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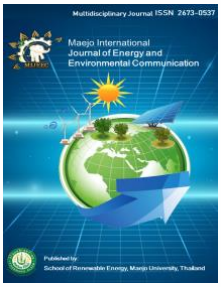

ARTICLE

# Evaluation of biomass growth, β-carotene, and lipid accumulation on *Dunaliella* sp. under the effect of various salinity concentrations

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ABSTRACT

Environmental factors highly influence microalgal strain growth and chemical compositions. Parameter salinity plays a key role in the growth of halotolerant microalgae, especially *Dunaliella* sp. Therefore, *Dunaliena* sp.'s resilience at various salinity concentrations was investigated by monitoring the development, biomass productivity, pigment concentration, and lipid accumulation. The growth and biomass productivity were found to be significantly higher at triple the concentration of salinity than the standard concentration. The lipids and pigments were extracted and analyzed by HPLC. The results show that algal growth and accumulation of beta-carotene and lipids were strongly influenced by salinity level in nutrient media. The pigment and lipid composition were analyzed rapidly by HPLC and confirmed the potential of *Dunaliella* sp. biomass for commercial applications.

## 1. Introduction

*Dunaliella salina* is a single-cellular microalgae that can store a lot of carotenoids, and the amount of β-carotene inside its cells might be more than 10% of their dry weight (Marin et al., 1998; Tsai et al., 2023). Thus, this species becomes more critical to be a source of β-carotene for the health of nutraceutical and pharmaceutical industries. Various Dunaliela microalgae species are well known, such as *D. bandawil*, *D. bioculata, D. primolecta, D. salina,* and *D. tertiolecta,* for high content of β-carotene. *Dunaliella* cells are spherical and look egg-shaped. The chlorophyll content is present in a cup-shaped with a pyrenoid center and surrounded by starchy seed (Bhuyar et al., 2020b; Kumaran et al., 2023).

The biochemical components of microalgal cells are subject to alterations due to external environmental factors, as microalgae possess flexible metabolic systems (Bhuyar et al., 2019, 2021b,c,d). Stress conditions that cause metabolic alterations in microalgae and result in distinct cell compositions include intense light, salinity, nitrogen deficiency, and extreme temperatures (Ren et al., 2021; Bhuyar et al., 2020a, 2021a). The cells can increase

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under optimal conditions such as sufficient nutrients, pH, temperature, and salinity. Environmental factor salinity plays a vital role in accumulating photosynthetic pigments (Palanisamy et al., 2023a, b). Under a high salinity environment, the cells were protected from microbial contamination, had high adaptability and toughness, and could survive in extreme environments (Naka and Kurahashi et al., 2024). Besides this, salt stress treatment is a much more convenient method to enhance beta-carotene accumulation. Thus, the potential of *Dunaliella* sp. to operate and has significant commercial value to cultivate in a shorter period. The ability of *D. salina* to flourish in harsh environments has long been taken advantage of in photoautotrophic production systems across the globe (Capa-robles et al., 2021). However, photoinhibition, photooxidative damage, and self-shading of cultures meant to create high cell densities result in low yield and productivity under this approach.

*D. salina* is mainly recognized as the most significant source of β-carotene production but has many other uses. β-carotene is a naturally occurring C40 carotenoid pigment with critical medicinal properties since it inhibits several tumors and cancers. Carotenoids, comprising carotenes and xanthophylls like lutein and β-carotene, are essential components of photosystem II. Thus, carotenoids prevent photo-oxidative damage to the photosynthetic machinery by decreasing reactive oxygen species (ROS) generation and deactivating them under extreme conditions.

Additionally, salinity is thought to have no real impact on *D. salina* cells' ability to accumulate beta-carotene. Depending on the microalgae species employed, salinity appears to influence beta-carotene synthesis in microalgae similarly to other parameters. Therefore, this study aims to investigate the growth of *Dunaliella* sp. under different salinity concentrations. The effect of salinity on the accumulation of lipid and β-carotene in cells was evaluated. It optimized the salinity concentration for higher biomass and β-carotene accumulation to enhance the value of *Dunaliella* sp. biomass application in industry.

## 2. Material and methods

### *2.1 Dunaliella* sp. and culture conditions

The *Dunaliella* sp. culture was obtained from Algae International Berhad, Port Dickson Farm. The microalgae were maintained in 10% of salinity under a laboratory environment. For the experiment, the f/2 media was prepared at different salinity concentrations of 15, 30, 45, 60, and 75% (NaCl w/v) by combining standard chemicals of f/2 media with modified concentrations. The sea salt (in grams) was added into the media in various ratios with one liter of media. The cultures were grown in 1L of Erlenmeyer flask under 200 µmol photon $m^{-2}s^{-1}$ LED light intensity with control temperature at 25±2°C. Three parallel groups were set up for each cultural situation. Every experimental combination underwent a triple assay. The initial concentration of *Dunaliella sp.* culture 1.2×105 is equivalently added into all the flasks with different salinities. All the flask cultures were aerated continuously with an air pump containing 0.04±0.02% of carbon dioxide. The culture growth was monitored for 14 days.

### 2.2 Growth and biomass measurement

The growth of *Dunaliella* sp. was determined by measuring the absorbance (optical density) reading by UV-Vis spectrophotometer at 660 nm (Jayakumar et al., 2021). The sample (1mL) was collected from the experimental flask in a cuvette to take optical density triplicate readings. During the early stage of the stationary phase, 10 ml of culture was collected and centrifuged at 45000 rpm for 5 minutes to separate the media from biomass. The harvested biomass was rinsed twice with distilled water to eliminate the salt substance and centrifuged again. The wet biomass was transferred into a heat chamber and dried at 75°C for 24 hours until obtaining constant weight.

### 2.3 Extraction of lipid

The dried biomass 2.5 g was soaked in 50 ml of hexane solvent overnight. The mixture was vortexed for 5 minutes. Then, the mixture was placed in water of ultrasound sonication at 60±2°C for 4 hours. The hexane solvent was separated from the biomass by centrifugation at 45000rpm, 20°C temperature for 10 minutes. The separated solvent was placed in a vacuum chamber until the solvent dried completely. The remaining lipid inside the breaker was weighed.

### 2.4 Pigment analysis

The pigment concentrations were measured by soaking *Dunaliella* sp. pellet in 80% acetone overnight. Then, the biomass from the acetone solvent was separated by centrifugation at 4000 rpm for 5 minutes. A spectrophotometer measured the supernatant absorbency at 412, 431, 460, and 480 nm. The following formula calculates the content of the pigment (µg/ml β-carotene). C*a*, C*b*, and C*c* represent chlorophyll a, b, and β-carotene, respectively.

$$Ca = 1.709A412 + 11.970A431 – 2.998A460 – 5.708A480$$
$$Cb = 0.171A412 – 0.230A431 + 11.871A460 – 13.248A480$$
$$Cc = 0.430A412 + 0.251A431 – 4.376A460 + 13.216A480$$

### 2.5 HPLC analysis

The pigments were analyzed by performing HPLC under an optimized analytical method. Analytical separations were performed using a reverse C18 column at a temperature of 25°C. The mobile phase: A) 5 mM ammonium acetate in water; B) 5 mM ammonium acetate in MeOH; C) ethyl acetate. The sample 50 µL was injected at 0.5mL/min flow rate with an optimized HPLC gradient.

### 2.6 Statistical analysis

Statistical analysis was carried out using Microsoft Excel software. Significant differences ($p<0.05$) between treatments were tested by one-way ANOVA (analysis of variance) ($P<0.05$).

## 3. Result and Discussion

### 3.1 Effect of salinity on the growth and biomass production of *Dunaliella* sp.

The growth of culture is highly influenced by the salinity of media, which can be confirmed based on the growth and biomass production shown in Figure 1. The highest growth was found at 30%, followed by 45% of salinity of media concentration. The cells in the culture took 2 days to become familiar with the salinity environment before rapidly growing. The biomass production was highest in 30% salinity (1.43±0.11g/L), followed by 45% salinity concentrations (1.32±0.10g/L). There is no significant difference between 30% and 45% salinity concentration ($p>0.05$). *Dunaliella* sp. could quickly adapt and produce high biomass in both concentrations compared to other salinity concentrations.

The lowest growth was found in extreme concentrations of salinity. A similar condition was discovered by Hu et al. (2024) in the cultivation of D. salina with seven salinity groups. The lower salinity groups show rapid growth after 2 days of cultivation; however, higher salinity groups initiated the culture to grow from day four. The cells require more time to adapt to a higher salinity environment. In contrast, Filho and Jesus (2010) conducted cultivation of *D. salina* under similar operational conditions and found that the growth rate was significantly decreased at high salinity. Therefore, it can adapt to high salinities essential for growth and biomass production.

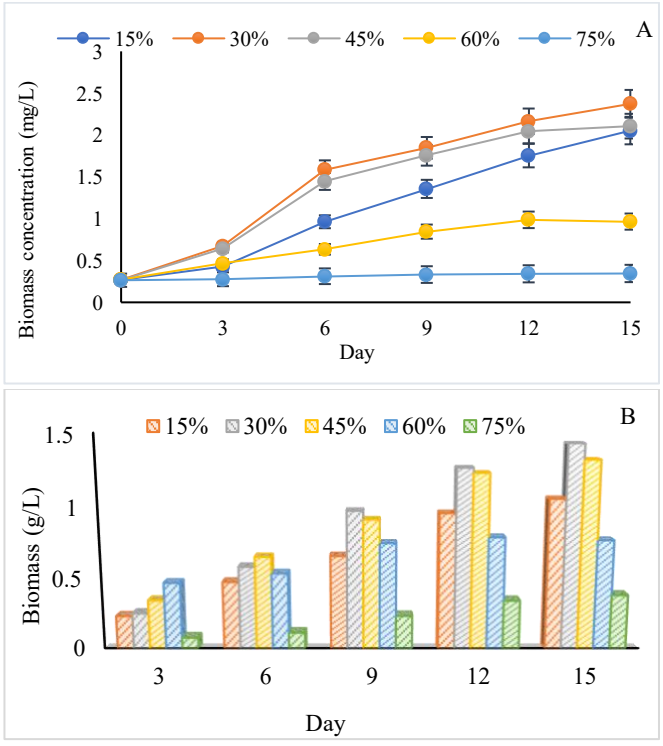


**Figure 1** The biomass concentration (mg/L) (A) and Biomass production of *Dunaliella* sp at different salinity concentrations (%) (B).

### 3.2 Effect of salinity in Beta carotene production

Figure 2 (A) shows that the highest beta carotene obtained from the *Dunaliella* sp. is 14.7±2.2µg mL-1 and 13.5 µg $mL^{-1}$ from salinity 45% and 30%, respectively. High salinity boosts the accumulation of Beta carotene even though the growth of culture is lower at a 45% salinity level. Chlorophyll content was higher at 30% than 45%, as shown in Figure 2 (B). *Dunaliella* sp.'s photosynthesis enhances pigment compounds' biosynthesis to grow at maximum chlorophyll content under optimum environmental conditions. Salinity is among the ecological stresses influencing *D. Salina*'s increased accumulation of β-carotene. Higher salinity (hypertonic) can lead to cell shrinkage, while lower salinity (hypotonic) might promote cell swelling. Authors Filho and Jesus (2010) found that beta carotene production steadily increased in the medium containing 0.5M salt concentrations. Meanwhile, 0.2M salt concentration shows an initial steady accumulation of beta carotene but then reaches the stationary phase. The combination of parameters of nutrient glycerol (12.5 mM) glycerol, 3.0 M salinity, and light intensity (50 µmol photons $m^{-2}s^{-1}$) under mixotrophic enhanced the growth and β-carotene accumulation in *Dunaliella salina* (Capa-Robles, 2021).

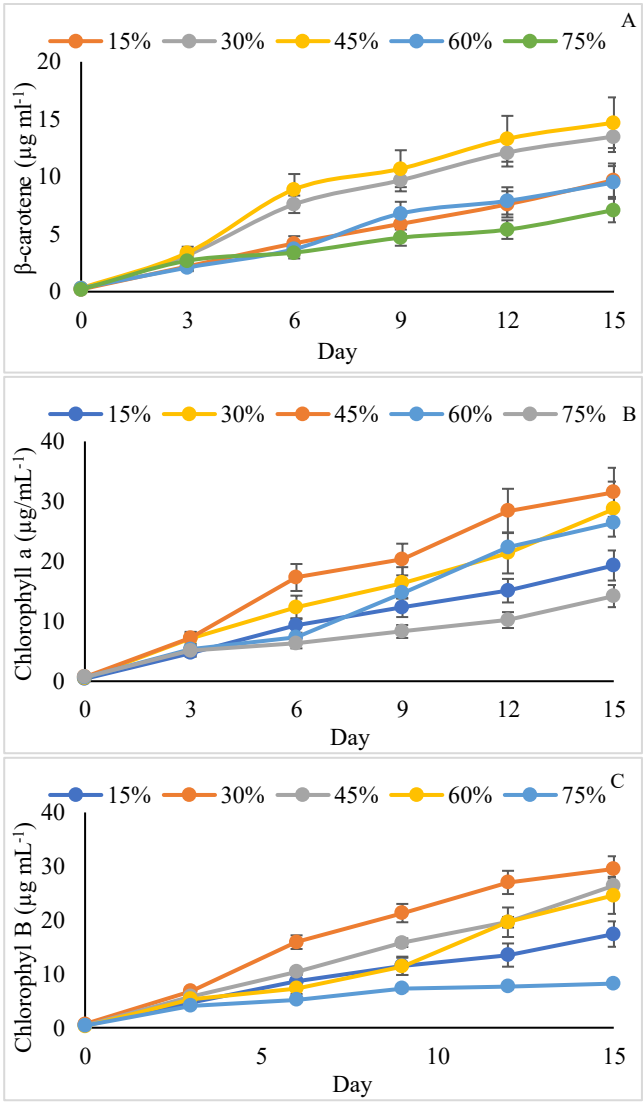


**Figure 2** The changes of pigments accumulation β-carotene (A), Chlorophyll A (B), and chlorophyll B (C) in different salinity concentrations

### 3.3 Lipid content of *Dunaliella* sp.

The lipid content accumulation extracted from *Dunaliella* sp.'s dried biomass under different salinities. The highest lipid content (18.45±1.3%) in 30% of salinity is shown in Figure 3. The extreme salinity concentration inhibits the growth and does not enhance the accumulation of lipid content. The optimum salinity condition ranges between 30 to 45%. Salinity enhances the maximum accumulation of lipids in the cells compared to other salinity concentrations. A similar study conducted by Gao et al. (2021) found the salinity of *D. salina*_YC led to a progressive increase in its total lipid content, the specific activity of superoxide dismutase, and beta-carotene concentration. Many studies have proved that nitrogen starvation can accumulate lipid content in many microalgae cells.

However, *Dunaliella* sp. does not show any accumulation of lipid content under nutrient starvation, according to (Chen et al., 2015). Another light factor influencing the lipid productivity in the *Dunaliella* sp. increased by 35.33% when the culture was grown under an ideal ratio of red and blue light (4:3) compared to white light. Byrd et al. (2017) found that the maximum total fatty acid content was up to 65% by dry weight in high salinity compared to control media in *Dunaliella* sp. Under brief high salinity stress conditions, these strains may assimilate some available glycerol as neutral lipids or triacylglycerols. Rismani et al. (2017) found that in D. salina cells, the maximum amount of total lipid was achieved at 3M NaCl. On the fifteenth day, this was almost equal to 143 mg. g dw-1, practically 14% more than at the beginning of the trial and 43% more than the control. There was no discernible variation in 3M NaCl's total fat content. Therefore, salinity factors highly influence lipid content accumulation in *Dunaliella* sp's biomass.

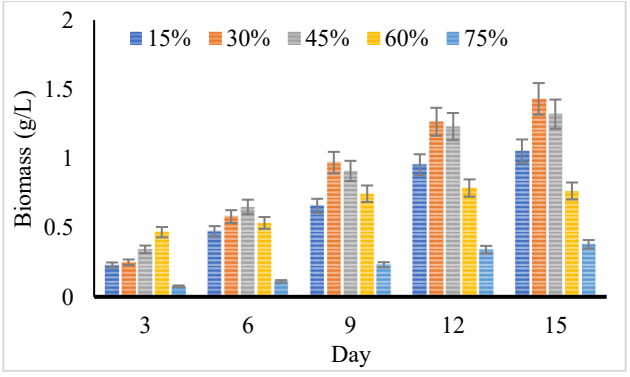


**Figure 3** Lipid content of *Dunaliella* sp. obtained from different salinity biomass.

### 3.4 HPLC analysis

The effect of salinity concentration on *Dunaliella* sp. pigment accumulation was noticed by HPLC analysis. The pigment quantification and identification were done in HPLC to confirm the pigment profile analysis. Table 1 shows the lists of carotenoids obtained from the HPLC analysis with an isocratic solvent system. A total of 10 pigments were found in the sample *Dunaliella* sp. These pigments had high potential for industrial applications for human health. Author Reshma et al. (2021) found higher chlorophyll a, b, and total carotenoid and β-carotene pigments at higher salinity. Therefore, salinity highly influences the growth and accumulation of pigments under optimum conditions.

**Table 1** The list of pigment analysis by HPLC

| Pigment | Molecular Formula | mg/g |
|---|---|---|
| Antheraxanthin | $C_{40}H_{56}O_3$ | 0.66 |
| Cryptoxanthin epoxide | $C_{40}H_{56}O_2$ | 0.53 |
| Epoxy lutein | $C_{40}H_{56}O_3$ | 3.47 |
| Flavoxanthin | $C_{40}H_{56}O_3$ | 0.74 |
| Fucoxanthin | $C_{42}H_{58}O_6$ | 2.56 |
| Neoxanthin | $C_{40}H_{56}O_4$ | 0.24 |
| Rubixanthin | $C_{40}H_{56}O$ | 0.62 |
| Chlorophyll b | $C_{55}H_{70}MgN_4O_6$ | 3.67 |
| Chlorophyll a | $C_{55}H_{72}MgN_4O_5$ | 13.24 |
| β-carotene | $C_{40}H_{56}$ | 6.64 |
| Total | | 32.37 |

## 4. Conclusion

The growth of *Dunaliella* sp. shows various performances in different salinities and is reflected in lipid and pigment biosynthesis accumulation. Therefore, greater salinity must be provided to optimize the maximum yield of biomass and pigments, especially β-carotene. Depending on the bioactive compound strain selection and condition of cultivation applied to obtain maximum biomass yield. The growth and pigments of *Dunaliella* sp. were improved under optimized cultured conditions of salinity. The salinity concentration is highly responsible for the accumulation of biomass and photosynthesis pigments in *Dunaliella* sp.

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