Photosynthetic profiling of a *Dunaliella salina* mutant DS240G-1 with improved *β*-carotene productivity induced by heavy ions irradiation

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Abstract: Carbon-ion irradiation is a technique for trait improvement in the microalgae, but the underlying mechanisms that how it altered the biomass, and photosynthetic pigments accumulation were unclear. One mutant (DS240G-1) was obtained from *Dunaliella salina* by heavy ion irradiation mutagenesis. Compared to the wild type, the biomass accumulation and maximum growth rate of DS240G-1 were increased by 34% and 55% respectively, and its *β*-carotene content was 21% higher than the wild type. Subsequent analysis of the chlorophyll fluorescence parameters indicated that higher *β*-carotene productivity was likely owing to the improved maximum quantum efficiency (F_v/F_m) and decreased thermal dissipation of photosynthesis in DS240G-1 than that of wild type during cultivation. In addition, the result of this study revealed that high content of ROS may induce *β*-carotene accumulation in mutant DS240G-1. Also, the total fatty acid (TFA) content in mutant DS240G-1 was 79% higher than that in wild type. Owing to its high *β*-carotene productivity and total fatty acid content, DS240G-1 could be considered as a promising candidate for microalgae *β*-carotene and biodiesel production. This work provided the first insight into the biological effects involved in carbon-ions irradiation on the photosynthetic activity of *D. salina*.

Keywords: microalgae, *D. salina*, carbon-ions irradiation, chlorophyll fluorescence, *β*-carotene

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1 Introduction

Microalgae have been considered as one of the major promising sources for third-generation biofuel production, due to their advantages such as short growth period, high photosynthesis efficiency, and high lipid content as well as the advantage that the co-production of valuable by-products[1-3]. The major compounds in microalgae involve fatty acid[4], protein, soluble polysaccharides, carotenoids, etc. *β*-carotene is one of the powerful antioxidants among carotenoids with many applications in nutraceuticals, food and feed industries because of its anti-aging, anti-inflammatory, sun proofing, and immune system boosting effects on organisms[5]. The unicellular green microalgae *Dunaliella salina*, with *β*-carotene content of 0.3%-10%, is recognized as the best biological source of natural *β*-carotene, and currently this species is the only commercialized microalgae for *β*-carotene production[6]. From a commercial point of view, the best strains of *D. salina* in mass cultivation should have the maximum specific growth rate and the highest *β*-carotene productivity under optimized conditions. However, the specific growth rate of *D. salina* is only 0.16-0.20 d^{-1}[6], resulting in low *β*-carotene productivity and this low growth rate makes the cultivation system vulnerable to biological contamination, consequently hinders large-scale cultivation.

Several attempts have been carried out to isolate mutants with straits by using chemical mutagens, ultraviolet (UV) or *γ* and X-rays exposure. For example, using UV irradiation, mutants of *D. bardawil* with higher potential for *β*-carotene synthesis under relatively low light intensities was obtained[7-9]. More recently, a novel mutant of *D. salina* named Zea1 was isolated by ethyl methyl sulfonate mutagenesis treatment[10] and the zeaxanthin content of *zea1* improved 30-fold than the wild type. Compared with other physical methods, such as X-rays and *γ*-rays whose linear energy transfer values (LET; the energy transferred per unit length, keV/*μ*m) were 0.2 and 2.0 keV/*μ*m, respectively[11], the LET value of the heavy-ion beam used in this study was extremely high, reaching 31 keV/*μ*m. Besides, the heavy-ion beam is well known due to its higher relative biological effectiveness (RBE) compared with low-LET *γ*-rays and X-rays. Moreover, the heavy-ion beam can also be controlled to deposit high energy at precise positions, in contrast to low-LET irradiation that may cause large deletions, translocations or rearrangements in the genome of a given organism[12,13]. Previous studies found that heavy ions irradiation increased the biomass of *Nannochloropsis* by 19% and the lipid productivity of *Desmodesmus* sp. by 20.6%[11,13], and the maximum quantum efficiency (F_v/F_m) of *Desmodesmus* sp. and *Nannochloropsis* after heavy ions irradiation was also higher than the wild strains[11,13]. The microalgae *Desmodesmus* sp. can

utilize nutrients such as nitrogen or phosphorous in wastewater and then produce high levels of total fatty acids, which would be a superior potential for high-quality biofuel production[3,14]. Therefore, the heavy ions irradiation may simultaneously increase biomass and by-products productivity in microalgae with improved photosynthesis activity. To the best of our knowledge, little has been reported using heavy ions irradiation on *D. salina* for efficient *β*-carotene production.

This work reported here is a first attempt to increase *β*-carotene productivity with the use of heavy-ion induced mutant produced from *D. salina*. Heavy-ion irradiation mutagenesis was applied in the wild type *D. salina* to improve its ability for *β*-carotene production. After mutation screening, one mutant (DS240G-1) was obtained, and then its biomass and *β*-carotene accumulation were investigated. Moreover, the underlying mechanism causing high biomass and *β*-carotene production were also analyzed in the DS240G-1 mutant. The chlorophyll fluorescence parameters (including the maximum quantum yield of Photosystem II (F_v/F_m), the actual quantum yield of PSII (ΦPSII), and non-photochemical quenching of PSII (NPQ), and pigments contents (*β*-carotene and chlorophyll-a content) of *D. salina* mutant were examined. Studying the effects of heavy ions exposure on the photosynthesis of microalgae will provide a better understanding of the biological effects of high-LET radiation on microalgae for *β*-carotene production.

2   Materials and methods

2.1   Microalgae medium and cultivation

The microalgae *D. salina* CCAP 19/18 was purchased from Culture Collection of Algae and Protozoa (Windermere, United Kingdom). The strain was maintained in the medium of optimized Artificial Sea Water (ASW), composing of 1.5 mol/L NaCl, 5 mmol/L KNO$_3$, 0.45 mmol/L MgCl$_2$·6H$_2$O, 0.05 mmol/L MgSO$_4$·7H$_2$O, 0.3 mmol/L CaCl$_2$·2H$_2$O, 0.13 mmol/L K$_2$HPO$_4$, 0.02 mmol/L FeCl$_3$, 0.02 mmol/L EDTA, 1 mL of trace elements stock per liter with 50 mmol/L H$_3$BO$_3$, 10 mmol/L MnCl$_2$·4H$_2$O, 0.8 mmol/L ZnSO$_4$·7H$_2$O, 0.8 mmol/L CuSO$_4$·5H$_2$O, 2 mmol/L NaMoO$_4$·2H$_2$O, 1.5 mmol/L NaVO$_3$, 0.2 mmol/L CoCl$_2$·6H$_2$O and the pH was adjusted to 7.5 by addition of Tris-buffer (40 mmol/L). *D. salina* seed culture was performed in 500 mL conical flasks at 50 μmol/(m^2·s) light intensity.

2.2   Carbon-ion irradiation

The carbon-ion irradiation was performed according to Wang et al.[15] After adjustment of the cell concentration to 1×10^6 cells/mL, the cell suspension was exposed to carbon-ion (^{12}C^{6+}) irradiation at doses ranging from 0 to 320 Gy (0, 30, 60, 90, 120, 180, 240 and 320 Gy), and there were at least three microalgae samples for each dose treatment. Irradiation was conducted at the Heavy-Ion Research Facility in Lanzhou (HIRFL), Institute of Modern Physics, Chinese Academy of Sciences. The initial energy was 80 MeV/u, and the average LET was 33 keV/μm[15]. After irradiation, the microalgal cells were diluted to an appropriate concentration, spread onto ASW-agar plates in triplicate, and cultured at (25±1)°C under illumination (100-120 μmol photons/m^2·s) to form colonies for mutants isolation.

2.3   Mutant isolation and screening

Colonies derived from the irradiated cells were selected according to their size and colors on the ASW-agar plates, and the methods were described as the Wang et al.[15] and Hu et al.[13] Firstly, individual colonies were transferred into 2 mL of ASW medium in the 24-well microplates and cultured for several generations to obtain purified monoclonal strains for amplification. The microalgae cells were then transferred to 10 mL of ASW medium in 15 mL glass tubes under low light (50 μmol/m^2·s) at 25°C for 7 d for further screening. On Day 6, OD$_{680\,nm}$ values of the cultures were measured with a spectrophotometer (Jasco V-530, JASCO Corporation, Japan). Afterwards, the 15 mL glass tubes were subjected to a high light intensity of 1000 μmol photons/m^2·s for another 7 d. The F_v/F_m values of microalgal mutants were then analyzed with a Water-PAM Chlorophyll Fluorometer (Water-PAM Heinz Walz GmbH, Effeltrich, Germany). Three replicates for each mutant were analyzed. After a large-scale screening, one mutant named DS240G-1 was obtained with high growth rate and photosynthesis efficiency.

2.4   Induction of intracellular *β*-carotene accumulation

A two-stage cultivation strategy was applied for *β*-carotene accumulation in algal cells. Once all cultures (DS240G-1 mutant and wild type) had reached the early stationary growth phase (after 7 d cultivation), microalgae cells were harvested by centrifugation, followed by washing with ASW. The harvested cells were then inoculated with the same concentration in a 500 mL bubble column bioreactor without nitrogen. Aeration enriched with 2% CO$_2$ was provided at 100 mL/min. In this second stage of cultivation, *β*-carotene accumulation was triggered by high light intensity of 1000 μmol photons/m^2·s[16]. After cultured for 7 d, the dry cell weight and pigment composition were measured. Triplicate experiments under each culture condition and triplicate sets of measurements were carried out.

2.5   Analytical methods

2.5.1   Growth analyses

Cell density was determined spectrophotometrically using a UV/VIS spectrophotometer (Jasco V-530, JASCO Corporation, Japan) at 680 nm. The microalgal dry weight (DW) was determined according to the method described by Cao et al.[17] Briefly, with pre-weighed Whatman GF/C filters, 10 mL culture broth was filtered and washed three times with 2 mL of 0.5 mol/L ammonium bicarbonate and then dried below 60°C for over 16 h until the weight was constant. The DW of the microalgae cells was calculated according to the difference between the final and initial filter weight and volume of the filtered sample.

The microalgal growth rate (μ_i, d^{-1}) was calculated by Equation (1).

$$\mu_i = \frac{LnDW_i - LnDW_{i-1}}{t_i - t_{i-1}} \tag{1}$$

The biomass productivity (P_i, g/L·d) was calculated as

$$P_i = \frac{DW_i - DW_{i-1}}{t_i - t_{i-1}} \tag{2}$$

where, DW_i and DW_{i-1} are the biomass concentration measured at time t_i and t_{i-1} (g/L), respectively; t_i and t_{i-1} are Day i and i–1 when the culture broth was sampled.

2.5.2   Main cell composition extraction and estimation

Approximately 5 mg dry biomass (for biomass fatty acid analysis) was added to 98%:2% (v/v) methanol:H$_2$SO$_4$ and incubated for 1 h at 70°C[18]. After methylation, deionized water and hexane were added to extract the fatty acid methyl esters (FAMEs). C17:0-TAG was added as the internal standard for quantification. Separation and identification of FAMEs were performed by using gas chromatography flame ionization detection (GC-FID, Agilent 6890) with a DB-23 capillary column (30 mm× 0.32 mm×0.25 μm). The injector temperature was 270°C, with a split ratio of 50:1. The program for the column temperature began

at 130°C for 1 min, followed by an increase to 170°C at a rate of 10°C/min, then another increase at a rate of 2.8°C/min to 215°C, where it was maintained for 1 min. The detection temperature was 300°C. FAMEs were identified by using the retention time and mass spectral matching. The protein content was determined using Markwell method, a modified Lowry method[19]. The starch content was measured according to the method described by Zheng et al.[20] Briefly, the algal pellet was re-suspended in 0.1 mol/L pH 4.4 acetate buffer, and autoclaved at 110°C for 15 min to solubilize the starch. Then 1.5 units of amyloglucosidase (Sigma-Aldrich, St, Louis, Mo, USA) were added and the solution was heated at 55°C for 1 h. The glucose was determined by the sulfuric acid-anthrone method. The blank assay for starch determination was carried out by adding equal amounts of reagents to a microalgae-free sample. Starch content (% DW) was calibrated by subtracting the glucose content from blank assay and multiplying 0.9.

2.5.3　Pigment extraction and determination

To determine the contents of pigments including chlorophyll (Chla and Chlb) and total carotenoids, 10 mg of dried biomass was extracted with 1 mL 90% (v/v) acetone, vortexed for 20 s, and then centrifuged at 10 000 g for 2 min. The above pigment extraction procedure was repeated several times until the algae were colorless. The absorbance at 645 nm (A_{645}), 662 nm (A_{662}), and 470 nm (A_{470}) of the extraction solution were measured respectively, using a UV/VIS spectrophotometer (Jasco V-530, JASCO Corporation, Japan). Chla (mg/L), Chlb (mg/L), and total carotenoids contents (mg/L) were calculated using the equations below:

$$Chla = 11.75\ (A_{662}) - 2.35(A_{645}) \tag{3}$$

$$Chlb = 18.61\ (A_{645}) - 3.96(A_{662}) \tag{4}$$

$$Total\ carotenoids = \frac{1000A_{470} - 2.270Chla - 81.4Chlb}{198} \tag{5}$$

$$Pigment\ content = \frac{Pigment\ concentration \times V}{M} \times 0.001 \times 100\% \tag{6}$$

2.5.4　*β*-carotene accumulation analysis

The modified spectrophotometric method was used to determine *β*-carotene content in biomass[21]. 1 mL of cell suspension was centrifuged at 10 000 r/min for 2 min. After centrifugation, the supernatant was discarded and 3 mL dodecane was added. The sample was shaken vigorously to re-suspend the algae pellets. Then, 9 mL of methanol was added to completely break up the cells and the tube was shaken vigorously again, then centrifuged for 2 min at 10 000 r/min. The dodecane-containing lipophilic carotenoids (upper layer) were measured with a spectrophotometer (Jasco V-530, JASCO Corporation, Japan) at 453 nm and 665 nm with dodecane as reference. The *β*-carotene concentration was calculated using Equation (7):

$$C_{\beta\text{-car}} = (A_{453} - A_{665}/3.91) \times 3.657 \times 3X \tag{7}$$

where, ($A_{453} - A_{665}/3.91$) is the absorbance of *β*-carotene corrected for chlorophyll contamination; 3.657 is the calibration factor derived from HPLC analysis of *β*-carotene concentration; 3 is the amount of milliliter of dodecane added for extraction; X is the dilution factor to measure absorbance on the spectrophotometer.

The content of *β*-carotene in the algae biomass was calculated according to Equation (8):

$$\beta\text{-carotene} = \frac{C_{\beta\text{-car}} \times 10}{DW} \tag{8}$$

where, $C_{\beta\text{-car}}$ is *β*-carotene concentration, mg/L; *β*-carotene is *β*-carotene content, %; and *DW* is cell dry weight, mg/L.

2.5.5　Chlorophyll fluorescence measurement

PSII activity of algal cells was measured by a chlorophyll fluorometer (Water-PAM Heinz Walz GmbH, Effeltrich, Germany). Firstly, the dark adaption of 10 min was performed before applying a saturating pulse (0.6 s, 1400 μmol/m^2·s) to measure the maximal PSII quantum yield (F_v/F_m), quantum yield of PSII (ΦPSII), nonphotochemical quenching (NPQ) and relative photosynthetic electron transport rate (rETR). F_v/F_m, ΦPSII, NPQ and rETR were determined according to the methods described by Yao et al.[22] and Wang et al.[15]

2.5.6　ROS Measurement

Confocal microscopy images of cellular ROS were captured following the method described by Zheng et al.[20] Briefly, *D. salina* cells (~2 million) were collected by centrifugation, washed once, and resuspended in 1× PBS buffer containing 5.0 μmol/L of the oxidant-sensing fluorescent probe H$_2$DCFDA (Thermo Fisher Scientific). After incubation at room temperature in the dark for 30 min, the samples were washed three times with 1× PBS buffer. The DCF fluorescence of 2 million cells was also collected with a fluorescence spectrophotometer (F-4500, Hitachi, Japan) using excitation at 504 nm and emission at 524 nm.

2.6　Statistical analyses

The One-way ANOVA analyses[17] were performed in Excel (version 2013, Microsoft) to make the significance analysis for the growth (dry weight), fatty acid content, protein content, starch content and *β*-carotene content of the microalgae WT and DS240G-1.

3　Results and discussion

3.1　Heavy-ion irradiation mutagenesis

The wild *D. salina* strain was treated with a wide dose range (0-320 Gy) of heavy-ion irradiation. As shown in Figure 1, the relationship between the survival rate of *D. salina* and the irradiation dose was fitted to a logistic curve equation, showing that the survival rate of *D. salina* decreased by approximately 69% to 27% with the increase of radiation dose. According to the previous report in mutagenesis breeding, high mutation frequency was usually found at low survival rates[15]. The results here indicated that low doses of irradiation (0-60 Gy) gave a high survival rate but a low probability of hyper-producing mutants. By contrast, high dose of irradiation (90-320 Gy) caused irreversible damage to cells and hindered growth. Hence, *D. salina* irradiated under 90, 120, 180, 240 and 320 Gy, respectively, were selected for further mutation screen in this study.

After carbon-ions irradiation treatment, microalgae colonies which appeared on ASW-agar plates were selected as putative mutants. The initial putative mutants screening through light microscopy revealed that the morphological characteristics (e.g. colony size, cell appearance and shape, etc.) of the assumed mutants were different from the wild-type algae cells. According to the previous study, a screening strategy of microalgae mutants with high yields of biomass and lipid-based on the determination of F_v/F_m value was developed[13]. Hence, the quantum efficiency of PSII (F_v/F_m) was select as an indicator to further characterization of the putative mutants by means of a chlorophyll fluorescence technology[13]. All algae mutants were subjected to the F_v/F_m value analysis under different illuminations by Water-PAM firstly. Hereafter, the algal growth and *β*-carotene yields of the mutants with high F_v/F_m values were quantified in 24-well microplates. From this method, the mutants which have F_v/F_m and *β*-carotene

content at least 10% higher than wild type were chosen for further study, while the other mutants were throw away[13].

After the large-scale screening, one mutant (DS240G-1) obtained from 240 Gy of heavy-ion irradiation was selected with a growth rate 50% higher than the wild strain. The photosynthetic efficiency and β-carotene contents of DS240G-1 were also 10% higher than those of wild type. Similar results had also been reported in heavy mutagenesis which got the desired mutant from the high cell mortality condition[11].
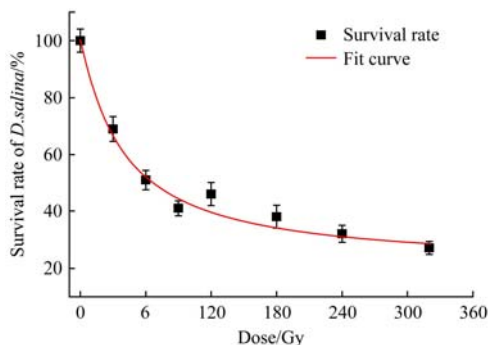
Note: Data are means of three repeated experiments and error bars indicate standard deviations.

Figure 1   Survival rate of *Dunaliella salina* CCAP 19/18 treated with different doses of carbon-ions irradiation

3.2   Growth and pigment characterizations of the DS240G-1 mutant

To further characterize the growth and pigment accumulation of DS240G-1, the growth curve, photosynthetic pigments content, and photosynthesis characteristics for the wild type and DS240G-1 were investigated in this study. Biomass production is one of the most important factors for microalgal commercialization[23]. As shown in Figure 2a and Table 1, the highest biomass dry weight

(1.10 g/L) and biomass productivity (0.14 g/L·d) of DS240G-1 were 34% and 40% higher than those of the wild type. The maximum specific growth rate of DS240G-1 was 0.90 d^{-1}, which was 55% higher than that of the wild type. These results indicated that DS240G-1 could be considered as a promising candidate for future commercialization.

Photosynthetic pigments are the major compositions of the light-harvesting complex which capture light energy in microalgae photosynthesis[23]. It was shown that the chlorophyll-a content of both wild type and DS240G-1 increased after inoculation, and reached their highest level on day 3, and then decreased in the following cultivation days (Figure 2b). The chlorophyll-a content of DS240G-1 was 14% higher than that of the wild type on day 3 (Figure 2b). A similar tendency was observed in the β-carotene content of these two strains (Figure 2c). The β-carotene content of DS240G-1 was 19% higher than wild strain on day 5. A possible explanation for higher photosynthetic pigment content is that enhanced pigment content might improve the light energy absorption ability, and then lead to the enhancement of the photosynthesis efficiency of microalgae, thus result in the biomass increasing in the DS240G-1 strain. Similar results that the enhancement of photosynthetic pigments content could increase the biomass had been reported in other microalgae strains[24-26].

Table 1   Growth analyses of *D. salina* wild type (WT) and DS240G-1 mutant for 7 d cultivation

	WT	DS240G-1
Dry weight/g·L^{-1}	0.82[**] (±0.03)	1.10[**] (±0.02)
Productivity/g·L^{-1}·d^{-1}	0.10[*] (±0.00)	0.14[*] (±0.00)
Specific growth rate/d[-1]	0.60[**] (±0.03)	0.93[**] (±0.02)

Note: Values are means (±SD) of n=3 cultivations per treatment, * represent the significant effect ($p<0.05$) and ** represent the very significant effect ($p<0.01$)

a. Growth curve

b. chlorophyll a (Chla)

c. β-carotene content
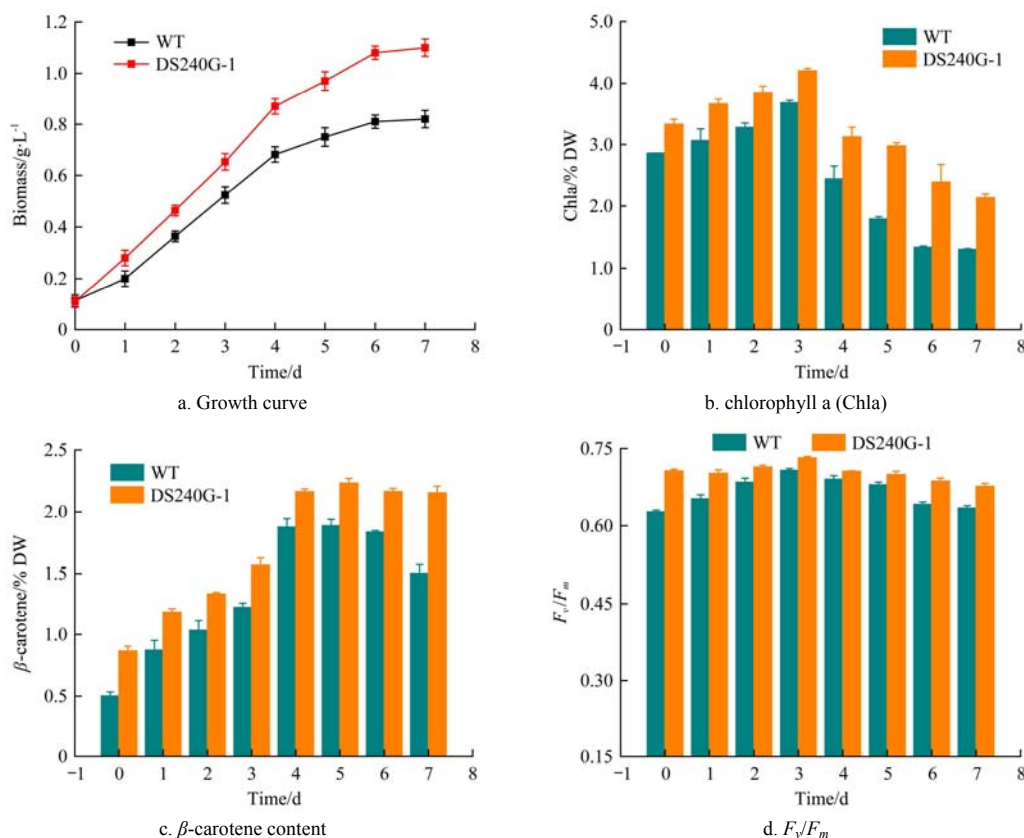
d. F_v/F_m

Note: Data are means of three replicates and error bars indicate standard deviations.

Figure 2   Growth curve, chlorophyll a (Chla), β-carotene content and F_v/F_m of *D. salina* wild type (WT) and DS240G-1

To further study the reason for high biomass production of DS240G-1, photosynthetic parameter F_v/F_m was measured in both DS240G-1 and wild-type strains (Figure 2d). The energy absorbed by light from the photosynthetic pigment of microalgae is converted into photosynthesis during the quantum conversion process of photosynthesis, chlorophyll fluorescence and heat dissipation[27]. F_v/F_m, reflecting the potential maximum quantum efficiency, can directly reflect the photosynthesis activity of PSII[28,29]. As shown in Figure 2d, the average values of F_v/F_m in DS240G-1 were 5% higher than that of the wild type during the entire culture process, which further verified the higher biomass was because of higher photosynthesis activity in DS240G-1.

3.3 *β*-carotene accumulation of the mutant DS240G-1 with high light

To further investigate the *β*-carotene accumulation of DS240G-1, a two-stage cultivation model was applied. Owing to separate growth and *β*-carotene production phases, the two-stage cultivation has been proved to improve overall *β*-carotene productivity in microalgae[9]. In this study, after 7 d cultivation in low light intensity and nitrogen-repletion conditions, microalgae culture was harvested and inoculated into the second-stage culture broth under an irradiance of 1000 *μ*mol photon/m^2·s with the same

cell biomass (0.5 g/L). As shown in Figure 3a, there was no significant difference (*p*>0.05) in biomass accumulation between the DS240G-1 and wild type during the cultivation period, and the biomass of DS240G-1 (0.78 g/L) was similar with the wild type (0.76 g/L) on Day 5. Furthermore, the maximal *β*-carotene content (8.75%) was obtained in DS240G-1 on Day 5 at the second-stage culture, which was 21% higher than that of the wild type (7.23%) (Figure 3b). Therefore, due to the significant increase of *β*-carotene content, the *β*-carotene yield of DS240G-1 (66.5 mg/L) was 18% higher compared to the wild type (56.4 mg/L) on Day 5 at the second-stage culture (Figure 3c).

To further investigate the reason for the high biomass productivity of DS240G-1 during *β*-carotene accumulation, the photosynthetic parameters including ΦPSII, NPQ and rETR were determined. As shown in Figure 3d, on day 5 with the highest *β*-carotene production, the ΦPSII values of DS240G-1 and the wild type decreased with increasing photosynthetically active radiation (PAR, 29-1416 *μ*mol photons/m^2·s). The ΦPSII values of DS240G-1 were higher than that of the wild strain over the entire investigated light step ranges (*p*<0.05). The above results showed that the photosynthetic efficiency of DS240G-1 was higher than that of the wild type during the *β*-carotene accumulation phase, which might result in its higher biomass productivity.
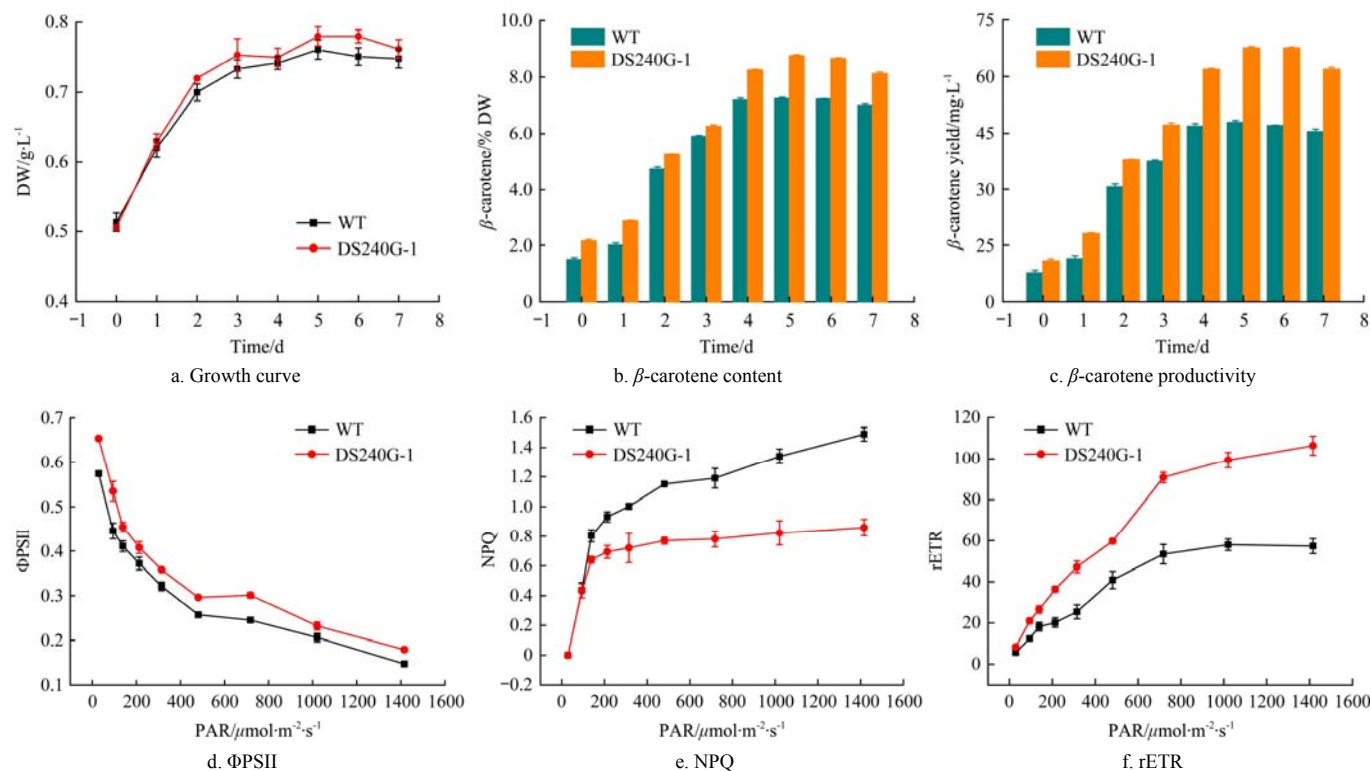
a. Growth curve   b. *β*-carotene content   c. *β*-carotene productivity

d. ΦPSII   e. NPQ   f. rETR

Note: Data are means of three repeated experiments and error bars indicate standard deviations

Figure 3   Cell biomass, *β*-carotene content and photosynthetic characteristics during *β*-carotene accumulation period of *D. salina* wild type (WT) and DS240G-1 mutant.   Growth curve, *β*-carotene content and *β*-carotene productivity, ΦPSII, NPQ, rETR.

NPQ reflects a protection process that thermally dissipates excess light energy that is not used for photosynthesis[15,30]. As shown in Figure 3e, the NPQ values of DS240G-1 and wild-type strain increased with increasing PAR. The NPQ values of DS240G-1 were lower than that of the wild-type strain over the light steps ranging from 139 to 1416 *μ*mol photons/m^2·s (*p*<0.05), and suggesting that DS240G-1 exhibited less thermal dissipation than the wild type strain at high levels of light steps. Also, the rETR is an approximation of the rate of electrons pumped through the photosynthetic chain[15,28]. As shown in Figure 3f, the rETR

values of DS240G-1 and wild type strain increased in response to increasing PAR and reached a maximum at 1416 *μ*mol photons/(m^2·s) and 1020 *μ*mol photons/(m^2·s), respectively. The rETR values of mutant DS240G-1were higher than that of the wild type strain over the light steps range 29-1416 *μ*mol photons/m^2·s (*p*<0.05), indicating that the mutant strain DS240G-1 showed higher photosynthetic electron transport rates than the wild type strain. In summary, high actual photosynthetic efficiency of PSII, low thermal dissipation ability, and high electron transport rate at all light steps (29-1416 *μ*mol photons/m^2·s) were observed for

mutant DS240G-1.   Besides, mutant DS240G-1 showed stronger resistance to high PAR than the wild-type strain.

The above results showed that the photosynthetic efficiency of DS240G-1 was higher than that of the wild-type strain during the β-carotene accumulation phase.   β-carotene yield is one of the key factors in microalgal β-carotene commercialization.   The increase in β-carotene yield depends on the improvements in both biomass and β-carotene content.   Similar results have been reported that higher biomass strains of *Nannochloropsis* and *Desmodesmus* sp. can be obtained through improving photosynthesis efficiency by heavy ions irradiation[11,13].   Taken together, it was suggested that increased photosynthesis efficiency of the DS240G-1 mutant resulted in its higher β-carotene yield than that of the wild type.

3.4   Changes in ROS content and cell biomass composition of the mutant with high light

When wild type and DS240G-1 were cultivated under high light (HL) conditions, the ROS content of DS240G-1 increased dramatically in the first-day cultivation.   From day 2 to day 7, the ROS content decreased with the increasing β-carotene content. The ROS content of DS240G-1 was higher than (20%-94%) that of the wild strain over the β-carotene accumulation stage.   Under stress conditions including high light irradiation, and nitrogen deficiency, ROS are generated in *D. salina*[20], which can lead to cellular damage.   In photosynthetic organisms, in order to avoid or relieve the damage caused by ROS, photosynthetic organisms have evolved different types of mechanisms to scavenge ROS[20].   In microalgae *D. salina*, β-carotene, as an integral component of microalgae photosynthetic apparatus pigment, can protect cells against the ROS formed from photo-oxidation[20,31].   The result of this study revealed that high content of ROS might induce massive β-carotene accumulation in mutant DS240G-1.

Furthermore, the total fatty acid, protein and starch contents were determined in wild type and DS240G-1 under high light conditions (Table 2 and Figure 4).   The protein content in wild-type strain was 21% higher than that in mutant DS240G-1, but the starch content in wild-type strain was 22% lower than that in mutant DS240G-1.   Also, the total fatty acid (TFA) content in mutant DS240G-1 (approximately 18% DW) was 79% higher than in wild type.   Owing to its higher β-carotene productivity and higher TFA content, DS240G-1 could be considered as a promising candidate for microalgae β-carotene and biodiesel production.

Table 2   Major biomass compositions of WT and DS240G-1

Strains	Protein/%DW	Starch/%DW	TFA/%DW
WT	48.51[*] (±4.10)	13.40 (±1.61)	9.85[**] (±0.83)
DS240G-1	40.13[*] (±1.62)	16.30 (±2.15)	18.43[**] (±0.61)

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

a. ROS

b. Biomass composition
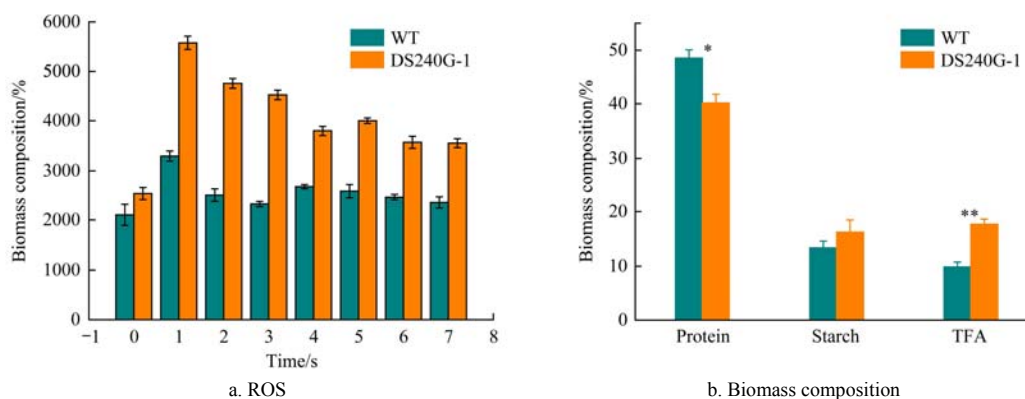
Note: Data are means of three repeated experiments and error bars indicate standard deviations, *represent the significant effect ($p<0.05$) and ** represent the very significant effect ($p<0.01$)

Figure 4   ROS and biomass composition characteristics during β-carotene accumulation period of *D. salina* wild type (WT) and DS240G-1 mutant

4   Conclusions

This work presented the first attempt to use carbon ions to induce mutagenesis of microalgae for enhanced β-carotene productivity.   In this study, the mutant (DS240G-1) with high β-carotene productivity (67.6 mg/L) was obtained from *D. salina*. Compared to the wild strain, its β-carotene content and TFA content increased by 21% and 79%, respectively, likely owing to the improved quantum efficiency of photosynthesis and ROS content under HL stress conditions, and indicated that the mutant strain could be considered as a valuable candidate for microalgal β-carotene production.   More work remains to be done to clarify the molecular mechanism of mutant (DS240G-1) strain after carbon ions irradiation.

Acknowledgements

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