

RESEARCH ARTICLE

β -carotene Production of UV-C Induced *Dunaliella salina* Under Salt Stress

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Abstract

Dunaliella salina is an important microalga for β -carotene production. In Thailand, the *D. salina* has not been commercially cultivated. Aim of this study was to screen high β -carotene producing mutants. *D. salina* KU11 cells were radiated with 0.4 mmol photons $m^{-2}\cdot s^{-1}$ UV-C for 22 min. After 3 weeks, colonies which survived after radiation were allowed to grow on agar plates; fifty colonies out of 19,720 UV-mutagenized strains turned to orange. These colonies were picked up and transfer to modified Johnson medium. Four mutants out of fifty were KU5, KU18, KU20 and KU37 significantly produced high β -carotene at the stationary phase approximately up to 1.62, 2.03, 1.32 and 1.21 folds, respectively, compared to the wild type strain. In all three consecutive cycles of cultivation, the mutant KU18 accumulated 2.4-8.5 folds β -carotene ($\mu g/ml$) and 2.1-4.8 folds β -carotene ($pg/cell$) higher than the wild type, whereas cell density of the mutant KU18 was not different from the wild type. This is the new record of β -carotene producing mutant isolated from saline soil in Thailand.

Keywords: *Dunaliella salina*; UV-C radiation; β -carotene; high salinity.

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INTRODUCTION

β -carotene is a major carotenoids pigment found in *Dunaliella* microalgae. The alga could produce β -carotene under extreme environmental stress, such as high light intensity, high salinity, extreme temperature and pH or nutrient deficiency¹. Carotenoids are natural pigments that play pivotal roles in many physiological functions. Carotenoids have also become popular as dietary supplements due to that carotenoids are powerful antioxidants and therefore it can provide protection against ailments, such as cardiovascular diseases, certain cancers, age-related macular degeneration, and neurodegenerative diseases e.g., amyotrophic lateral sclerosis². Recently, microalgae were studied due to their abilities in producing novel bioactive metabolites, including carotenoids that provided health and cosmetic benefits³. Carotenoid usage has been studied for long time. However, most focus on carotenoids obtained from several microalgae, vegetables, fruits, and higher plants. In Thailand, cultivation area of *D.salina* in commercial scale did not widely spread out due to the limit of β -carotene accumulation in the strain³. Genetic improvement of wild species of *D. salina* may play important role on the increase of the β -carotene content.

UV radiation causes DNA mutation in bacteria, algae and plants, by inducing dimer formation between nucleotide bases, oxidative mutation and generation of several photo-products that affect replication and transcription of the DNA, causing mutations and death⁴.

UV-C radiation induced mutation of several genera of green algae especially in Chlamydomonadales including *Chlamydomonas* and *Dunaliella*. UV-C radiation affected on growth and photosynthesis which resulted in accumulation of starch and pigment^{5,6,7,8}. Sirikha-chornkit et al.⁸, studied utilize of UV-C to isolate *Dunaliella* mutant. These mutants produced total carotenoid higher than the original wild type did.

Recently, Sharma et al.⁶, presented an approach, using a small dose of UV-C radiation, to rapidly induce unsaturated fatty acids and carotenoid biosynthesis in *Dunaliella salina* and *Haematococcus pluvialis*. Total carotenoids and β -carotene were doubled in 24 h on *D. salina* when UV-C radiation of 50 mJ/cm² was utilized whereas the astaxanthin yield of *H. pluvialis* was increased

five times in 48 h at 30 mJ/cm² of UV radiation. This new technique represented a convenient, timesaving and low cost method for commercial microalgal carotenoids production. Moreover Masi & Melis⁹, reported effect of supplemental UV-B radiation during growth in *D.salina* was investigated at the cellular level, supplemental UV-B radiation induced a doubling of cell volume, a phenomenon attributed to a slow-down in the rate of cell division. Recently, *D. salina* was one of algal species which were potentially sources for beta-carotene production owing to high commercial value as an anti-oxidant; hence the researches involving *D. salina* were intensively conducted worldwide. However, there was few researches involving *D. salina* studied in Thailand.

Aim of this study was to screen β -carotene producing strains of *Dunaliella salina* after UV-C radiation. Growth and β -carotene production of the mutants was compared to the wild type. We hypothesize that the β -carotene production in the UV-C mutants is higher than in the wild type strain.

MATERIALS AND METHODS

Strain and Culture Condition

Dunaliella salina KU 11 was isolated from saline soil samples collected from the northeastern part of Thailand and was maintained on modified Johnson's medium¹⁰. The pure culture was grown at 25°C under continuous illumination of 50 mmol photons m⁻²·s⁻¹ cool-white fluorescent light. This wild type strain was sub-cultured every 15 days.

Mutagenesis by UV-C and Mutant Screening

For UV-C mutagenesis, the *Dunaliella salina* KU 11 was prepared to the density of 5'10⁶ cells/ml. Cells were then spread onto 1.5% agar plates and kept in the dark for 2 h, and were exposed to 0.4 mmol photons m⁻²·s⁻¹ UV-C irradiation for 22 min, petri disc was wrapped in aluminum foil for overnight to prevent light-induced DNA repair. Cell were grown at 50 mmol photons m⁻²·s⁻¹. After three weeks, when cells started to turn orange, they were selected for the further study.

Growth and pigment accumulation in wild type and mutants

Fifty mutants were obtained from the 19,720 colonies, from the previous study, and colonies were grown in modified Johnson's medium containing 1.5M NaCl under light intensity

200 mmol photons $m^{-2}\cdot s^{-1}$

KU18 the mutant which provided the highest β -carotene was selected to culture for three consecutive cycles compared to the wild type (KU11). Algae were grown in modified Johnson's medium containing 2.5M NaCl and 0.5 g/l KNO_3 under light intensity 200 mmol photons $m^{-2}\cdot s^{-1}$ for 14 days.

Cell number was counted every 2 days using a haemocytometer. The specific growth rate (μ) was calculated from the growth curve using a fitting program for the data from logarithmic growth phase. The μ was computed from the following formula (1):

$$\mu = \frac{\ln X_2 - \ln X_1}{(t_2 - t_1)} \quad (1)$$

Where X_1 and X_2 are cell concentrations at time t_1 and t_2 ¹¹.

The algal cells were collected every 2 days for Chlorophyll a concentration of the sample was determined according to Arnon¹², Total Carotenoid concentration was determined according to Lichtenthaler¹³, and β -carotene analysis. To determine the β -carotene content, the samples were extracted with 80% acetone and analyzed by the reverse phase HPLC (Column: VertiSepTMBIO C30, 5 μ m, 4.6' 250 mm, mobile phase: Methanol/Tertiary methyl butyl ether, 80/20 (v/v), flow rate: 1.0 ml/min). The β -carotene was identified by its retention time and quantified by the absorbance spectra in comparison with a β -carotene standard. (Sigma)

Statistical Analysis

All the data analysis was processed by Microsoft Excel 2016 t test. The statistical significance was determined at 95% level of confidential limit.

RESULTS

Mutagenesis by UV-C and Mutant Screening

Algal colonies were initially irradiated by UV-C. Survival rate was 1% from the original 19,720 colonies. Three weeks later, only 50 colonies began to turn orange and were selected for β -carotene accumulation. The biomass productivity was calculated for 12th days from all those selected colonies which were cultured under 1.5M NaCl modified Johnson's medium, under light intensity of 200 mmol photons $m^{-2}\cdot s^{-1}$. The highest dry weight content was achieved in KU18 (0.79 \pm 0.02 g/L) followed by KU5 (0.62 \pm 0.04 g/L), KU20(0.53 \pm 0.03 g/L) and KU37 (0.41 \pm 0.01 g/L) (Fig. 1). While the wild type cells still green color, the mutant cells were changed from green to orange (Fig. 2-3). High chlorophyll a, total carotenoid and β -carotene content were found in the mutants 5, 18, 20 and 37 compared to the wild type strain (Table 1). KU18 provided the best pigment accumulation (Fig. 4) and the result of β -carotene accumulation (pg/cell) was also the highest of the selected colonies (Fig. 5).

Growth and pigment accumulation in wild type and mutant KU18

Owing to the best ability to produce β -carotene, the *D. salina* KU18 mutant was selected for growth and pigment accumulation study. The mutant KU18 showed similar growth pattern in all three consecutive cycles of cultivation. The specific growth rate (μ) of the mutant KU18 was 0.198-0.258 which did not differ from the wild type (Table 2). The cell number gradually increased and reached the exponential phase in 6 days. The exponential phase of growth period was observed from 6th-8th days of cultivation. Growth of the

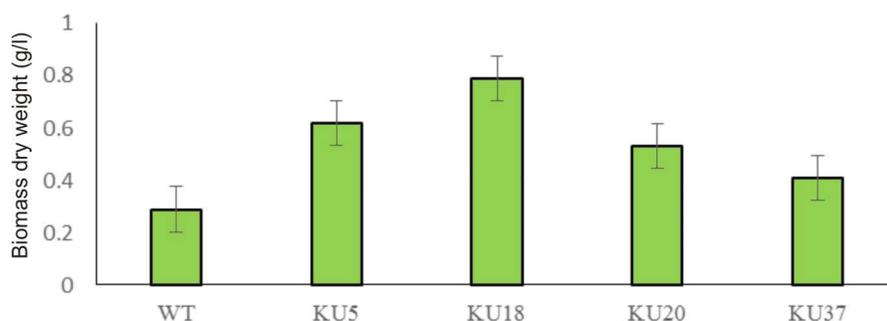
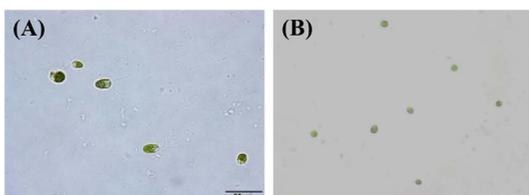
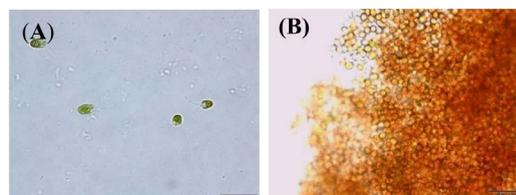


Fig. 1. Mutant screening and biomass yield of *D. salina* of each strain cultured in 1.5 M NaCl modified Johnson's medium under light intensity 200 mmol photons $m^{-2}\cdot s^{-1}$.

Table 1. Chlorophyll a, Total carotenoid and β -carotene content in the mutants (mt) 5,18,20 and 37 at the 12th day of cultivation, wild type (wt)

Isolates	wt	mt 5	mt 18	mt 20	mt 37
Chlorophyll a (mg/ml)	3.81±0.27	12.64±0.44	12.89±1.14	12.02±0.58	11.97±0.48
Total carotenoid (mg/ml)	0.39±0.08	2.66±0.09	2.99±0.10	2.35±0.27	2.13±0.11
β -carotene (mg/ml)	0.12±0.03	1.74±0.11	2.15±0.12	1.44±0.15	1.33±0.09

**Fig. 2.** *D. salina* KU 11 cells grown in liquid modified Johnson's medium containing 2.5 M NaCl at 3rd day (A) and 12th day (B) of incubation, observed under microscope.**Fig. 3.** *D. salina* mutants KU 18 cells grown in liquid modified Johnson's medium containing 2.5 M NaCl at 3rd day (A) and 12th day (B) of incubation, observed microscope

mutant KU18 reached stationary phase after 8 days of the cultivation (Fig. 6).

The *D. salina* mutant KU18 showed significant difference in the chlorophyll and total carotenoid ratio at all three consecutive cultivation cycles. The highest chlorophyll content was found in the culture grown in the third cultivation cycle, in which the highest chlorophyll content was 11.05 $\mu\text{g/ml}$. The highest total carotenoid content (mg/ml) reached the maximum content at the 12th day

after inoculation in the third cycle of consecutive cultivation (4.72 $\mu\text{g/ml}$, 3.84 pg/cell) (Fig. 7 and Table 2).

In all three consecutive cycles of cultivation, the mutant KU18 accumulated 2.4-8.5 folds β -carotene ($\mu\text{g/ml}$) and 2.1-4.8 folds β -carotene (pg/cell) higher than the wild type, whereas cell density of the mutant KU18 was not different from the wild type (Table 2).

Table 2. Growth and pigment production of KU18 mutant at 12th days of cultivation. The number in brackets of mutant KU18 represents the fold numbers compared to the wild type in modified Johnson's medium containing 2.5M NaCl under light intensity 200 mmol photons $\text{m}^{-2}\cdot\text{s}^{-1}$ for 14 days.

Strain	1 st cultivation cycle		2 nd cultivation cycle		3 rd cultivation cycle	
	wt-1	mt 18-1	wt-2	mt 18-2	wt-3	mt 18-3
Cell density ($\times 10^4/\text{cell}$)	129	121*	118	113*	111	123*
Specific growth rate (μ)	0.258	0.249*	0.200	0.198*	0.252	0.241*
Doubling time (td)	2.68	2.79*	3.47	3.50*	2.75	2.88*
Chlorophyll a ($\mu\text{g/ml}$)	1.03	5.61*(5.4)	0.54	6.64*(12.3)	4.12	11.05*(2.7)
Chlorophyll a (pg/cell)	0.80	4.56*(5.7)	3.01	8.30*(2.8)	3.71	8.99*(2.4)
Total Carotenoid ($\mu\text{g/ml}$)	0.76	3.53*(4.6)	0.36	3.29*(9.1)	2.09	4.72*(2.3)
Total Carotenoid (pg/cell)	0.59	2.87*(4.8)	2.12	3.96*(1.9)	1.88	3.84*(2.0)
β -carotene ($\mu\text{g/ml}$)	0.49	2.20*(4.5)	0.24	2.04*(8.5)	1.30	3.06*(2.4)
β -carotene (pg/cell)	0.38	1.82*(4.8)	1.12	2.43*(2.2)	1.17	2.49*(2.1)

*Significantly different as determined by t test ($\alpha = 0.05$, $n=3$)

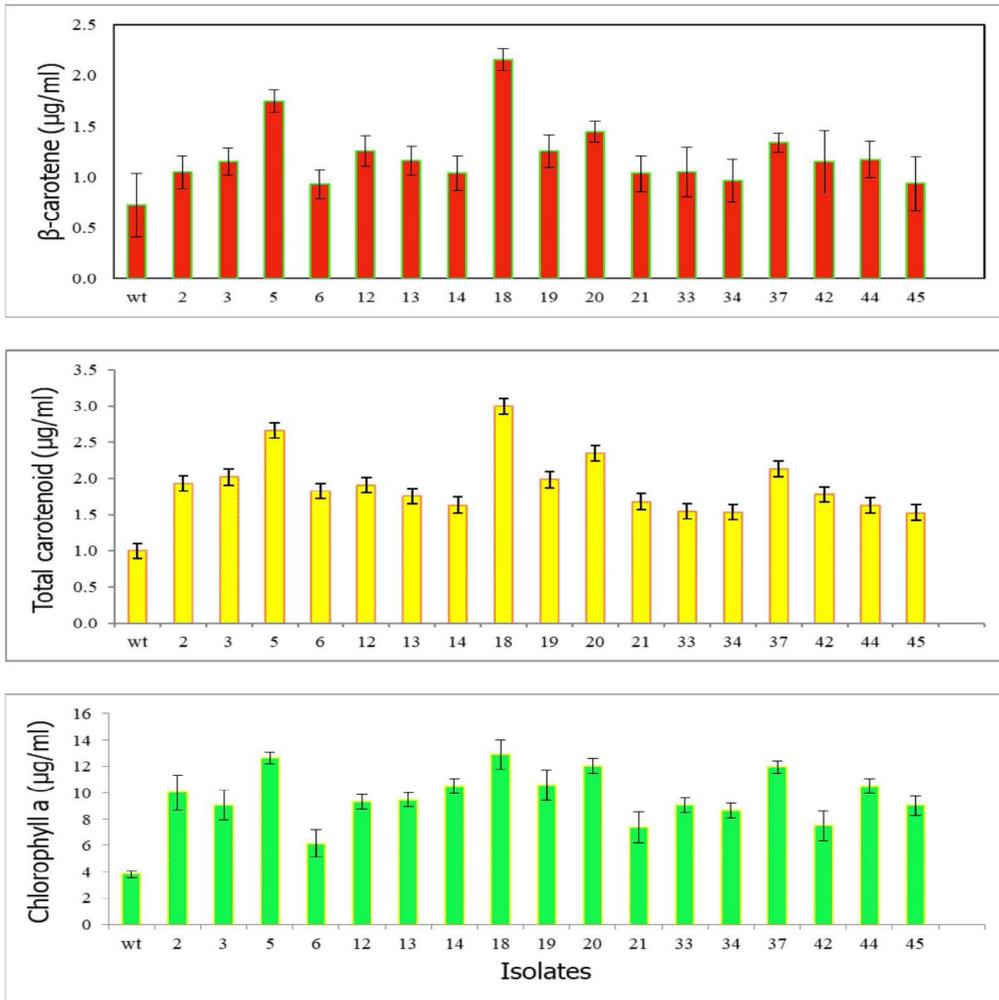


Fig. 4. Pigment concentrations of 17 selected survival mutants grown in modified Johnson's medium under light intensity 200 mmol photons $m^{-2}\cdot s^{-1}$ at 12th day of cultivation

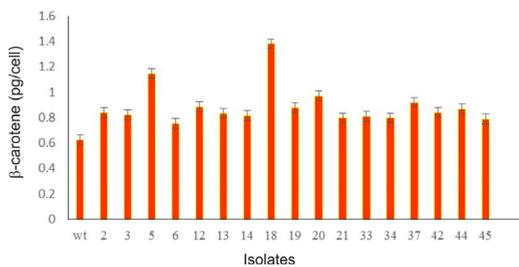


Fig. 5. Bar diagram represents β -carotene content (pg/cell) of 17 selected survival mutants grown in modified Johnson's medium under light intensity 200 mmol photons $m^{-2}\cdot s^{-1}$ at 12th day of cultivation

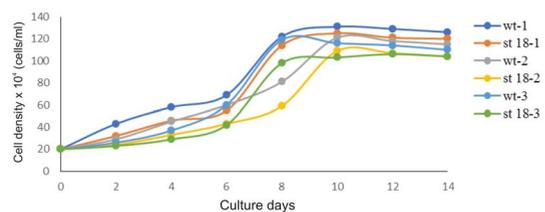


Fig. 6. Growth of wild type (wt) and mutants (mt) in the first cultivation cycle (wt-1, mt KU18-1), the second cultivation cycle (wt-2, mt KU18-2) and the third cultivation cycle (wt-3, mt KU18-3) in 2.5M NaCl modified Johnson's medium under 200 mmol photons $m^{-2}\cdot s^{-1}$.

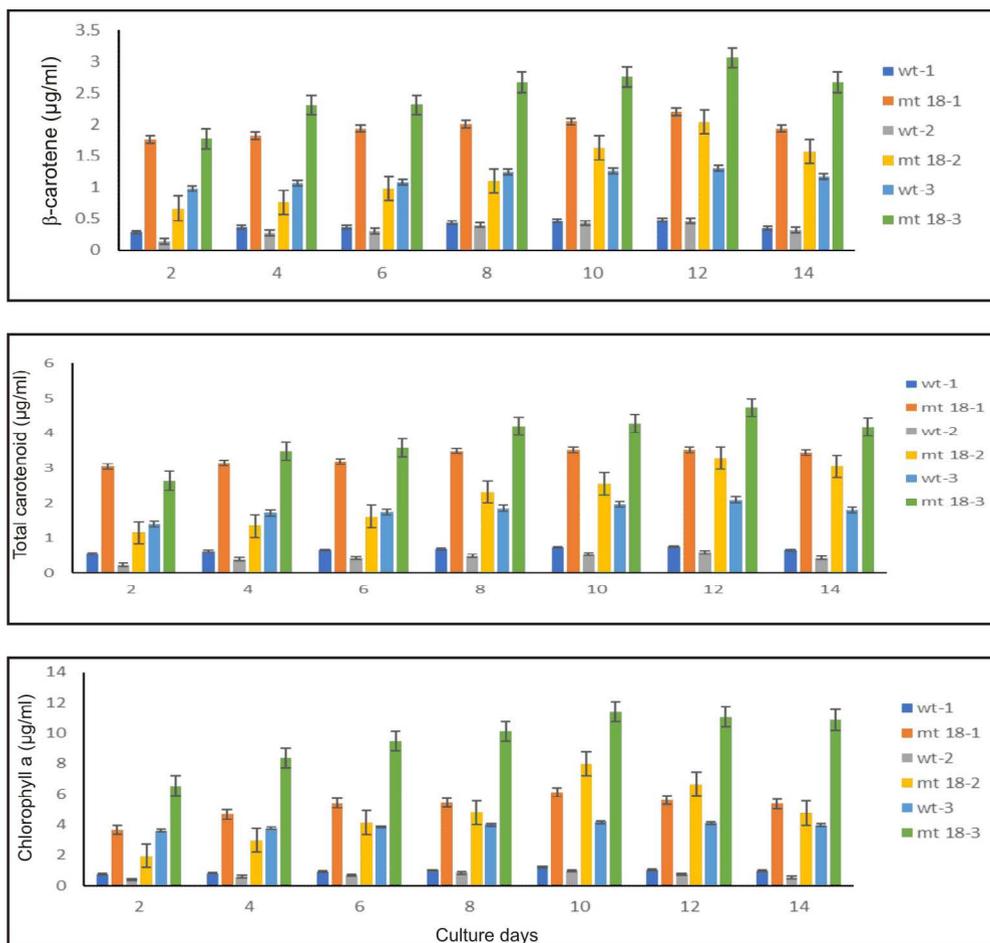


Fig. 7. Pigment of wild type and KU18 mutant strain in the first cultivation cycle (wt-1, st18-1), the second cultivation cycle (wt-2, st18-2) and the third cultivation cycle (wt-3, st18-3) in 2.5M NaCl modified Jonson's medium under 200 mmol photons $m^{-2}\cdot s^{-1}$.

DISCUSSION

This study aimed to screen β-carotene producing strains of *Dunaliella salina* after UV-C radiation. Growth and β-carotene production of the mutants was compared to the wild type. The result indicates that *Dunaliella salina* KU18 was a mutant strain because it was selected from very large populations (19,720 colonies) with 1% survival rate. Moreover, the KU18 produced higher total carotenoid and β-carotene than the wild type in all three consecutive cultivation cycles. However, role of the UV-C on expression of β-carotene gene was not reported in this study. Similar result was reported by Sharma et al.⁶, who reported that continuous UV-C radiation for 24 h rapidly induced

carotenoid biosynthesis in *D. salina* and the total carotenoids and β-carotene were doubled in 24 h. UV-C has been successfully used to generate microalgae mutants¹⁴. Sirikhachornkit et al.⁸, found that the UV-C induced total carotenoid content of *D. tertiolecta*. UV-C played an important role on the induction of expression of genes that are encoding the antioxidant.

Nikookar et al.¹⁵, reported that plant or alga increased its chlorophyll content during stress because during stress, plant or alga cytosol contained several strong oxidative agents, such as superoxide free radical. The oxidative agents stimulated plant or alga cell to synthesis more chlorophylls and carotenoids to protect cell or

tissue from further damage by stress conditions¹⁶. Jahns & Holzwarth¹⁷ suggested that increasing in chlorophyll was probable a result of xanthophyll accumulation to protect photosystem II. Unlike ordinary plant which chlorophyll synthesis negatively affected by salt stress, green algae could increase chlorophyll content. However, high nitrogen concentration probably promoted chlorophyll synthesis, since pyrrole ring, a precursor in chlorophyll synthesis. Hypersaline condition also evoked oxidative stress in alga cytosol; salt stress induced synthesis of several terpene derivatives which were precursors to carotenoids and xanthophyll¹⁴.

In this study, the KU18 produced higher β -carotene in 2.5M NaCl concentration than that in 1.5M NaCl concentration. As reported early by Prieto et al.¹⁸, the highest β -carotene production was obtained in the 2.5M NaCl concentration. β -carotene content affected by salinity¹⁹. They found that the highest carotenoid contents per cell were obtained when two species of *D. salina* and *D. bardawil* were cultured in media containing 2M NaCl. In the study of Radakovits et al.¹⁸, carotene contents varied from 0.2 to 11.4 pg/cell at the optimum concentration of 3.0M NaCl. In general plant as well as algae synthesized secondary metabolites stress conditions. All plant and algae protected themselves by synthesis of terpene compounds and these terpenoids were precursors to the synthesis of carotenoids and xanthophyll⁷. In hypersaline condition *Dunaliella* could accumulate up to 14% dried weight of β -carotene. In cytosol of this algae also contained high concentration of starch, glycerol and fatty acid, in order to regulate osmotic pressure driven by salt stress. Accumulation of β -carotene as well as glycerol in cytosol was thought to be as a carbon sink in alga cell^{2,6,20}. Moreover, *Dunaliella* was sensitive to UV radiation and UV which increased production of fat, oil and β -carotene^{12,21,22,23}.

The mutant KU18 accumulated 1.38 and 2.49 pg β -carotene/cell in the medium containing 1.5 and 2.5M NaCl, respectively (Figure 5 and Table 2). To test for the β -carotene accumulation efficiency between the mutant KU18 and the wild type strain, the light intensity was limited to 200 mmol photons $m^{-2}\cdot s^{-1}$. Under this condition, the accumulation efficiency of β -carotene in the mutant KU18 is more effective than that in the

wild type strain. Similarly, Kim et al.¹, reported that development of a *Dunaliella tertiolecta* Strain with increased β -carotene content using random mutagenesis under light intensity 160 μ mol photons $m^{-2}\cdot s^{-1}$.

CONCLUSION

The 0.4 mmol photons $m^{-2}\cdot s^{-1}$ UV-C was successfully used to induce mutation of *D. salina* KU11 strain with the increase in β -carotene production. The mutant was proved to be sustained. In all three consecutive cultivation cycles, the β -carotene content in the *D. salina* mutant KU18 remained higher than the wild type strain.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

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