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ORIGINAL RESEARCH

# Effect of phosphorus and sodium chloride levels on growth performance, carotenoid accumulation and isomerization to *9-cis* β-carotene in Thai *Dunaliella salina* NUAC09

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**Abstract** *Dunaliella* is known as a commercial of β-carotene-producing microalgae. *D. salina* NUAC09 was previously isolated, and its characterization and beta-carotene-producing efficacy were investigated for further application. This study aimed to examine the effect of phosphorus (0, 0.043, 0.053 and 0.07 g $KH_2PO_4$/L) and sodium chloride (0.5, 1, 1.5, 2 and 2.5 M NaCl) on growth performance, accumulations of pigments, antioxidant activity and *cis*, *trans* β-carotene isomers ratio of Thai *D. salina* NUAC09. The strain NUAC09 was done using morphological investigation and phylogenetic analyses based on Internal Transcribed Spacer (ITS) sequences. Morphological features and the ITS phylogenetic trees revealed that the NUAC09 could be identified as *D. salina*. The strain NUAC09 was then separately grown under various phosphorus and NaCl levels as described above. Results showed that maximum cell density, chlorophyll *a* content, total carotenoid content, and biomass dry weight was observed in cells grown under 0.07 g $KH_2PO_4$/L and 2.5 M NaCl. HPLC analysis revealed that two β-carotene isomers, *all-trans* and *9-cis* β-carotene were detected at 20.8 and 21.4 min retention time, respectively. The maximum *all-trans* β-carotene (178, 238 mg/g DW) and *9-cis* β-carotene (99, 156 mg/g DW) content and the highest antioxidant activities (72.4 and 74.6 % inhibition) were observed in the absence of phosphorus (0 g $KH_2PO_4$/L) and 0.5 M NaCl, respectively. Overall, our results indicate that phosphorus and NaCl levels affect the growth, total carotenoid, antioxidant activity and β-carotene isomer content of NUAC09, suggesting a strategy to boost *9-cis* isomer production by adjusting the nutrients in the growth medium.

**Keywords** *Dunaliella* . Phosphorus . NaCl . β-carotene isomer . 9-cis β-carotene . Antioxidant activity

## Introduction

A reddish-orange pigment β-carotene is commonly present in many vegetables, fruits, and microalgae. This pigment is the main source of pro-vitamin A and is utilized in several industrial production processes owing to its antioxidant properties (Pourkarimi et al. 2020). The application of the pigment is not only limited to nutrition supplements, but also pharmaceutical and cosmetic industries (Davidi and Pick 2017).

There are two types of structural isomers of β-carotene are *cis*- and *trans*-isomers. Recently, the best-known *cis*-isomer exhibited better antioxidant properties than the *trans*-isomer (1.3 to 2.4) (Levin and Mokady 1994; Hu et al. 2008; Relevy et al. 2015; Weinrich et al. 2019). However, most of the β-carotene industrially produced and used to date came from chemical synthesis which was only *trans*-isomer. In

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contrast, the natural extract of β-carotene provides *cis*- and *trans*-isomers (Xu et al. 2018). This has led most consumers to pay more attention to the β-carotene obtained from natural sources, rather than chemical synthesis. The health concerns were that synthetic β-carotene might have residual harmful substances (Weinrich et al. 2019).

*Dunaliella* algae are one of the richest sources for natural carotenoids and accumulates a high content of β-carotene (up to 10% of the dry biomass), comprising *all-trans*, *9-cis*, *13-cis*, and *15-cis* isomers. Among these isomers, *9-cis* β-carotene makes up 50% total β-carotene, approximately (Xu et al. 2018). As this isomer is receiving more attention, *Dunaliella* is currently upscaled for commercial production. The synthesis of *9-cis* β-carotene requires 8-step chemical synthesis. Therefore, it's price was relatively high compared to that of *all-trans* β-carotene when commercialized, as the market price by Sigma-Aldrich (Merck) suggests €6/g for the *all-trans* form, compared to €500,000/g for *9-cis* β-carotene (Harvey and Ben-Amotz 2020). The antioxidant activity from *D. salina* extract inhibits different cancers by neutralizing reactive oxygen species (ROS), which causes cellular cytotoxicity (Singh et al. 2016). Various stressors can impact the accumulation of β-carotene in *Dunaliella*. By altering culture conditions, the microalgae are subjected to stressors such as nutrient concentrations (excess or deficit) (Mai et al. 2017), temperature fluctuations (Wu et al. 2016), light intensity (Xu and Harvey 2019) and NaCl fluctuation (Ahmed et al. 2017). The key factors that play important roles in β-carotene production in *Dunaliella* are phosphorus and NaCl which participate in various metabolic processes during algal growth and development, such as photosynthesis, pigments biosynthesis, cell membrane construction, signal transduction and various enzyme activities. (Pourkarimi et al. 2020).

To the best of our knowledge, there are no reports on whether this method influences *cis*-β-carotene synthesis, especially in *Dunaliella* strains. The aim of this present study was to examine growth characterizations, β-carotene isomerization, and antioxidant activity of Thai *Dunaliella salina* strain NUAC09 under various phosphorus and NaCl concentrations.

## Materials and methods

### Algae strain and cultivation conditions

The pure *Duanliella salina* NUAC09 was obtained from microalgae bank of Fisheries Science Program, Faculty of Agriculture Natural Resources and Environment, Naresuan University. A preliminary study found that the NUAC09 strain had higher carotenoid production than other strains. Therefore, it was selected for use in the present study. The pure culture was maintained in both agar and liquid D media containing 1.0 M NaCl (Pick et al. 1986). The culture was incubated in an ambient temperature of 25±2 °C with a continuous photoperiod at 100 µmol PPF $m^{-2}s^{-1}$ provided by white fluorescent lamps. The strain was sub-cultured every 14 days.

### Morphological characterization

The morphometrical characteristics of *Dunaliella salina* NUAC09 were analyzed using a light microscope (Nikon, Japan) and calculated using ImageJ software (Dokumcuoglu and Yilmaz 2020). Morphological characters such as shape, coloration of the cell, flagella occurrence and characteristics of stigma were based on a standard method (Borowitzka and Siva 2007).

### Molecular identification

DNA was extracted using the phenol-chloroform method, followed by PCR amplifications (Wu et al. 2000). To support the morphological identification, the Internal Transcribed Spacer (ITS) of Thai strain was amplified following method described by Preetha et al. (2012). *Dunaliella*-specific primer IT4 (5' GGAAGTAAAAGTCGTAACAAG-3') and ITS5 (5'-TCCTCCGCTTATTGATATGC-3') were used for PCR amplification and sequencing. The ITS sequences obtained in this study were aligned with reference sequences retrieved from GenBank using ClustalW in BioEdit (Preetha et al. 2012). Phylogenetic analyses were conducted using Maximum Likelihood (ML) and Neighbour joining (NJ) methods in MEGA (11.0.10) (Tamura et al. 2021). Bootstrap values for ML and NJ were calculated with 1,000 replications.

### Optimization of NaCl levels and Phosphorus concentrations for β-carotene production

The strain was grown in Johnson medium (Johnson et al. 1968) containing 1.0 M NaCl as the basal medium until the population reached $10^6$ cells/mL in inoculum. The cells were harvested at that point by transferring 10% of the culture into a 200 mL glass cylindrical tube containing stress medium. To test the suitable NaCl condition for high β-carotene accumulation by the *D. salina* NUAC09, the cultures were grown with 5 different NaCl levels including 0.5 (T1), 1.0 (T2; control), 1.5 (T3), 2.0 (T4) and 2.5 (T5) M NaCl. For optimizing the phosphorus concentration, *D. salina* NUAC09 isolate was cultured in Johnson medium containing different phosphorus concentrations as followed: 0.00 (T1), 0.035 (T2; control), 0.053 (T3) and 0.070 (T4) g KH2PO4/L. An initial cell density of $0.2\times10^6$ cells/mL was used for all treatments.

### Determination of growth, pigment content and dry weight

The cells were collected every two days and cell density enumeration was performed under light microscope (NIKON, YS2, Japan) using hemocytometer (Neubauer, Germany). The specific growth rate ($\mu$) and doubling time ($t_d$) of microalgae were calculated as described in Wu et al. (2016).

All treatments were subjected to chlorophyll *a* and total carotenoid assays in triplicates every two days from each stress condition. A total of 2 mL of algal culture were centrifuged at 8,000 rpm for 10 min. Cell pellet was extracted for pigments with 2 mL of ice-cold 100% acetone. The chlorophyll *a* and total carotenoid contents in the supernatants were quantified at 664, 647 and 452 nm wavelength by VIS spectrophotometer (V–5100, China), followed by calculation according to Wu et al. (2016)**.** At the end of experiments, the dry weight of the algae was determined. The specific growth rate (SGR) was calculated following Xu et al. (2018) using the equations: $DW = SW/Vt$; $\mu = \ln(N_2/N_1)/(t_2-t_1)$; Where DW was dry weight (g/L); SW was sample dry weight (g); Vt was sample volume (L); $\mu$ was the specific growth rate (SGR, $\mu$/d); $N_2$ and $N_1$ were biomass (g/L) at the final and initial time points ($t_2$ and $t_1$, respectively).

### Composition of β-carotene isomers

The composition of pigments extracted from different strains was analyzed using high performance liquid chromatography (HPLC) with diode array detection (DAD) (Sher et al. 2018). Standards of *all-trans* β-carotene obtained from Sigma-Aldrich, Inc. (Merck KGaA, Darmstadt, Germany). Carotenoids and chlorophylls were extracted from freshly harvested cell pellets at the end of experiments using methyl tert-butyl ether (MTBE) and Methanol (MeOH) (20:80) as extraction solvents. Briefly, a total of 15 mL of algal culture was centrifuged at 3,000 rpm at 18 °C for 5 min and the pellet was extracted with 10 mL MTBE-MeOH (20:80) and sonicated for 20 s. Then, 1-2 mL of the supernatant was filtered through 0.45 µm syringe filter into amber HPLC vials. It was then analyzed using a YMC18 column with Diode-Array Detection (DAD) at 25 °C, and isocratic elution with 80% methanol: 20% ethanol at a flow rate of 1 mL/min, pressure of 60 bar, and was identified based on the retention time (RT) of standard *all-trans* β-carotene. The quantities of *9-cis* and *all-trans* β-carotene in the biomass were determined from the corresponding standard curves, as described by Xu et al. (2018) and Weinrich et al. (2019).

### Antioxidant activity assay (DPPH radical scavenging activity)

DPPH radical scavenging activity was assayed as previously described (Nguyen et al. 2016). In summary, 0.2 mL of DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (0.05 µM) was added to 1.8 mL of each fraction (100 µg/mL) and incubated at room temperature for 15 min prior to reading absorbance at 517 nm. Ascorbic acid was used as a standard control.

### Statistical analysis

All data was analyzed by one-way ANOVA using R version 3.5.1 (R Development Core Team, 2009). All significant levels were set at $P < 0.05$.

## Results

### Species identification

The morphology of the NUAC09 was examined under a light microscope. A vegetative cell of NUAC09 was green, spherical, ovoid, or pear-shaped with cell size ranging from 14.5-16.1 µm in length and 8.7-10.2 µm in width. The cell contained almost equal biflagellate (Fig. 1A-B). Meanwhile, we observed that cyst forms changed to bilateral, asymmetrical, 12-20 µm in diameter and light yellow to brownish in color under nutrient starvation (Fig. 1C-D). The phylogenetic trees based on ITS sequences from the studied strains and reference sequences from GenBank were constructed. The phylogenetic trees derived from ML and NJ analyses were nearly identical in topology; therefore, only the ML tree has been shown for clarity of illustration. The results showed that the strain was identified as *Dunaliella* which were divided into four clades: *D. salina, D. teriolecta, D. parva* and *D. viridis*. Moreover, the ITS tree revealed that strain NUAC09 was grouped to *D. salina* clade together with CCAP 19/30 and CCAP 19/18 with ML=87 and NJ=88 bootstrap values, respectively (Fig. 1E). Thus, NUAC09 could be identified as *D. salina*.

### Effect of different phosphorus and NaCl levels on cell growth, pigment, and biomass

In the present study, the effect of different phosphorus concentrations (0.00, 0.035, 0.053 and 0.070 g $KH_2PO_4$/L) on cell growth, pigment content and biomass of *D. salina* NUAC09 was investigated for 14 days.

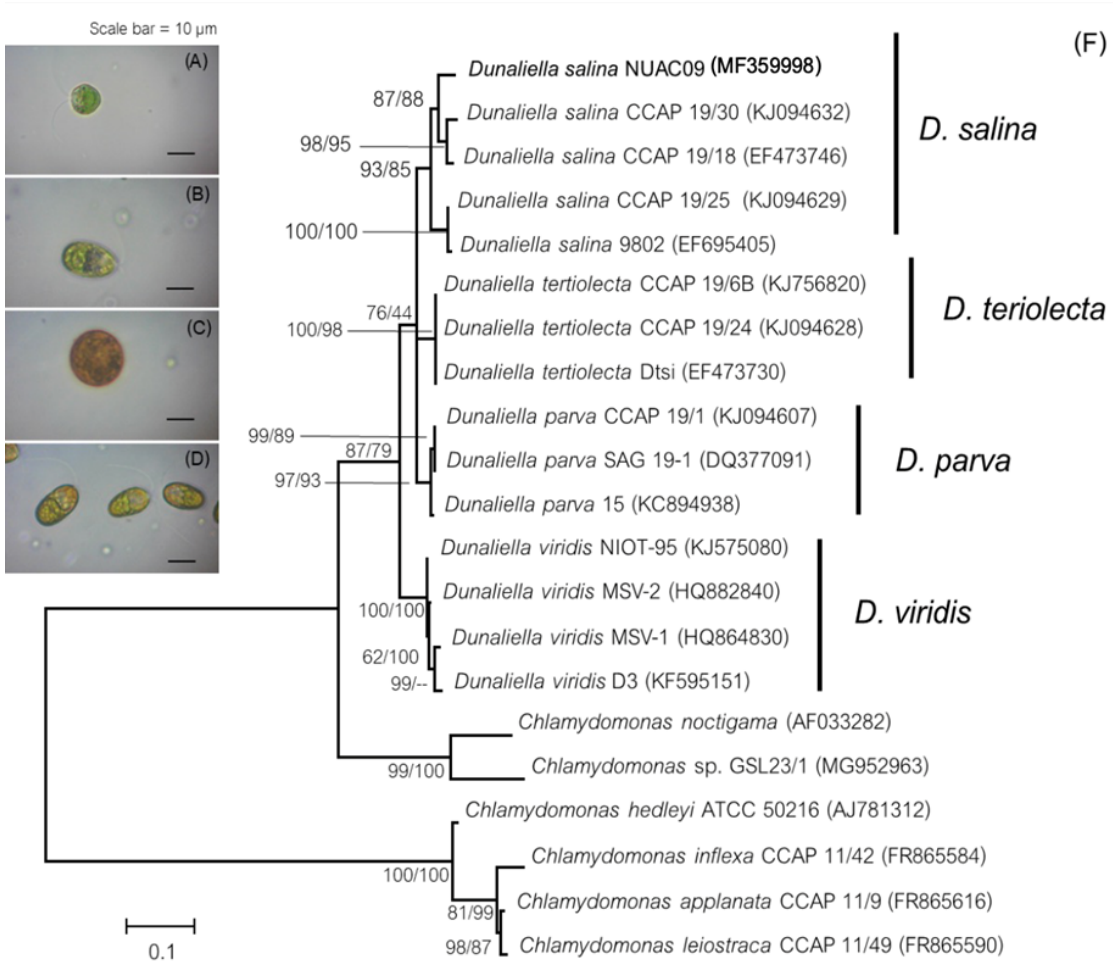


**Fig. 1** Morphology (A-B: vegetative cells, C-D: cysts or red cell) and phylogenetic tree (F) analyses based of *D. salina* NUAC09 on ITS region-Maximum likelihood. ML and NJ bootstrap values are presented (based on 1,000 replicates) with support greater than 50%. *Chlamydomonas* species were used as outgroup.

During day 4th to 10th, it was shown that cells grown in the presence of phosphorus demonstrated relatively high cell density compared to the phosphorus-deficiency condition (T1; 0.0 g $KH_2PO_4$/L). Algal cells grown in the highest phosphorus concentration (0.07 g $KH_2PO_4$/L), showed maximum cell density compared to other treatments. After day 14th, 0.070 g $KH_2PO_4$/L supported the highest cell density (4.42±0.28×10$^6$ cells/mL) of NUAC09 ($P < 0.05$) compared to the other $KH_2PO_4$ concentrations. (Fig. 2A and Table 1).

The chlorophyll *a* content of *D. salina* NUAC09 is presented in Fig. 3A and Table 1. The Chlorophyll *a* content of the algae cultured at all phosphorus levels (0.000-0.070 g $KH_2PO_4$/L) obviously increased after inoculation and was highest on day 8th of cultivation (Fig. 3A), and then gradually decreased until the end of cultivation (day 14th), where the chlorophyll *a* (µg/mL) from 0.070 g $KH_2PO_4$/L was higher than that in the control group (0.035 g $KH_2PO_4$/L) by 1.55 times (35.7%). Similarly, maximum productivity and specific growth rate were obtained from treatment 0.070 g $KH_2PO_4$/L group (2.13±0.00 g/L and 0.57±0.03 $\mu$/d, respectively) (Table 1). The results clearly showed that the total carotenoid content increased when the phosphorus concentration increased (Fig. 3B). The highest cellular carotenoid content (40.86±8.40 µg/mL) of *D. salina* NUAC09 was recorded from treatment 0.70 g $KH_2PO_4$/L at day 14th. Furthermore, the color of the algal cultures gradually changed with different $KH_2PO_4$ concentrations. The cell color of algae cultured in 0.035-0.070 g $KH_2PO_4$/L was light green in the early stage (Fig. 4A). It then turned yellowish-green on day 6th (Fig. 4B), and the cell color changed to dark orange on day 14th (Fig. 4C). Under phosphorus-deficient condition (0.00 g $KH_2PO_4$/L), the cell color was light green in the early stage (Fig. 4A) and turned yellow from day 6th onwards (Fig. 4B).

In the present study, the effects of different NaCl concentrations (0.5, 1.0, 1.5, 2.0 and 2.5 M) on cell growth, pigment production, and biomass were observed. The growth of *D. salina* NUAC09 at different NaCl concentrations was significantly different ($P < 0.05$). The cell density of NUAC09 was improved at high NaCl concentration (0.5-2.5 M). After day 14th, the cell density of the 2.5 M NaCl group (6.09±0.04×10$^6$ cells/mL) was noticeably higher than that of the other groups ($P < 0.05$), and this treatment

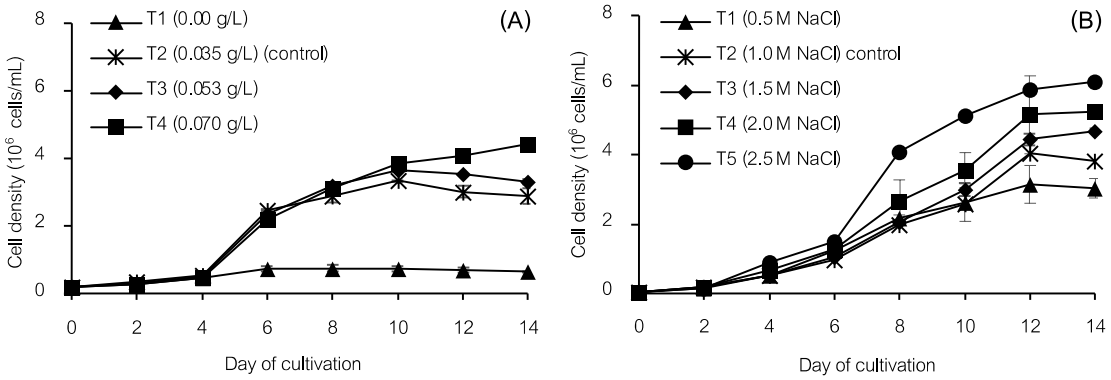


**Fig. 2** Cell density (10$^6$ cells/mL) of *D. salina* NUAC09 grown in Johnson medium under different phosphorus (A) and NaCl (B) levels.

**Table 1** Maximum values of growth parameters and pigment content of *D. salina* NUAC09 grown in Johnson medium containing different phosphorus concentrations.

| Parameters | Concentration of phosphorus (g $KH_2PO_4$/L) | | | |
|---|---|---|---|---|
| | T1 (0.000 g/L) | T2 (0.035 g/L) | T3 (0.053 g/L) | T4 (0.070 g/L) |
| Cell density (10$^6$ cells/mL) | 0.76±0.11$^d$ | 3.34±0.02$^c$ | 3.65±0.00$^b$ | 4.42±0.28$^a$ |
| Chlorophyll *a* (µg/mL) | 1.40±0.10$^d$ | 7.70±0.31$^b$ | 6.79±0.00$^c$ | 11.98±0.35$^a$ |
| Chlorophyll *a* (pg/cell) | 1.69±0.05 | 1.49±0.27 | 1.36±0.03 | 1.47±0.09 |
| Carotenoid (µg/mL) | 11.17±0.79$^d$ | 21.87±0.75$^c$ | 28.08±2.41$^b$ | 40.86±8.40$^a$ |
| Carotenoid (pg/cell) | 16.06±0.75$^c$ | 7.61±0.72$^b$ | 8.46±0.56$^b$ | 9.20±1.33$^a$ |
| Biomass content (g/L) | 0.61±0.00$^d$ | 2.00±0.00$^c$ | 2.06±0.00$^b$ | 2.13±0.00$^a$ |
| Specific growth rate ($\mu$/d) | 0.38±0.02$^b$ | 0.66±0.10$^a$ | 0.62±0.07$^a$ | 0.57±0.03$^a$ |
| DPPH (% inhibition) | 72.4±6.90$^a$ | 50.4±1.90$^b$ | 59.0±2.70$^b$ | 63.6±2.10$^c$ |
| $IC_{50}$ (µg/mL) | 0.48±0.00$^d$ | 0.69±0.01$^a$ | 0.59±0.00$^b$ | 0.55±0.00$^c$ |

Remark: Averages ± standard deviations in the same row followed by the different letters are significantly different ($P < 0.05$)

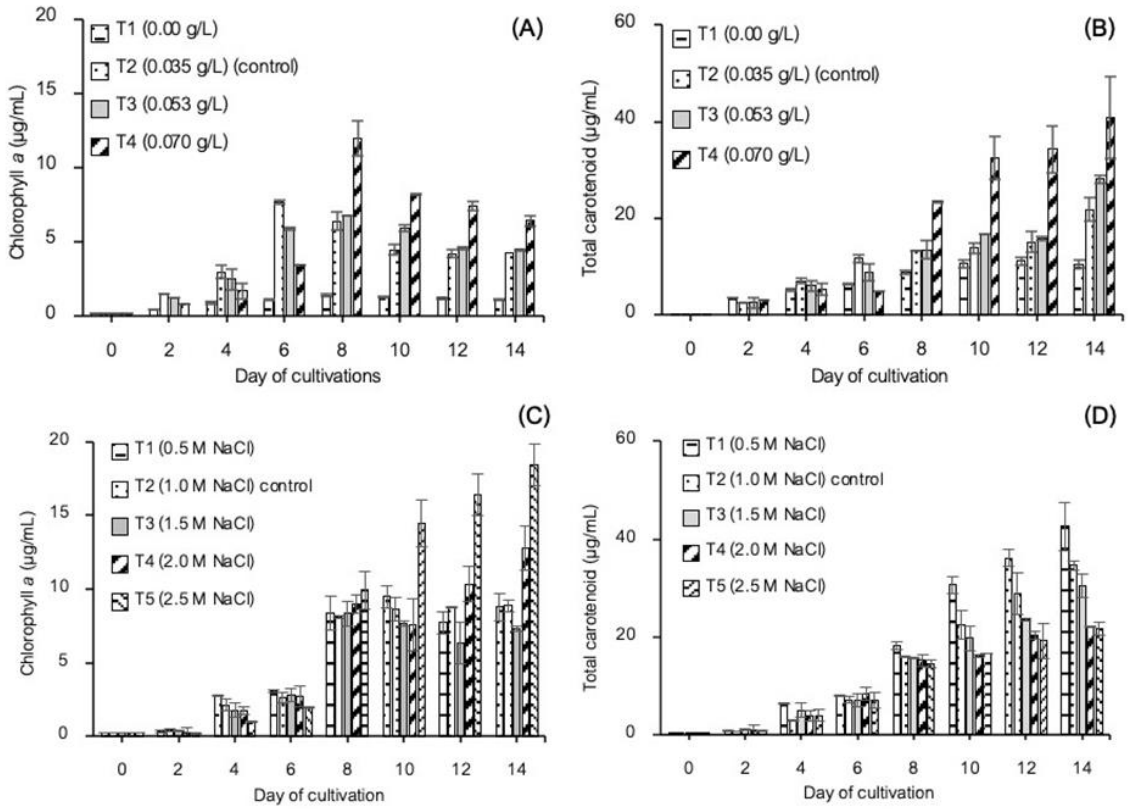


**Fig. 3** Pigment concentrations representing Chlorophyll *a* and total carotenoid content in *D. salina* NUAC09 grown in Johnson medium under different phosphorus (A and B) and NaCl (C and D) levels.

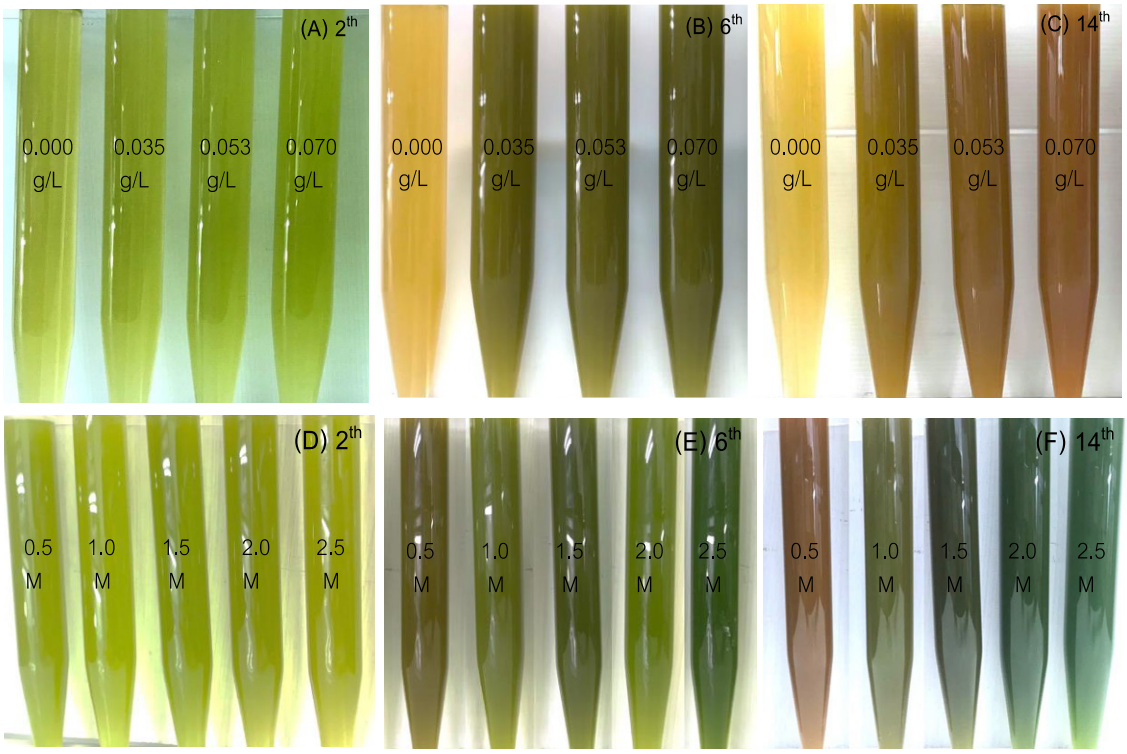


**Fig. 4** Photographs of *D. salina* NUAC09 grown in Johnson medium with different phosphorus (A-C) and NaCl (D-F) concentrations after initiation representing 2nd (A, D), 6th (B, E), and 14th (C, F) days post-inoculation.

showed maximum growth throughout the experiment. The lowest cell density was observed in the 0.5 M NaCl (3.03±0.29×$10^6$ cells/mL). (Fig. 2B and Table 2).

The results clearly showed significant differences in the chlorophyll *a* content of algae grown at all NaCl concentrations ($P < 0.05$). Chlorophyll *a* content varied according to NaCl concentration. It was revealed that both the productivity (Fig. 3C) and cellular content of chlorophyll *a* (Table 2) in the 2.5 M NaCl treatment was significantly higher than that in the other treatments on day $10^{th}$ to $14^{th}$. It was then followed by treatment 2.0, 1.5, 1.0 and 0.5 M NaCl, respectively. Similarly, the maximum biomass (1.56±0.09 g/L) was also obtained in the 2.5 M NaCl.

In contrast, the total carotenoid content was inversely affected by NaCl concentration. A significant difference was initially observed on day $8^{th}$ post-inoculation in the 0.5 M NaCl treatment and remained significantly higher than that of the other treatments throughout the experiment ($P < 0.05$). The result clearly showed that total carotenoid accumulation substantially increased from 21.60±1.37 to 42.67±4.94 µg/mL with decreasing NaCl levels from 2.5 to 0.5 M. (Fig. 3D and Table 2). Furthermore, the cell color changed from light green to dark green as the culture period and NaCl concentration increased (Fig. 4D and E), but the cells were orange at the end of day $14^{th}$ in 0.5 M NaCl (Fig. 4F).

Effect of different phosphorus and NaCl concentrations on β-carotene isomers

HPLC analysis revealed that two β-carotene isomers, *all-trans* and *9-cis*, were detected at 20.8 min and 21.4 min retention time respectively, clearly showing that the content of *all-trans* and *9-cis* β-carotene increased with decreasing phosphorus concentration ($P < 0.05$). The highest cellular content of *all-trans* (178.0±0.5 mg/g DW) and *9-cis* β-carotene (99.5±7.0 mg/g DW) were found from the culture without phosphorus concentration (0.00 g $KH_2PO_4$/L) (Table 3). The production of each β-carotene isomer at various NaCl concentrations is shown in Table 4. Corresponding to the effect of phosphorus, the total isomer content increased when NaCl levels decreased ($P < 0.05$). After 14 days, the highest *all-trans* (238.2±69.9 mg/g DW) and *9-cis* β-carotene (156.5±39.8 mg/g DW) in 0.5 M NaCl (Table 4).

Therefore, the growth medium containing 0.00 g $KH_2PO_4$/L of phosphorus experiment and 0.5 M NaCl of salinity experiment were suitable for the β-carotene isomer yield of NUAC09 strain.

**Table 2** Maximum values of growth parameters and pigment content of *D. salina* NUAC09 grown in Johnson medium containing different NaCl concentrations.

| Parameters | Concentrations of sodium chloride (M NaCl) | | | | |
|---|---|---|---|---|---|
| | T1 (0.5 M) | T2 (1.0 M) | T3 (1.5 M) | T4 (2.0 M) | T5 (2.5 M) |
| Cell density ($10^6$ cells/mL) | 3.03±0.29$^{d}$ | 3.83±0.05$^{c}$ | 4.69±0.06$^{b}$ | 5.24±0.11$^{b}$ | 6.09±0.04$^{a}$ |
| Chlorophyll *a* (µg/mL) | 8.77±0.96$^{c}$ | 8.86±0.36$^{c}$ | 7.34±0.17$^{c}$ | 12.81±1.52$^{b}$ | 18.43±1.45$^{a}$ |
| Chlorophyll *a* (pg/cell) | 2.93±0.55$^{a}$ | 2.31±0.12$^{b}$ | 1.56±0.03$^{c}$ | 2.47±0.40$^{ab}$ | 3.03±0.26$^{a}$ |
| Carotenoid (µg/mL) | 42.67±4.94$^{a}$ | 34.70±0.93$^{b}$ | 30.43±2.33$^{b}$ | 22.10±0.21$^{c}$ | 21.60±1.37$^{c}$ |
| Carotenoid (pg/cell) | 14.22±2.56$^{d}$ | 9.08±0.48$^{cd}$ | 6.49±0.44$^{c}$ | 4.26±0.50$^{b}$ | 3.55±0.25$^{a}$ |
| Biomass content (g/L) | 0.58±0.11$^{d}$ | 0.81±0.12$^{c}$ | 1.13±0.21$^{b}$ | 1.15±0.14$^{b}$ | 1.56±0.09$^{a}$ |
| Specific growth rate ($\mu$/d) | 0.63±0.00$^{a}$ | 0.56±0.05$^{ab}$ | 0.52±0.05$^{b}$ | 0.56±0.04$^{ab}$ | 0.62±0.04$^{a}$ |
| DPPH (% inhibition) | 74.6±0.80$^{a}$ | 73.6±3.50$^{a}$ | 73.3±0.80$^{a}$ | 66.5±1.10$^{b}$ | 66.9±0.5$^{b}$ |
| $IC_{50}$ (µg/mL) | 0.27±0.00$^{b}$ | 0.28±0.01$^{ab}$ | 0.29±0.02$^{a}$ | 0.30±0.00$^{a}$ | 0.28±0.01$^{ab}$ |

Remark: Averages ± standard deviations in the same row followed by the different letters are significantly different ($P < 0.05$)

**Table 3** Concentrations of β-carotene (*all-trans* and *9-cis*) isomers in *D. salina* NUAC09 grown in Johnson medium containing different phosphorus concentrations.

| Treatments | Concentration of phosphorus (g $KH_2PO_4$/L) | Concentration of isomer (mg/g DW) | |
|---|---|---|---|
| | | *all-trans* β-carotene | *9-cis* β-carotene |
| T1 | 0.00 g/L | 178.0±0.5$^{a}$ | 99.5±7.0$^{a}$ |
| T2 (control) | 0.035 g/L | 111.3±4.9$^{b}$ | 93.7±2.4$^{a}$ |
| T3 | 0.053 g/L | 62.7±1.2$^{c}$ | 47.4±2.7$^{b}$ |
| T4 | 0.070 g/L | 61.3±5.9$^{c}$ | 39.8±1.6$^{c}$ |

Remark: Averages ± standard deviations in the same column followed by the different letters are significantly different ($P < 0.05$)

**Table 4** Concentrations of β-carotene (*all-trans* and *9-cis*) isomers in *D. salina* NUAC09 grown in Johnson medium containing different NaCl concentrations.

| Treatments | Concentration of sodium chloride (M NaCl) | Concentration of isomer (mg/g DW) | |
|---|---|---|---|
| | | *all-trans* β-carotene | *9-cis* β-carotene |
| T1 | 0.5 M | 238.2±69.9[a] | 156.5±39.8[a] |
| T2 (control) | 1.0 M | 54.7±19.3[b] | 38.8±16.6[b] |
| T3 | 1.5 M | 45.1±0.6[b] | 26.1±0.6[b] |
| T4 | 2.0 M | 64.4±15.3[b] | 33.5±8.7[b] |
| T5 | 2.5 M | 41.1±8.8[b] | 18.0±3.6[b] |

Remark: Averages ± standard deviations in the same column followed by the different letters are significantly different (P <0.05)

Effect of different phosphorus and NaCl concentrations on activity of antioxidant content

The antioxidant activity of crude extracts from *D. salina* NUAC09 under different phosphorus (0.00-0.070 g $KH_2PO_4$/L) and NaCl concentration (0.5-2.5 M NaCl) was determined by DPPH inhibition. The antioxidant activity value with 72.4±6.9% DPPH inhibition at 0.00 g $KH_2PO_4$/L (Table 1), with 74.6±0.8% DPPH inhibition at 0.5 M NaCl (Table 2). The DPPH result (% inhibition) revealed that decreasing phosphorus and NaCl concentrations increased antioxidant activity (P <0.05). Especially, the best antioxidant activity and 50% inhibitory concentration ($IC_{50}$) were obtained from treatment 0.00 g $KH_2PO_4$/L (72.4±6.9% inhibition and $IC_{50}$ 0.48±0.0 µg/mL).

Similarly, it has been shown that NaCl levels affect antioxidant activity. The crude extract from treatment with 0.5 M NaCl showed maximum activity of 74.6±0.8% DPPH inhibition and 0.27±0.0 µg/mL $IC_{50}$ when compared with the control group (P <0.05). Therefore, the stress condition (phosphorus deficit and low NaCl) for algal culturing appeared to be related to antioxidant capacity.

## Discussion

In this study, the morphology of *Dunaliella* strain NUAC09 was consistent with that of *D. salina*. In accordance with a study by Preetha et al. (2012), *Dunaliella* has two flagella of equal length and a single cup-shaped chloroplast, which in marine and halophilic species has a central pyrenoid. Moreover, the morphometric information of strain tested was in the range of the length (2.8 to 40.0 µm) and width (1.5 to 20.0 µm) reported in the original description of *D. salina* and the previous studies (Preetha et al. 2012; Borovkov et al. 2019). Corresponding to the morphology results, phylogenetic analyses of the ITS region confirmed that strain NUAC09 was grouped in the *D. salina* clade. The *D.* strain NUAC09 was identified as *D. salina* based on a combination of morphological characteristics and phylogenetic analyses.

Phosphorus is essential macronutrients needed to promote algal growth and they regulate metabolic activities if supplied in an acceptable form (Yaakob et al. 2021). Various phosphorus concentrations in the microalgae cultivation medium may influence growth and pigment content (Masojídek et al. 2013). Phosphorous is an essential nutrient that makes up slightly less than 1% of the total algal biomass and is required at approximately 0.03-0.06% in the medium to sustain algae growth (Yaakob et al. 2021). Our results showed that increased phosphorus concentration affected cell density, specific growth rate, pigment content, and biomass. In concordance with the previous study, *D. salina* KU11 isolated in Thailand presented the highest growth (0.57 µg/day), biomass (0.24 g/L) and β-carotene content (17.4 mg/L) when increasing phosphorus concentrations at 0.01 to 0.03 g $KH_2PO_4$/L (Wongsnansilp et al. 2016). Song et al. (2016) reported that the cell density (~$3.0\times10^6$ cells/mL), specific cell growth rate (0.12 $\mu$/day) and chlorophyll content (~1.2 µg/mL) of *D. terilecta* from China was obtained in 0.10 g $Na_2HPO_4$/L. Furthermore, another green alga such as *Scenedesmus obliquus* and *Chlorella* sp., the best of cell density (~$2.3\times10^6$ cells/mL), chlorophyll content (~16 µg/mg) and biomass (~1.0 g/L) when increased phosphorus concentration between 0.01-0.30 g/L (Hamouda and Abou-El-Souod 2018; Kim et al. 2012; Chen et al. 2011). However, phosphorus deficiency affects the growth, pigment content, and biomass. In this study, the chlorophyll content and carotenoid content in high phosphate concentration were significantly higher than those in low phosphate concentration when compared on a culture volume basis, and a positive correlation was shown between the cell density and pigment. Therefore, the increased phosphorus content affected the growth,

pigment content, and biomass of *Dunaliella*.

NaCl is the most important environmental factor that affects the development, pigment content, and organic composition of halophilic microalgae (Charioui et al. 2016). The present study suggests that the growth parameters (cell density, chlorophyll *a*, SGR, and biomass content) increased with increasing NaCl concentration in the growth media. Corresponding to Mofeed (2015), *D. salina* from Egypt exhibited the highest cell density ($8.97\times10^6$ cells/mL) at 2.0 M NaCl concentration. The highest cell density ($7.17\times10^7$ cells/mL), total carotenoid (5.16 µg/mL) and biomass content (1.23 g/L) of *D. salina* SA134 from China was observed at the 2.0 M NaCl (Ahmed et al. 2017) culture condition. Furthermore, in cell division of *D. tertiolecta* was most rapid between 0.1 and 0.5 M NaCl and growth rates were reduced 30 to 55% at NaCl extremes (0.05 and 3.0 M NaCl) (Jahnke and White 2003). However, some studies found that initial NaCl concentrations higher than 1.0 M markedly inhibited *Dunaliella* cell growth (Takagi et al. 2006; Chen et al. 2009; Rad et al. 2011). This shows that different strains of *Dunaliella* exhibit different growth patterns, indicating that *Dunaliella* can tolerate a wide range of salinity. The carotenoid/chlorophyll ratio reflects the ability of plants to endure adversity (Sairam and Srivastava 2002), indicating that salinity stress causes different degrees of damage to algae (Haghjou et al. 2014). Additionally, it has been reported that an increase in carotenoid content is often accompanied by a high level of reactive oxygen species in microalgal cells exposed to abiotic stresses, such as salinity, which is higher or lower than the optimum level. In this study, carotenoid production per culture volume of NUAC09, the strain collected from the salt pan in Thailand, was negatively related to NaCl levels. Thus, it is plausible that NUAC09 could exhibit stress due to low salinity levels. Corresponding to a study done by Fazeli et al. (2006) and Khatoon et al. (2020) which showed that low salinities of 0.5 M and 0.7 M NaCl favoured total carotenoids content production by *D. tertiolecta* and *D. salina* when compared to higher salinity on a culture volume basis. In conclusion, salinity is a factor that affects the growth and pigment production of NUAC09, and appropriate salinity studies are necessary for future production strategies.

A remarkable result was found for the assaying isomers, indicating that phosphorus and NaCl levels affected the β-carotene isomer content in *D. salina* NUAC09. Our strain is in line with the results of other studies (Ben-Amotz et al. 1987; Lin et al. 2010; Xu et al. 2018; Weinrich et al. 2019), who reported two main β-carotene isomers (*all-trans* and *9-cis*) in *Dunaliella*. Phosphorus deficiency and low levels of NaCl increased the isomer content. The maximum *all-trans* and *9-cis* β-carotene content from a previous study showed that most microalgae have a large accumulation of β-carotene under stressed growth conditions, such as high-intensity light, high salinity, and nutrient deprivation (Moslemipetroudi et al. 2021). This could be because of the phytoene synthase enzyme (PSY), which is a key regulator of the biosynthesis of isomers (*trans* and *cis*) in *Dunaliella*. Darvidi et al. (2015) reported that enhancing phytoene synthase (PSY) genes expression could affect the conversion of *all-trans* to *9-cis* β-carotene. Other studies have suggested that the catalytic conversion of *all-trans* β-carotene to *9-cis* β-carotene leads to nutrient starvation in *D. salina* and *D. bardawil* (Shaish et al. 1993; Davidi et al. 2015; Xu and Harvey 2019). Similarly, Davidi and Pick (2017), identified and estimated the gene expression of β-carotene in *D. bardawil* and found that the β-carotene isomer pathway occurs during nitrogen deprivation in the strain. Jian-guo et al. (1996) reported that *D. salina* from China cultured in Johnson medium with shortage of macro nutrients (N and P) represented increasing ratio of *cis* to *all-trans* β-carotene isomer (60.8% to 39.3%). Hu et al. (2008) measured the β-carotene isomers of *D. salina* and reported that the *all-trans* and *9-cis* β-carotene isomers were 138.3 and 124.7 mg/g DW. Gomez et al. (2003) working on *D. bardawil* also reported an increase of *9-cis/all-trans* β-carotene ratio with increase in salt concentration and 0.5 M NaCl favored on *9-cis* production. Furthermore, *Heterochlorella luteoviridis* had the highest *all-trans* and *9-cis* β-carotene isomers with 0.39 and 0.19 mg/g DW obtained at 0.036 g $NaNO_3$/L (Menegol et al. 2017). Finally, the current study showed that with decreased phosphorus concentration and NaCl levels, *9-cis* β-carotene synthesis was increased in *D. salina* NUAC09. Therefore, this result can be applied to large-scale cultures to induce *cis*-isomer β-carotene synthesis, and *9-cis* β-carotene should be further investigated through single or a combination of factors that could enhance the synthesis.

Our results showed that the antioxidant enzyme activity of DPPH increased with decreasing phosphorus and NaCl levels. Based on extensive studies, it has been concluded that antioxidant activity is positively correlated with β-carotene production during stress conditions (Murthy et al. 2005; Hu et al. 2008; Tran et

al. 2014; Singh et al. 2016) by declining photosynthetic carbon fixation, and hence, accumulation of lipids with carotenoids as a cellular response to low carbon (Srinivasan et al. 2018). The highest antioxidant activity in our results was found in the 0.0 g $KH_2PO_4$/L and 0.5 M NaCl growing conditions. This would result from the intracellular defense mechanism against free radicals under unfavorable conditions for algae (Srinivasan et al. 2018). Similarly, 12 strains of *Dunaliella* from the USA under different NaCl and nutrient (N and P) depleted conditions yielded algal extracts, which were effective in suppressing DPPH free radicals ranging between 20-60% inhibition (Nguen et al. 2016). Srinivasan et al. (2018) suggests that microalgae cultures under nutrient depletion (N, P, K) improved the activities of antioxidant enzymes (SOD, CAT and APX) when compared to nutrient completed condition. Hamouda and Abou-El-Souod (2018) mentioned that increased phosphorus concentration (0.0-0.01 g/L) influenced the highest of antioxidant (35% DPPH) of *Scenedesmus obliquus*. In contrast, Singh et al. (2016) found that the antioxidant enzyme activity of DPPH was significantly increased (27.8-57.5%) at 3.0 and 3.5 M NaCl when compared to 1.0 M NaCl (15%). It was concluded that phosphorus and NaCl affected the antioxidant activity of the NUAC09 strain. However, the interaction with other factors should be examined to enhance the efficacy of antioxidant activities of the *Dunaliella* strain crude extract.

## Conclusion

The results showed that the morphological features of NUAC09, including cell dimension, structure, and shape, were closely related to the description of the genus *Dunaliella*. Correspondingly, ITS phylogenetic trees showed that strain NUAC09 was grouped into the *D. salina* clade. Hence, all the morphological and molecular results indicated that NUAC09 could be classified as *D. salina*. It was clearly shown that increasing phosphorus concentrations and NaCl levels in the growth medium could positively influence growth parameters (cell density, chlorophyll *a*, SGR, and biomass). However, in the case of pigments, a positive correlation was observed only between phosphorus levels and total carotenoid content per culture volume. Decreasing NaCl levels in the medium would induce total carotenoid accumulation on a volumetric basis. Interestingly, the maximum values of the *9-cis* β-carotene isomer content (99.5-156.5 mg/g DW) and DPPH scavenging activity (72-75% inhibition) were obtained in the experiment with both phosphorus deficient and low NaCl. The present study can be used as a guideline for β-carotene production at a commercial scale.

**Competing interests** The authors declare that they have no competing interests.

**Authors' contributions** All authors have contributed equally to data collection, data analysis, writing, reviewing, editing, and finally, approving the final manuscript.

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