β-Carotene Production from *Dunaliella salina* Cultivated with Bicarbonate as Carbon Source

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Introduction

*Dunaliella salina* is a halo-tolerant microalgal species that accumulates high levels of β-carotene [1, 2]. Although it has been used in commercial production of carotene for several decades, its production cost is still very high, limiting its application to only high-value nutraceuticals [3]. For products of lower value but much larger market size, such as animal and aquaculture feed additives, the current production cost of *Dunaliella* is much too high to be economically feasible, and needs to be significantly reduced.

Carbon accounts for about 50% of microalgae's dry weight, and its supply is very important in the development of cost-effective microalgal cultivation processes. In conventional cultivation systems, inorganic carbon is usually supplied as gaseous CO$_2$, which has caused serious technological difficulties in photobioreactor (PBR) design and operation [4,5,6], ultimately resulting in high production cost. Recently, bicarbonate has been suggested as a better approach to supplying carbon, with the advantages of easy transportation, handling, and storage. In such conditions, an aeration-free PBR can be used as a low-cost cultivation system [7-10].

There have been some reports on employing sodium bicarbonate to stimulate β-carotene accumulation in *D. salina* [8, 9, 11, 12]. However, β-carotene production reached in these studies was much lower than the conventional cultivation with CO$_2$, amounting to only 8.25 ± 0.01 mg/l [12], 7.10 ± 0.08 mg/l [9], and 20.43 ± 2.84 mg/l [8], respectively. These levels are too low to be used as routine practice in large-scale production. As a matter of fact, when bicarbonate is used as carbon source, the optimal culture condition for β-carotene accumulation may be different from that with CO$_2$. In this situation, it is necessary to disclose the effects of different factors on cell growth and β-carotene accumulation with bicarbonate as carbon source.

Bicarbonate has been considered as a better approach for supplying CO$_2$ to microalgae cells microenvironments than gas bubbling owing to cost-effectiveness and easy operation. However, the β-carotene production was too low in *Dunaliella salina* cultivated with bicarbonate in previous studies. Also, the difference in photosynthetic efficiency between these two carbon sources (bicarbonate and CO$_2$) has seldom been discussed. In this study, the culture conditions, including NaHCO$_3$, Ca$^{2+}$, Mg$^{2+}$ and microelement concentrations, were optimized when bicarbonate was used as carbon source. Under optimized condition, a maximum biomass concentration of 0.71 g/l and corresponding β-carotene content of 4.76% were obtained, with β-carotene yield of 32.0 mg/l, much higher than previous studies with NaHCO$_3$. Finally, these optimized conditions with bicarbonate were compared with CO$_2$ bubbling by online monitoring. There was a notable difference in $F_{v}/F_{m}$ value between cultivations with bicarbonate and CO$_2$, but there was no difference in the $F_{v}/F_{m}$ periodic changing patterns. This indicates that the high concentration of NaHCO$_3$ used in this study served as a stress factor for β-carotene accumulation, although high productivity of biomass was still obtained.

Keywords: *Dunaliella salina*, β-carotene, bicarbonate, microelement, carbon source
sensitive to stress conditions for microalgae, and has been established as a quantitative indicator that shows certain stress levels. For example, the value of \( Fv/Fm \) can reflect fatty acid accumulation in response to nitrogen depletion [15]. \( Fv/Fm \) was also used as an indicator in \emph{Dunaliella} sp. in previous study to disclose the mechanism of \( \beta \)-carotene accumulation in response to different stresses [12]. Thus, the \( Fv/Fm \) was monitored in this study, to compare the stress the cells experienced in cultivation with either bicarbonate or CO\(_2\), which finally affects the \( \beta \)-carotene accumulation.

The present study, therefore, investigated the effects of various factors on \emph{D. salina} growth and \( \beta \)-carotene production when sodium bicarbonate is used as carbon source. Different concentrations of sodium bicarbonate, as well as Ca\(^{2+}\) and Mg\(^{2+}\) were investigated for \emph{D. salina} growth and \( \beta \)-carotene accumulation. Central composite design experiments were carried out for studying the effect of microelements on biomass and \( \beta \)-carotene content of \emph{D. salina}. Finally, maximal PS II quantum yield and \( \beta \)-carotene content were analyzed and compared between bicarbonate and CO\(_2\)-based cultivation.

**Materials and Methods**

**Strain and Medium**

The microalgae \emph{D. salina} CCAP 19/18 was purchased from Culture Collection of Algae and Protozoa agencies (UK), and it was maintained in Artificial Sea Water (ASW). The nutrient medium was 1.5 M NaCl, 5 mM KNO\(_3\), 4.5 mM MgCl\(_2\)-6H\(_2\)O, 0.5 mM MgSO\(_4\)-7H\(_2\)O, 3 mM CaCl\(_2\)-2H\(_2\)O, 0.13 mM K\(_2\)HPO\(_4\), 0.02 mM FeCl\(_3\), 0.02 mM EDTA, 25 mM NaHCO\(_3\), 1 ml of trace elements stock with 50 mM H\(_2\)BO\(_3\), 10 mM MnCl\(_2\)-4H\(_2\)O, 0.8 mM ZnSO\(_4\)-7H\(_2\)O, 1.0 mM CuSO\(_4\)-5H\(_2\)O, 2 mM NaMoO\(_4\)-2H\(_2\)O, 1.5 mM NaVO\(_3\), 0.8 mM CoCl\(_2\)-6H\(_2\)O, and the pH was adjusted to 7.5 by addition 40mM of Tris-buffer [16]. Before inoculation, microalgae were cultivated in batch mode to promote fast growth in 500 mL conical flasks with light intensity of 40 \( \mu \)mol/m\(^2\)/s\(^{-1}\) and alternating 12 h/12 h light/dark cycles. After inoculation, the initial cell concentration in each horizontal photobioreactor (PBR) was about 0.2 \times 10\(^6\) cells/ml\(^{-1}\). The horizontal PBRs were polystyrene boxes of 12 cm \times 12 cm, with a working volume of 250 ml and a light path of 20 mm. Light was provided by white LEDs, with intensity on the top surface of the PBR controlled at 200 \( \mu \)mol/m\(^2\)/s\(^{-1}\) and under 12 h/12 h light/dark cycles. Cultivation temperature was controlled at 25 ± 0.5°C in the illumination incubator. Each experiment below was carried out in triplicate.

**Growth Analysis**

Cell numbers were counted daily using a hematocytometer. Dry weight (DW, g/l) was measured by using preweighed Whatman GF/C filters [17, 18]. Ten milliliter cultures were filtered and washed three times with 2 ml 0.5 M ammonium bicarbonate and then were dried below 60°C for over 16 h until the weight was constant. The DW of the microalgae cells was calculated according to the final weight and volume of the filtered sample. Analysis of microalgal \( \beta \)-carotene was based on methods described in Mojaat [19]. For \( \beta \)-carotene content measurement, 10 mg of dried biomass was extracted with 1 ml acetone and vortexed for 20 s. Then, it was centrifuged at 10,000 \times g for 10 min. This extraction was repeated twice. The extracts were filtered by 0.45-\( \mu \)m pore size (PTFE) membrane syringe filters (1.7 cm\(^2\)). All extracts were treated using amber glass vials with screw caps to protect carotenoids from degradation under light. The \( \beta \)-carotene analysis was carried out by High-Performance Liquid Chromatography (HPLC, Agilent Technologies 1100, USA). The mobile phase was 10% acetonitrile and 90% methanol. The flow rate was 1 ml/min\(^{-1}\), and the detection wavelength was 452 nm. The standard sample of \( \beta \)-carotene was purchased from Sigma (Sigma-Aldrich, USA).

**Experimental Design and Data Analysis**

**Influence of sodium bicarbonate concentrations on \emph{D. salina} growth.** To test the effect of NaHCO\(_3\) concentrations on \emph{D. salina} CCAP 19/18, six concentration gradients were selected, i.e., 25, 50, 100, 200, 300, and 500 mM. The concentrations of NaCl, Ca\(^{2+}\), and Mg\(^{2+}\) in the culture medium were 1.5 M, 3.0 mM, and 5.0 mM, respectively.

**Influence of Ca\(^{2+}\) and Mg\(^{2+}\) concentrations on \emph{D. salina} growth.** When optimizing the concentrations of Ca\(^{2+}\) and Mg\(^{2+}\) for \emph{D. salina} growth, gradients of Ca\(^{2+}\) and Mg\(^{2+}\) concentrations were from 0.3 to 3.0 mM, and from 0.5 to 5.0 mM, respectively, as listed in Table 1. Optimized NaHCO\(_3\) concentration (200 mM) was adopted for all cultures with various Ca\(^{2+}\) and Mg\(^{2+}\) concentrations.

**Central Composite Design**

With a Plackett-Burman (PB) design, three significant microelements listed in Table 2 were screened from FeCl\(_3\)-6H\(_2\)O, H\(_2\)BO\(_3\), ZnSO\(_4\)-7H\(_2\)O, CoCl\(_2\)-6H\(_2\)O, CuSO\(_4\)-5H\(_2\)O, MnCl\(_2\)-4H\(_2\)O, NaMoO\(_4\)-2H\(_2\)O, and NaVO\(_3\), as
displayed in Table S1. A central composite design was used to investigate their effects on dry cell weight and \textit{D. salina} β-carotene yield. The design matrix was a 2$^4$ full factor design combined with five central points, and eight axial points where one variable was set at an extreme level while other variables were set at their central point levels. The coded and real values of each parameter are shown in Table 2. Based on Table 3, the responses (cell dry weight and β-carotene yield) were correlated as a function of variables by a second-order polynomial equation, i.e.,

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j$$

(1)

where \(Y\) is the predicted response, \(\beta\) is the coefficient of the equation, and \(X_i\) and \(X_j\) are the coded levels of variables \(i\) and \(j\), respectively. The software Design-Expert (Stat-Ease Inc., USA) was adopted for this correlation through non-linear regression. An \(F\)-test was used to evaluate the significance of the models.

\textbf{NaHCO$_3$ vs. CO$_2$ Cultivation with Online Monitoring}

To confirm the experimental results obtained from the NaHCO$_3$, \textit{D. salina} was cultivated with a multi-device-equipped flat-plate photobioreactor (PBR)-Algal station [15] in both NaHCO$_3$-based (optimal NaHCO$_3$ concentration) and CO$_2$-based (2%) cultivations. Light was provided by white LEDs, with intensity on the top surface of the PBR controlled at 200 $\mu$mol/m$^2$/s$^{-1}$ and under 12 h/12 h light/dark cycles. Light path of the PBR was 20 mm and total cultivation volume was 1 L. The cultivation temperature was automatically controlled at 25 ± 0.5°C. Cultures in the PBR were agitated with 0.2-μm membrane-filtered air at 200 ml/min (NaHCO$_3$-based culture) or with 2% CO$_2$ (CO$_2$-based culture). Maximal PS II quantum yield \((F_v/F_m)\), OD, and pH value were recorded online.

\textbf{Results}

\textbf{Effect of NaHCO$_3$ Concentration on Cell Growth and β-Carotene Accumulation}

Growth curves for \textit{D. salina} cultivated under a series of NaHCO$_3$ concentrations are shown in Fig. 1A, indicating \textit{D. salina} could grow well in medium with NaHCO$_3$ as carbon source. Cell densities of 25, 50, and 100 mM NaHCO$_3$ cultures increased rapidly during the first 3 days of cultivation, and were higher than those of 200, 300, and 500 mM cultures. After that, cell density decreased in cultures with NaHCO$_3$ concentrations lower than 100 mM. The lowest cell density was observed in 25 mM NaHCO$_3$ and the highest cell density was recorded in 200 mM NaHCO$_3$ culture, which was 1.65 × 10$^6$ cells/ml$^{-1}$ on Day 7. As far as the DW is concerned, the highest DW was obtained on Day 7 with 200 mM NaHCO$_3$ concentration (0.66 ± 0.02 g/l), whereas the lowest DW of 0.35 ± 0.01 g/l was obtained in culture with 500 mM NaHCO$_3$ (Fig. 2). It was interesting that \textit{D. salina} was able to grow in culture with 500 mM NaHCO$_3$, although 200 mM NaHCO$_3$ enabled the fastest growth.

Correspondingly, pH variations of these cultures were displayed in Fig. 1B. The pH values of the six cultures spiked on Day 1 then increased slowly afterwards. At Day 7, the highest and lowest pH values were obtained in culture with 25 mM NaHCO$_3$ (pH = 10.3) and 500 mM NaHCO$_3$ (pH = 9.8), respectively. For cultures with ≥100 mM NaHCO$_3$,
pHs in these cultures were below 10.0 during the whole cultivation time. When correlating pH with microalgal growth, cultures with higher pH resulted in lower growth rates, indicating that elevated pH inhibited growth of *D. salina*, although it was able to grow at pH value higher than 10.0. Moreover, these results indicated that NaHCO₃ had a strong buffering effect on pH.

When correlating NaHCO₃ concentration with DW, the cellular β-carotene content levels of *D. salina* grown under different NaHCO₃ concentrations were displayed in Fig. 2. The highest β-carotene content of 4.20 ± 0.12% DW was obtained in culture with 200 mM NaHCO₃, and the corresponding β-carotene yield was 27.72 ± 1.65 mg/l. In contrast, the lowest β-carotene content of 2.1 ± 0.11% was obtained in 25 mM culture, reaching β-carotene yield of 9.77 ± 0.45 mg/l. In culture with 500 mM NaHCO₃ concentration, although high β-carotene content of 3.7 ± 0.11% was obtained, the overall available β-carotene yield was only 12.95 ± 0.46 mg/l owing to limited microalgal growth. From these results, it was implied that NaHCO₃ concentration could greatly influence cell growth and β-carotene accumulation of *D. salina*, and 200 mM was optimal for both biomass and β-carotene production in this study.

**Effect of Ca²⁺ and Mg²⁺ Concentrations on Cell Growth and β-Carotene Accumulation**

The correlation between Ca²⁺, Mg²⁺ and growth of *D. salina* was shown in Fig. 3A. Cell numbers ranged from 1.40 to 1.65 × 10⁶ cells/ml under different Ca²⁺ and Mg²⁺ concentrations, and significant differences were observed among all cultures on Day 7 (*p* = 0.03 < 0.1). The highest cell number was obtained in culture with 3.0 mM Ca²⁺ and 5.0 mM Mg²⁺, while the lowest cell number was obtained in culture with 0.3 mM Ca²⁺ and 0.5 mM Mg²⁺. pH values of all cultures on Day 7 displayed insignificant differences (*p* = 0.12 > 0.1), with values around 9.8 (Fig. 3B).

As shown in Fig. 4, DW was positively correlated with Ca²⁺ and Mg²⁺ concentrations, with the highest and lowest DW (0.69 g/l and 0.61 g/l) respectively at the same culturing conditions regarding cell numbers (Fig. 3A). In contrast, β-carotene content was negatively correlated with Ca²⁺ and Mg²⁺ concentrations, with the highest and lowest β-carotene content (respectively 4.5% and 4.1%) obtained at culturing conditions opposite to those of cell density and DW. It was noteworthy that β-carotene yield obtained in cultures with different Ca²⁺ and Mg²⁺ were around the same levels (26.2 to 27.5 mg/l), displaying no significant differences (*p* = 0.87 > 0.10). Thus, low concentration Ca²⁺ and Mg²⁺ of 0.3 mM and 0.5 mM respectively, are adequate for β-carotene accumulation.

![Fig. 1. Effect of different concentrations of NaHCO₃ on (A) *D. salina* cell density; (B) pH of the culturing broth. Values represented as mean ± SD (n = 3).](image1)

![Fig. 2. Effect of NaHCO₃ concentrations on DW, β-carotene content, and β-carotene yield of *D. salina*. Values represented as mean ± SD (n = 3).](image2)
Effect of Micronutrients on Cell Growth and β-Carotene Accumulation

Optimization of significant factors. For central composite design, the central point values and level ranges of three significant factors were selected according to the PB design results (Table S1). As shown in Table 3, the central point in the central composite design was repeated five times, and standard deviation of these five replicates used to determine the experimental errors were 0.008 g/l for DW, and 0.07% for β-carotene content. The experimental data of DW and β-carotene content in Table 3 were correlated as functions of the three variables by a second-order polynomial equation using the Design-Expert software. The coefficient values in Eq. (1) and their p-

Table 4. The values of coefficients in the second-order polynomial and the associated statistical test for DW and β-carotene.

| Variable | DW | β-carotene |
|----------|----|------------|
|          | F-value | p-value | Estimate | F-value | p-value | Estimate |
| Model    | 12.06 | 0.0068 | 0.7 | 12.11 | 0.0067 | 3.96 |
| A- FeCl₃·6H₂O | 36.44 | 0.0018 | -0.029 | 3.05 | 0.0412 | 0.1 |
| B- CoCl₂·6H₂O | 3.75 | 0.1104 | -9.19E-03 | 5.52 | 0.0657 | 0.14 |
| C- NaVO₃ | 7.6 | 0.04 | 0.013 | 4.7 | 0.0824 | -0.13 |
| AB | 2.15 | 0.2027 | 9.83E-03 | 7.66 | 0.0395 | -0.23 |
| AC | 6.01 | 0.0579 | -0.016 | 3.09E-03 | 0.9578 | -4.61E-03 |
| BC | 3.14 | 0.1366 | -0.012 | 1.348E-07 | 0.9997 | -0.00003048 |
| A² | 27.2 | 0.0034 | -0.018 | 70.58 | 0.0004 | 0.36 |
| B² | 15.77 | 0.0106 | -0.014 | 15.27 | 0.0113 | 0.17 |
| C² | 5.23 | 0.0799 | -7.82E-03 | 0.004034 | 0.9518 | 0.002685 |

Fig. 3. Effect of Ca²⁺ and Mg²⁺ concentrations on (A) cell number of *D. salina*; (B) pH of the culturing broth. Values represented as mean ± SD (*n* = 3).

Fig. 4. Effect of Ca²⁺, Mg²⁺ concentrations on DW and β-carotene accumulation of *D. salina*. Values represent as mean ± SD (*n* = 3).
values and F-values were listed in Table 4. The optimal value of the three variables were derived by Design-Expert software, and the maximum dry cell weight of 0.71 g/l was obtained in culture with 1.85 μM FeCl3·6H2O, 1.6 μM CoCl2·6H2O, and 1.48 μM NaVO3 concentrations. The maximum β-carotene content of 4.76% was obtained in culture with 5.92 μM FeCl3·6H2O, 2.23 μM CoCl2·6H2O, and 2.05 μM NaVO3. From p-levels in Table 4 and the response surfaces in Fig. 5, it is evident that microelements, especially Fe3+ and Co2+ and their concentrations, can have significant influences on *D. salina* growth and β-carotene accumulation.

Three-dimension surface responses were plotted to illustrate the relationship between the variables and their responses. Because the statistical analysis indicated that FeCl3·6H2O and CoCl2·6H2O concentration had more significant effects on the responses than NaVO3 concentration (p-level, Table 4), the responses (DW and β-carotene content) were plotted as the functions of FeCl3·6H2O and CoCl2·6H2O. As shown in Fig. 5A, low concentrations of FeCl3·6H2O and CoCl2·6H2O led to an increase in DW, but β-carotene content increased with augmentation of CoCl2·6H2O and FeCl3·6H2O concentrations (Fig. 5B).

**Verification of Optimized Culture Conditions**

The optimal conditions determined from the central composite design were verified by comparing the experimental data obtained at these conditions with that predicted from central composite design (Eq. (1) and Table 4). Three verification experiments were conducted, which were respectively optimal for DW, β-carotene content, and both DW and β-carotene content (Table 5). For DW experiment under optimized condition, the experimental data was 0.77 ± 0.01 g/l, while the predicted value was 0.86 g/l, indicating 9–10% deviation. As for the condition optimal for β-carotene content, experimental data was 4.78% ± 0.04, while the predicted value was 4.73%, indicating a deviation of less than 5% (Table 5). With the second-order polynomial equation obtained in this study, the calculated DW (before optimizing the microelements) was 0.67 g/l, and the β-carotene content was 4.2%. After the microelement optimization, DW was calculated as 0.78 g/l, and β-carotene content was 4.78% (Table 5). These experiments verified the effectiveness of the model developed in this study.

**Comparisons between Cultivations with NaHCO3 and CO2**

After 5 days of cultivation with supply of two different carbon sources, the growth showed significant differences in both OD680 (p < 0.01) and DW (p < 0.01). The initial ODs were similar (0.78 for NaHCO3-based and 0.79 for CO2-based) and then increased to 5.65 ± 0.17 and 6.58 ± 0.23, respectively (Table 6), and the corresponding final DWs were 0.89 ± 0.10 and 1.09 ± 0.08 g/l, respectively (Table 6). The productivity in cultures

**Table 5. Comparison of experimental and predicted DW and β-carotene content at optimal culture conditions.**

| Culture conditions | DW (g/l) | β-carotene content (%) |
|--------------------|---------|------------------------|
| Optimal for DW     |         |                        |
| 200 mM NaHCO3, 0.45 mM MgCl2·6H2O, 0.05 mM MgSO4·7H2O, 0.3 mM CaCl2·2H2O, 1.85 μM FeCl3, 1.5 μM NaVO3, 1.6 μM CoCl2·6H2O | Predicted | 0.86 | 4.11% |
| All other conditions were as described in section Materials and Methods | Experimental data | 0.77 ± 0.01 | 4.23% ± 0.01 |
| Deviation (%) | -10.00 | +2.90 |
| Optimal for β-carotene content |             |                        |
| 5.92 μM FeCl3, 2.0 μM NaVO3, 2.2 μM CoCl2·6H2O, all other conditions were as described for "optimal DW" | Predicted | 0.76 | 4.86% |
| Experimental data | 0.69 ± 0.02 | 4.78% ± 0.04 |
| Deviation (%) | -9.20 | -1.60 |
| Optimal for DW & β-carotene |             |                        |
| 5.92 μM FeCl3, 2.0 μM NaVO3, 2.2 μM CoCl2·6H2O, all other conditions were as described for "optimal DW" | Predicted | 0.78 | 4.73% |
| Experimental data | 0.71 ± 0.05 | 4.52% ± 0.04 |
| Deviation (%) | -9.00 | -4.60 |
with NaHCO₃ and CO₂ were 0.18 and 0.21 g/l⁻¹/d⁻¹, respectively. Clearly, the CO₂-based mode provided a better growth environment for D. salina, resulting in a 14.2% and 22.4% higher OD and DW than the NaHCO₃-based mode. The pH varied from 6.7 ± 0.1 to 8.3 ± 0.1 in the CO₂-based mode, while the pH varied from 8.0 ± 0.11 to 9.5 ± 0.1 in the NaHCO₃-based mode (Fig. 6B).

Moreover, the 200 mM bicarbonate had positive effects on the productivity of target value chemicals, β-carotene, showing the highest carotenoid concentration in the NaHCO₃-based condition, 4.7% (41.5 ± 0.2 mg/l) (Fig. 6D, Table 6), this value was significantly higher than that with CO₂-based condition, 2.2% (23.8 ± 0.3 mg/l). Also, the difference between the DW and β-carotene content in Figs. 6 and 2 should be due to microelement optimization, as evidenced in Fig. 5 and Table 4.

The changing patterns in \( F_v/F_m \) are depicted in Fig. 6C, and the \( F_v/F_m \) of D. salina changed periodically following changes in light under two carbon supply conditions, but exhibited similar patterns. Notably, the \( F_v/F_m \) followed 'sine' trends during the whole culture light/dark period. As for 2% CO₂-based condition, during the light period, the \( F_v/F_m \) decreased quickly from 0.73 to the lowest value 0.68 within the first 3 h and then increased gradually to the highest value (0.77) until the darkness period. During the 10 h darkness period, the \( F_v/F_m \) decreased gradually but was significantly higher than that in the 14 h illumination period. For the NaHCO₃-based culture condition, it was found that lowest \( F_v/F_m \) values were significantly lower than CO₂-based mode (\( p = 0.0003 \)), and the lowest value of \( F_v/F_m \) in the CO₂-based mode was 0.05 higher than in the NaHCO₃-based culture.

Table 6. Comparison of biomass, β-carotene yield, biomass productivities in cultivation with different carbon sources.

| Treatment   | Day | OD680 (mg/l) | Dry weight (g/l) | β-carotene yield (mg/l) | Biomass productivity (g/l/d⁻¹) |
|-------------|-----|-------------|------------------|------------------------|-------------------------------|
| 2% - CO₂    | 0   | 0.78 (0.01) | 0.16 (0.02)      | 1.04 (0.03)             | 0.21                          |
| 2% - CO₂    | 5   | 5.65* (0.17)| 1.09* (0.08)     | 23.8* (0.5)             | 0.18                          |
| 200 mM NaHCO₃| 0   | 0.79 (0.02) | 0.16 (0.02)      | 1.04 (0.03)             | 0.21                          |
| 200 mM NaHCO₃| 5   | 6.58** (0.23)| 0.89* (0.10)     | 41.5** (0.2)            | 0.18                          |

Values are mean (±SD) of \( n = 3 \) cultivations per treatment, *represent the significant effect (\( p < 0.05 \)) and ** represent the very significant effect (\( p < 0.01 \)).

Fig. 6. NaHCO₃-based cultivation vs. CO₂-based cultivation by online monitoring (A) Growth curve of D. salina; (B) pH curve; (C) \( F_v/F_m \) curve; (D) β-carotene content. The shaded areas indicate the standard error of the line values.
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### Discussion

Although several studies have indicated that \textit{D. salina} grew well in high concentration of bicarbonate, this study is the first one that reported \textit{D. salina} can accumulate a good amount of \(\beta\)-carotene under such cultivation conditions. Table 7 compared the \(\beta\)-carotene content and \(\beta\)-carotene yield of \textit{Dunaliella} strains in available literature. For \textit{D. salina} CCAP 19/18, the strain used in this study, it accumulated only 2.26 mg/l \(\beta\)-carotene when cultured with 20 mM NaHCO\(_3\) [20]. Also, the \(\beta\)-carotene yield obtained in this study is much higher than previous studies with other strains in \textit{Dunaliella}. For example, it is about 4-fold of the yield of \textit{D. salina} V-101 with 100 mM NaHCO\(_3\) [12], and about 1.6-fold of \textit{Dunaliella} sp. with 60 mM NaHCO\(_3\) [7]. Actually, 200 mM NaHCO\(_3\) supported better cell growth than other concentrations, but it is lower than that with 2% CO\(_2\) (Table 6). Since 200 mM bicarbonate resulted in a decreased value of \(F_{v}/F_{m}\) (Fig. 6C), it is considered as a stress, but this is favorable for \(\beta\)-carotene production. However, the commonly observed stagnant growth under stress was not found in this study, since the obtained DW of 0.71 ± 0.05 g/l\(^{-1}\) is quite comparable with those observed without stress [15]. Thus, this study provided a feasible approach for \(\beta\)-carotene production from \textit{D. salina} with bicarbonate as carbon source.

Precipitation appeared in culture with 200 mM NaHCO\(_3\) along with 3.0 mM Ca\(^{2+}\) and 5.0 mM Mg\(^{2+}\), since they react with excessive CO\(_3\)\(^{2-}\) at high pH (equilibrium HCO\(_3\)\(^{-}\) + OH\(^-\) → CO\(_3\)\(^{2-}\) + H\(_2\)O). Reducing the concentration of Ca\(^{2+}\) and Mg\(^{2+}\) to 0.3 mM and 0.5 mM, respectively, was proved to be an effective approach to avoid precipitation, and showed no significant reduction of biomass and \(\beta\)-carotene accumulation. In order to reduce production cost, seawater with NaHCO\(_3\)-supply may be used for \textit{D. salina} cultivation, in which Ca\(^{2+}\) and Mg\(^{2+}\) concentration is about 9-12.5 mM, and 80.5 mM, respectively [21, 22]. These are much higher than 0.3 mM and 0.5 mM, thus excessive Ca\(^{2+}\) and Mg\(^{2+}\) need to be removed via pretreatment. Precipitation with carbonate may be used as a simple method, and CO\(_2\) bubbling could regenerate bicarbonate afterwards, which may provide inorganic carbon as in this study [23].

This study first reported the effect of microelements on \(\beta\)-carotene accumulation in \textit{D. salina} when NaHCO\(_3\) is used as carbon source. The result indicated FeCl\(_3\)-6H\(_2\)O has a negative effect on cell biomass and \(\beta\)-carotene content with bicarbonate as carbon source. These findings were in accordance with previous research with CO\(_2\) as carbon source, and the amount of FeCl\(_3\)-6H\(_2\)O supplied in this study may induce the generation of active oxygen molecules, and result in a negative effect on biomass and positive effect on \(\beta\)-carotene accumulation [11, 19]. As shown in this study, CoCl\(_2\)-6H\(_2\)O has negative effect on cell growth but positive effect on \(\beta\)-carotene accumulation. There was no report on this topic when \textit{D. salina} is cultivated with CO\(_2\), but the study on \textit{Platymonas subcordiformis}, \textit{Chaetoceros curvisetus} and \textit{Skeletonema costatum} did show that Co\(^{2+}\) inhibition to cell growth in that it affects the interactions among the thylakoid membrane protein-pigment complexes, and obstructs the reaction center of PSII [24]. Also, it was reported that Co\(^{2+}\) contributes to the accumulation of carotenoids in \textit{Parvula viridis}, since it is an oxidative stress-inducing factor to react with hydrogen peroxide through a Fenton-type reaction to generate hydroxyl radicals [25], and these findings are in accordance with this study. This study also indicated that NaVO\(_3\) concentration had positive effect on biomass but had no significant effect on \(\beta\)-carotene accumulation when cultured with NaHCO\(_3\), and there was no previous report on this in \textit{D. salina} cultivated with CO\(_2\). It was reported that 2.5 mM NaVO\(_3\) promoted astaxanthin production in \textit{H. pluvialis}, and the possible mechanism is the inhibited expression of PTPases (Protein Tyrosine Phosphatases) by NaVO\(_3\) [26-28]. However, the NaVO\(_3\) concentration used in this study was only 2.62 μM, which may be too low to induce \(\beta\)-carotene accumulation.

The mechanism of improved \(\beta\)-carotene content under NaHCO\(_3\)-based cultivation was unknown and there is little information available on the responses of photosynthetic electron flow, especially in photosystem II (PSII) to NaHCO\(_3\)-based in \textit{Dunaliella}. In the present study, the variation of \(F_{v}/F_{m}\) exhibited similar patterns under two carbon supply conditions, but the NaHCO\(_3\)-based culture resulted in lower \(F_{v}/F_{m}\) values than that of CO\(_2\). Also, the pH value was higher at NaHCO\(_3\)-based culture than that of CO\(_2\). Higher \(\beta\)-carotene accumulation may be attributed to both high concentration NaHCO\(_3\) and high pH. It was reported that higher extracellular NaHCO\(_3\) concentration leads to a higher intracellular pH, which may damage or inhibit the enzymes involved in

| Microalgae | Initial cell density | L/D cycle | Light intensity (μmol/m\(^2\)/s\(^{-1}\)) | Culture time (d) | \(\beta\)-carotene content (mg/l) | \(\beta\)-carotene yield (mg/l) | Carbon source | Reference |
|------------|---------------------|-----------|-----------------------------------------|------------------|---------------------------------|--------------------------|-------------|----------|
| \textit{D. salina} V-101 | - | 16/8 | 50 | 7 | 0.05% | 8.25 ± 0.01 | 100 mM NaHCO\(_3\) | Ramachandran Svasanini et al., 2018 [12] |
| \textit{D. salina} CCAP 19/30 | - | 12/12 | 200 | 7 | - | 1.2 | 10 mM NaHCO\(_3\) | Yanan Xu et al., 2016 |
| \textit{D. salina} UTEX 2538 | - | 12/12 | 1000 | 5 | - | 13.2 | 10 mM NaHCO\(_3\) | Yanan Xu et al., 2018 (Xu et al., 2018) |
| \textit{Dunaliella} sp. | 0.1 | - | 340 | 17 | - | 20.43 ± 2.84 | 60 mM NaHCO\(_3\) | Ga-Yeong Kim et al., 2017 [7] |
| \textit{Dunaliella} sp. | 0.1 | 16/8 | 22 | 28 | 0.18% | 7.10 ± 0.08 | 150 mM NaHCO\(_3\) | Srinivasan et al., 2015 [9] |
| \textit{D. salina} CCAP 19/18 | 0.2 × 10\(^5\) | 12/12 | 200 | 7 | 4.50% | 32.0 | 200 mM NaHCO\(_3\) | This study |

Table 7. \(\beta\)-carotene accumulation by strains of \textit{Dunaliella} under varied cultivation conditions.
photosynthesis and reduce the efficiency of PSII photosystem \( F_{v}/F_{m} \) \[29\]. Also, it was reported that higher NaHCO\(_3\) concentration (above 0.6 mM) in the culture could inhibit the extracellular carbonic anhydrase (CA) activity, which is an important enzyme catalyzing the reversible dehydration of HCO\(_3\)\(^-\) to CO\(_2\) and decline of CA activities has significant inhibition of effective quantum efficiency of PSII, and thus reduce the value of \( F_{v}/F_{m} \) \[30\]. It was reported that decreased PSII activity results in the increase of ROS concentration, and \( \beta \)-carotene is synthesized to scavenge the ROS \[31\]. This may be the reason why increased \( \beta \)-carotene content was observed in culture with high concentration of NaHCO\(_3\). However, the connection between ROS and \( F_{v}/F_{m}\) under NaHCO\(_3\) stress in microalgae is still unknown and further in-depth research is needed to disclose the mechanism.

From the above results, FeCl\(_3\)·6H\(_2\)O, NaVO\(_3\) and CoCl\(_2\)·6H\(_2\)O concentrations significantly influenced \( D.\) \( salina\) biomass production with NaHCO\(_3\) as carbon source, which was not reported in cultivation with CO\(_2\). The notable difference in \( F_{v}/F_{m}\) value between cultivations with bicarbonate and CO\(_2\) indicates that NaHCO\(_3\) acts as a stress factor for \( \beta \)-carotene production more than CO\(_2\), and may make it useful in an easy and effective \( \beta \)-carotene induction method.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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