Burhaniye (located at 39°28'29.9"N 26°52'13.3"E) and Seyfe Lake/Kirsehir (located at 39°14'12.8"N 34°21'56.2"E) in Turkey. The samples were added to Daigo's IMK (FUJIFILM Wako Chemicals U.S.A. Corporation) liquid medium and incubated at 20  $\pm$  2 °C in the incubator (IKA shaker) for three weeks. Green microalgae cells were diluted by transferring to fresh medium several times for two to four weeks. Single colonies obtained from singular cells by repeated sub-culturing on agar (1.5%) plates as described by Andersen (2005). After several re-cultivations, a single colony was transferred into sterile tube in liquid medium. The cultured isolate was maintained at 22°C and a light intensity of 40  $\mu\text{mol photons/m}^2\text{s}$ .

### Growth Condition

*Dunaliella* species were cultured in Daigo's IMK medium as inoculum in 300 mL erlenmeyer flasks containing 150 mL of a liquid medium with adding the sea salt, and pH of the medium was arranged to 7.5. Batch cultures were kept at 22°C on an orbital shaker (IKA KS 4000 ic) at 110 rpm under continuous light with an intensity of 40  $\mu\text{mol photons/m}^2\text{s}$  for 15 days.

### Morphological Identification

Two different *Dunaliella* species were successfully isolated and continued in Daigo's IMK medium under laboratory conditions. *Dunaliella* species were discriminated by means of their morphological features cell shape, cell color, cell length, width, flagella length, and growth conditions. Bright-field microscopy photos from green microalgae were performed using a BX53 microscope (Olympus) equipped with a XC 30 camera.

### Molecular Identification

**DNA purification** Isolation of chromosomal DNA of the species of *Dunaliella* was performed with the ZR Fungal/Bacterial DNA MiniPrep (ZymoResearch).

**PCR amplification** 18S rDNA amplification was performed in 50  $\mu$ L reactions using primers SSUF-SSUR ([5'-TGGTTGATCCTGCCAGTAG-3']-[5'-TGATCCTCCG-CAGGTTACAC-3']; M1F-M2R ([5'-CGGGATCCGTAGTCATATGCTTGTCTC-3']-[5'-CG GAATTCCTTCTG-CAGGTTACAC-3']) and M1F-M3R([5'-CGGGATCCGTAGTCATATGCTTGTCTC-3']-[5'-GGAATTCGG AAACCTTGTACGAC-3']) (Olmos *et al.* 2000). The amplification was fulfilled using 35 cycles in a BioRAD thermocycler, with an annealing temperature of 54°C for the reactions. One cycle consisted of 1 min at 95°C, 1 min at 54°C and 2 min at 72°C. DNA and PCR products were analyzed by 1 % agarose gel electrophoresis in TBE buffer (Tris-Boric acid-Ethylenediaminetetraacetic acid (EDTA)) and stained with SYBR safe and visualized under UV illumination. **Phylogenetic Analysis** The analysis of the PCR sequence was made by RefGen Biotechnology Company (<http://www.refgen.com/>) in Turkey.

### Cultivation Conditions

Control of *Dunaliella salina* and *D. polymorpha* were grown in bubble column photobioreactor (PBR) (2 L), containing 1800 mL of Daigo's IMK medium including 21 g/L artificial sea salt at 20  $\pm$ 2°C under using photoperiod at 18:6 h (Light:Dark) cycle for 18 days. The light intensity of 50  $\mu$ molphotons/m<sup>2</sup>s provided by cool-white fluorescent and the aeration rate was at 2 L/min. *Dunaliella* species grown for control conditions under the light intensity of 50  $\mu$ molphotons/m<sup>2</sup> s were harvested during mid-log phase of growth. The microalgae cells were used as inoculants with a concentration of 20% (v/v) for photobioreactors experiments. The microalgae cell was counted using a Neubauer chamber, then specific growth rate and doubling time were measured from the logarithmic phase of growth curve as (specific growth rate)  $\mu = (ln X_2 - ln X_1)/(t_2 - t_1)$

where X<sub>n</sub>, cell numerousness on specific time point; t<sub>n</sub>, specific sample survey time (days). Doubling time (dt) was also calculated as  $dt = 0.693/\mu$  (Sener *et al.*, 2022).

The *Dunaliella* cultures were harvested at the beginning of stationary phase using centrifuged. Harvested *D. salina* and *D. polymorpha* were respectively grown to inoculate in the medium in two different (bubble column and stirred column photobioreactors) PBRs. Two different PBRs were used in the first 1 L only bubble column PBR and the second same volume of PBR having air bubbling aeration system with

magnetic stirrer (IKA) at a stirring rate of 100 rpm (Figure 1). Two PBRs were prior autoclaved to use. Two different mixing systems was used with the ventilation rate of 1 L/min controlled using flow meter (RST electronic Ltd, LZM-6T Turkey). Illumination was provided under the continuous light on both side bottles by LED lamp (Cata 10W CT-5254, Velman Fixed Luminaires BG-T5001 9W linear) with a light intensity of 300  $\mu$ mol photons/m<sup>2</sup>s. For 18 days cultivated cultures were harvested and then biomasses were dried using freeze drying (Christ- Alpha 1-2 LDplus).



**Figure 1.** Microalgae cultivated in different Photobioreactors (PBRs) system

### Analytic Methods

Two milliliters of the culture were centrifuged at 8000 rpm for 10 min. The pellets were extracted with 2 mL of 100% (v/v) methanol at 35 °C for 30 min in the ultrasonic bath (HYDRA ultrasonic). After the test tubes were centrifuged, the pigment contents (chlorophyll-a, chlorophyll-b and total carotenoids) were evaluated by spectrophotometre in methanol extracts at 480, 652, 665 and 750 nm. The amounts of the pigments were ultimately calculated by the following equations 1-3:

$$\text{Chl} - a \left( \frac{\text{mg}}{\text{L}} \right) = -8.10 \times (A652) + 16.57 \times (A665) - A750 \quad \text{Eq. 1}$$

$$\text{Chl} - b \left( \frac{\text{mg}}{\text{L}} \right) = 27.44 \times (A652) - 12.17 \times (A665) - A750 \quad \text{Eq. 2}$$

$$\text{Total Car} \left( \frac{\text{mg}}{\text{L}} \right) = 4 \times A480 - A750 \quad \text{Eq. 3}$$

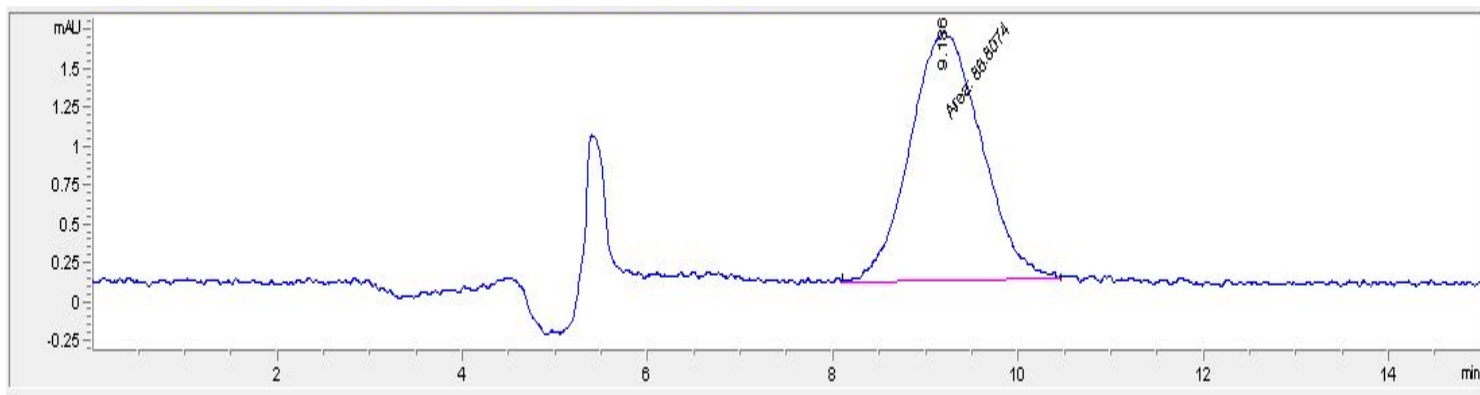
The content of total carotenoids and chlorophylls were calculated according Wellburn method (Ajala and Alexander, 2020).

### Total Protein, Carbohydrate, Lipid and B-Carotene Measurement

Total protein content was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard. Total carbohydrate content was measured based on the phenol-sulphuric acid reaction of carbohydrate (Dubois et al., 1956) by D-glucose as a standards ranging in concentration from 0 to 150 µg/mL. Lipid was extracted from lyophilized cell biomass using a modified Bligh and Dyer's method (Bligh and Dyer, 1959) as described by (Sahin et al., 2019). Total lipids were dosed gravimetrically.

β-carotene extraction: 0.01g dried weight of algal mass was extracted with 10 mL methanol (MeOH) sonicated in ultrasonic bath for 15 min. The extraction was cleared by centrifugation at 6000 rpm at 4°C for 10 min, then 2 mL of the supernatant was filtered through 0.45 µm syringe filter into HPLC vials. The β-carotene extraction solution was analyzed by an Agilent 1260 Infinity HPLC system with DAD detector an Agilent 5 µm, 250\*4.6 mm C18 column (Figure 2). Carotenoids were extracted from lyophilized cells using MeOH as extraction solvent. In the mobile phase 100%, solvent A was as methanol and hexane (75:25, v/v). The flow rate was 1 ml/min, 0-14 min.

Each results were obtained with three biological replicates and all data were shown as mean ± standard deviation. It was considered as significant when  $p < 0.05$ .



**Figure 2.** β-carotene detected using HPLC-DAD



## Results and Discussion

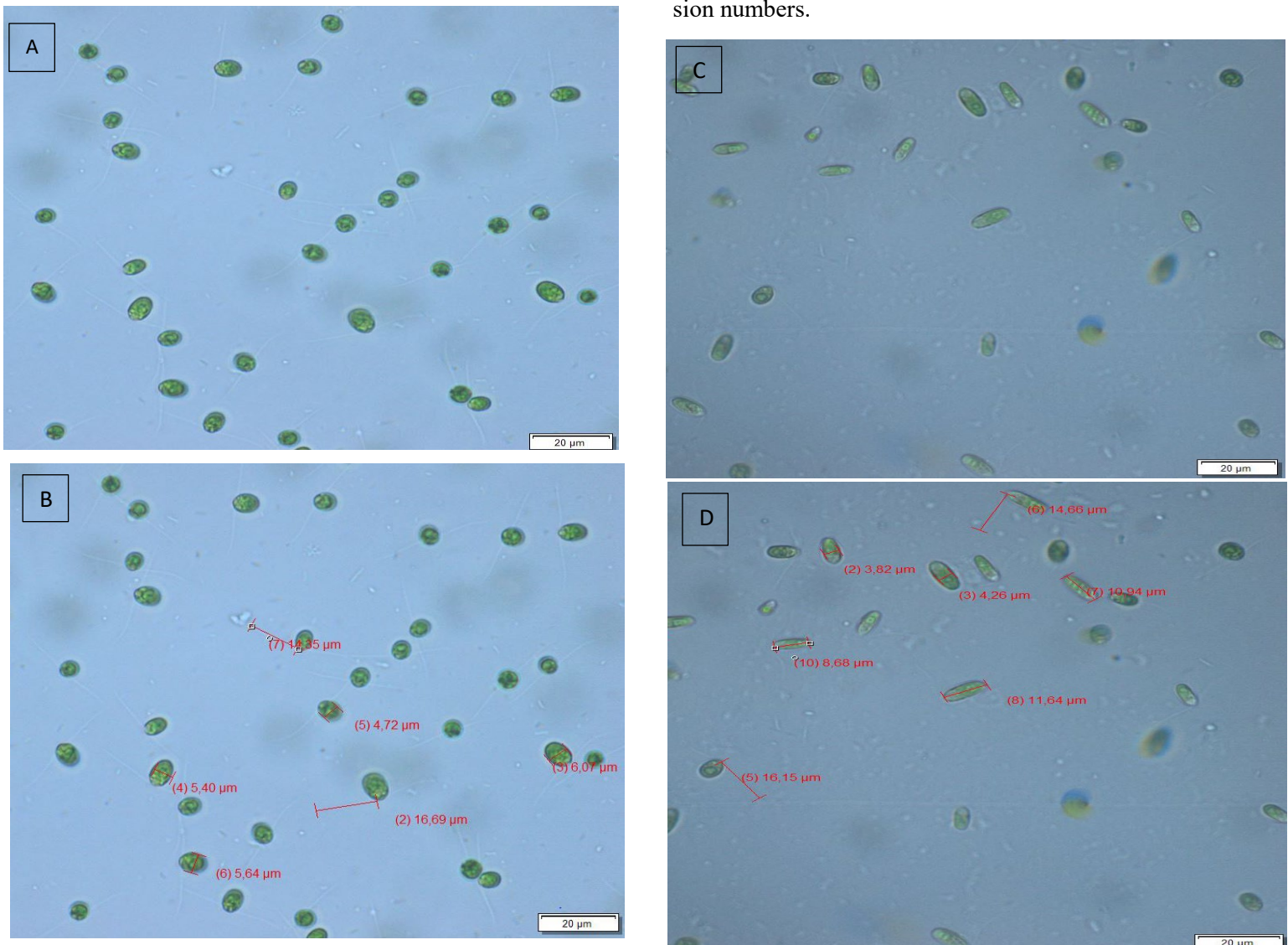
### Morphological and Molecular Identification

The morphologic taxonomy of *Dunaliella* has not been easily determined due to the cell morphological changeability and non-existing of the rigid polysaccharide cell wall (Emami et al., 2015 and Elleuch et al., 2019). The identification of species relying on only morphological characteristics is able to be troublesome. For this reason, reliable and accurate methods can be used to evaluate molecular variation.

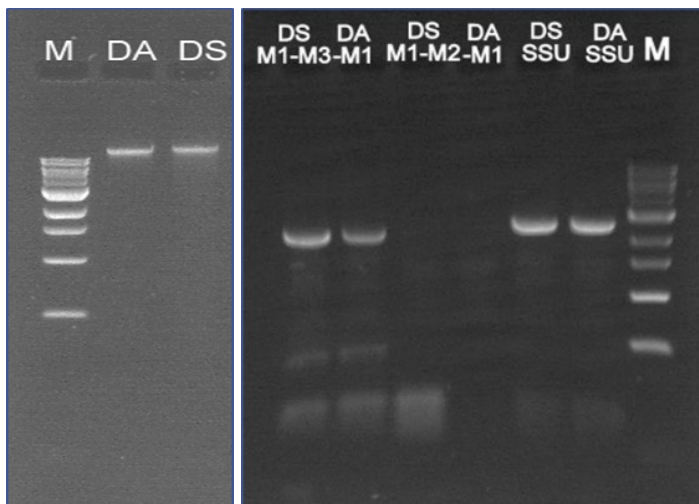
Morphological identification; *Dunaliella salina* Teodoresco (1905); Cells oblong, pyriform, ellipsoidal to cylindrical with round anterior and posterior regions with two equal long flagella; chloroplast situated in the basal region; each cell 10.0-15.5 mm long and 6-9 mm wide; flagella

16-21 mm long. *Dunaliella polymorpha* Butcher (1959a); Cell generally green, radially symmetrical, mostly oval, ellipsoidal or cylindrical, 8-12  $\mu\text{m}$  long, 5-8  $\mu\text{m}$  wide. Flagella length about 1.5 times the cell length. Stigma small and medial.

Phylogenetic analysis of green algae evaluated using combined SSU rDNA gene sequence alignment and bright-field microscopical observations. Two different isolates of *Dunaliella* have been exposed to the comparison of 18 S rRNA regions for amplification. BLAST examined on NCBI-nucleotide database resulted in the highest similarity to *D. salina* (GenBank acc. no: KR340579, KR340580). Furthermore, the 18S rDNA gene sequence of *D. polymorpha* had been deposited in the GenBank with KR340581, KR340582 the accession numbers.



**Figure 3.** A, B: *Dunaliella polymorpha*; C, D: *Dunaliella salina* light microscope photographs



**Figure 4.** DNA and PCR products from *Dunaliella polymorpha* (DA) and *D. salina* (DS); M: size markers (1 kp DNA ladder) A: DNA isolation of DA and DS; B: 18S rDNA of DA and DS; (primers SSUF-SSUR, M1F-M2R, M1F-M3R)

Olmos et al., (2000) was reported that the primers pair (M1, M2) could let the amplification of the tallness of 18S rDNA in microalgae of *Dunaliella*. On the other hand, the report made M3 oligonucleotide analyzing from the 3' termini of *D. salina* and then proven homological features with entire strains.

Phylogenetic analysis of green algae was evaluated using combined SSU rDNA gene sequence alignment and bright-field microscopically observations. In terms of the conspecific of the single-celled microalgae, stems from SSU rDNA sequence similarities could be not the whole time coherent with those from DNA base (mol % GC) values. Both of the species characteristic by their surprisingly range of DNA base composition values emerged SSU rDNA sequence the highest similarities among strains of *Prototheca zopfii* or *Chlorella sorokiniana* (Ueno et al., 2003 and Krienitz et al., 2011).

The isolated and identified indigenous strains of *Dunaliella salina* (EGEMACC 84) and *Dunaliella polymorpha* (EGEMACC 22) were joined to Ege University Microalgae Culture Collection (EGEMACC-<http://www.egemacc.com/>), Turkey. Cryopreservation of strains according to Day and Stacey (2007) will be applied in the future for alternative long-term storage by the culture collection.

### Microalgae Growth Conditions

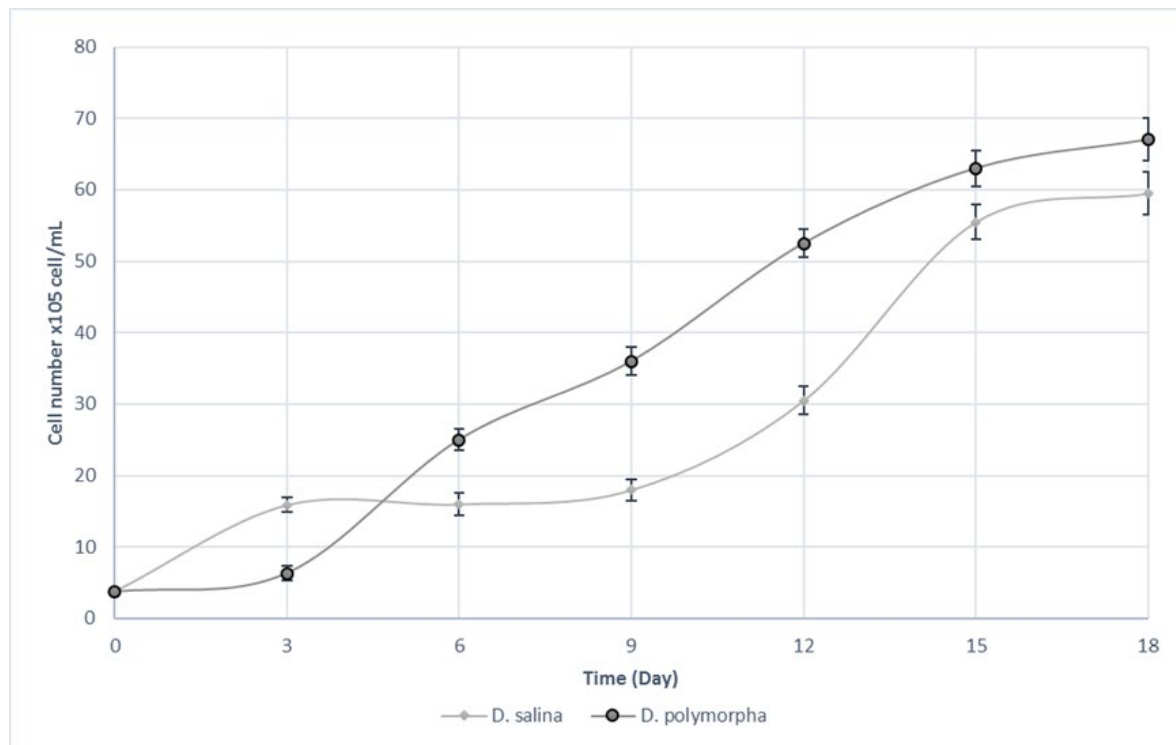
*Dunaliella salina* and *D. polymorpha* were respectively grown in the Daigo's IMK Medium for 18 days in bubble column PBR used to control. Kanamoto et al., 2021 reported that

the marine microalgae *Pavlova* spp. cultivated the highest biomass production and highest fucoxanthin accumulation compared with f/2 and Walne's media, the use of grown in 50% seawater enriched with either 2× Daigo's IMK medium. The presence of seawater elements in Daigo's IMK medium was determined in the highest biomass (0.92 g dry cell weight/dcw)/L and the fucoxanthin concentration (2.62 mg/g dcw) after the cultivation. According to Colusse and colleagues (2020), the economical evaluation of media and biochemical analyses on biomass growth using different culture (F/2, Conway, and Johnson) media were investigated in *D. salina*. *D. salina* were grown in Daigo's IMK medium made with artificial seawater for dissolving 22 g/L sea salt (Sener et al., 2022). The cell cultivated under optimum conditions was illustrated in Figure 5. The specific growth rate of *D. polymorpha* in the growth phase was higher ( $\mu_{max} = 0.281$  and  $dt=2.46 \text{ day}^{-1}$ ) than that of *D. salina* ( $\mu_{max}= 0.218$  and  $dt=3.18 \text{ day}^{-1}$ ) at the light intensity of  $50 \mu\text{mol photons/m}^2\text{s}$  at 18:6 h (L:D). Khadim et al., 2018 used the same photoperiod of 16:8h L:D for *D. salina* inoculum preparation. Ricardo et al., 2018 showed that *D. salina* reached the highest densities at low salinities (100 and 500 mM NaCl) under a continuous light regimen. When exposed to 500 mM NaCl at 18:6 h L:D period, carotenes such as neoxanthin and violaxanthin obtained the furthest ample pigment. *Chlorella vulgaris* was cultivated at different light:dark periods. After, the maximum growth rate was 16:8 h L:D cycle (Kendirlioglu et al., 2015).

In the study of two-phase cultivation of carotenogenic microalgae *D. salina* and *D. polymorpha* in Turkey, their biochemical characteristics were studied and their production potential was determined. Given in Figure 5, the exponential phase cells removed from the medium and inoculated at irradiation  $300 \mu\text{mol photons/m}^2\text{s}$  in both bubble column and stirred column photobioreactors (PBRs). *D. salina* and *D. polymorpha* were cultivated under stress condition in bubble column and stirred column PBRs. The use of only a bubble could bring about weak mass transfer leading to the decreased contact area between liquid and gas (Kunjapur and Eldridge 2010). In each bioreactor configuration category, certain conditions for optimal cultivation are applied for the selected strain of microalgae. Also, green microalgae in different PBRs under  $300 \mu\text{mol photons/m}^2\text{s}$  light intensity were evaluated to determine the protein, carbohydrate, lipid, carotenoid concentration and  $\beta$ -carotene content. *D. salina* in stirred PBR gave the highest lipid ( $334.79 \pm 0.02 \text{ mg/L}$ ), carbohydrate ( $40.94 \pm 0.04 \text{ mg/L}$ ), protein ( $137 \pm 0.013 \text{ mg/L}$ ), carotenoid ( $96.7 \pm 0.02 \text{ mg/L}$ ) and  $\beta$ -carotene content ( $21.18 \pm 0.03 \mu\text{g/mL}$ ) by comparing with other cultivation systems given in Table 1. The maximum biomass concentration of *D. poly-*

*morpha* 0.906 mg/L was found at light intensity 50  $\mu\text{mol photons/m}^2$  while the lipid content ( $276.70 \pm 0.01$  mg/L) and  $\beta$ -carotene content ( $17.51 \pm 0.02$   $\mu\text{g/mL}$ ) was obtained under higher light intensity. Gharajeh et al., 2020 reported that the lipid, protein, carbohydrate, and pigment content of three isolates, *Dunaliella* sp. ABRIINW-B1, -G2/1 and -I1 were measured as produced respectively 42, 36 and 47% lipid content as well as the occurrence of high lipid and low carbohydrate (4–7%). The protein contains for *Dunaliella* species varies as about 40% *Dunaliella* (Hosseini Tafreshi & Shariati

(2009)), 30–43% in *D. salina* (Muhaemin & Kaswadji (2010)) and 57% in *D. salina* (Berker, 2007). The lipid content for *Dunaliella* species varies as 23% in *D. primolecta*, 6–25% in *D. salina*, 17–67% in *Dunaliella* sp., 16–71% in *D. tertiolecta* (Ahmed et al., 2017). Ishika et al. 2018 reported that under high salinity (up to salt saturation (250 ppt)) cultivated and determined the average lipid and average carbohydrate content of *Dunaliella salina* 56.2% and 13.7%, respectively. Although total lipid, protein content was not very high, this study was nearly those of previous studies (Table 1).



**Figure 5.** Control groups of the growth kinetic of *Dunaliella salina* and *D. polymorpha* cultivated under optimum condition by cell counting

**Table 1.** Effects of aeration and agitation in photobioreactors (PBR) on dry biomass, protein, carbohydrate, lipid, carotenoid and  $\beta$ -carotene content in *Dunaliella salina* and *D. polymorpha*

	Dry cell mass (g/L)	Protein content (mg/L)	Carbohydrate content (mg/L)	Total Lipid content (mg/L)	Carotenoid (mg/L)	$\beta$ -carotene content ( $\mu\text{g/mL}$ )
Control <i>D. salina</i>	0.801	186 $\pm$ 0.027	75.04 $\pm$ 0.05	213.24 $\pm$ 0.02	82.8 $\pm$ 0.02	10.94 $\pm$ 0.02
<i>D. salina</i> Bubble column PBR	0.666	132 $\pm$ 0.014	33.24 $\pm$ 0.02	264.34 $\pm$ 0.02	77.9 $\pm$ 0.03	13.49 $\pm$ 0.04
<i>D. salina</i> stirred PBR	0.663	137 $\pm$ 0.013	40.94 $\pm$ 0.04	334.79 $\pm$ 0.02	96.7 $\pm$ 0.02	21.18 $\pm$ 0.03
Control <i>D. polymorpha</i>	0.906	149 $\pm$ 0.001	89.12 $\pm$ 0.035	204.57 $\pm$ 0.03	83 $\pm$ 0.01	14.05 $\pm$ 0.04
<i>D. polymorpha</i> Bubble column PBR	0.799	110 $\pm$ 0.016	39.65 $\pm$ 0.02	276.70 $\pm$ 0.01	94.6 $\pm$ 0.02	17.51 $\pm$ 0.02
<i>D. polymorpha</i> stirred PBR	0.697	113 $\pm$ 0.01	30.13 $\pm$ 0.016	268.58 $\pm$ 0.04	88.6 $\pm$ 0.01	15.35 $\pm$ 0.03

Mean  $\pm$  standard deviation



This study showed that the variance of *Dunaliella* species and cultivation conditions significantly altered the metabolite concentrations in the cells. The effects of nitrogen, sulfur, and phosphorus limitations, different light intensities, and different CO<sub>2</sub> concentrations on growth and lipid accumulation were investigated for *D. salina*. According to Yuan et al., 2019 when high light intensity enhanced carbohydrate accumulation, low light intensity was beneficial to lipid accumulation under N-limited conditions. Ahmed et al., 2017 considered *D. salina* owned high lipid accumulation for the production of biofuel, industrial, and pharmaceutical purposes. *Dunaliella* cells have a lack of rigid cell walls made of cellulose; moreover, the disruption of cells is much speedier than that in green microalgae. However, *Dunaliella* cells can be easily damaged from stress-causing rupture of the air bubbles at the culture surface and mixing agitation of culture medium in PBRs. Ajala and Alexander 2020 reported that the productivity of algae affects the hydrodynamic effects of aeration and agitation in the PBR. In the current study, aeration and both stirring and aeration were performed to the *Dunaliella salina* and *D. polymorpha* cultures to state the biomass concentration and biochemical composition in different cultivation conditions.

## Conclusion

This study has detected in two different photobioreactors how two identified indigenous *Dunaliella* strains play a role for the accumulation of carotenoids and biochemical compounds in two different photobioreactors. Carotenoid productivity in cells is known to enhance with high light intensity and different mixing systems in green microalgae. Nevertheless, among the newly isolated *D. salina* and *D. polymorpha*, much more productivity of  $\beta$ -carotene content was not determined under applied high light intensities and different mixing systems in this study. The biochemical composition performance of the newly isolated strains show a different cultivation strategy needed for all strains. In this study, strain selection from *Dunaliella* species emphasizes the investigation of their biochemical characteristic for the commercial production of carotenoids on human health products and animal feed.

## Compliance with Ethical Standard

**Conflict of interests:** The authors declare that for this article they have no actual, potential or perceived conflict of interests.

**Ethics committee approval:** Ethics committee approval is not required for this study.

**Funding disclosure:** -

**Acknowledgments:** The author is thankful to Associated Professor Dr. Esra Imamoglu for providing language help. The Author thanks Dr. Zinar Pinar Gumus for performing the HPLC-DAD analysis.

**Disclosure:** -

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