Comparison of the responses of two *Dunaliella* strains, *Dunaliella salina* CCAP 19/18 and *Dunaliella bardawil* to light intensity with special emphasis on carotenogenesis

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*Dunaliella salina* and *Dunaliella bardawil* are well known for carotenogenesis, the overproduction of carotenoids, under stress conditions. The effect of high light (HL) and low light (LL) on the growth, morphology, photosynthetic efficiency, and the β-carotene and zeaxanthin production of *D. salina* CCAP 19/18 and *D. bardawil* was investigated and compared. Both strains showed similar growth kinetics under LL growth condition, but *D. salina* CCAP 19/18 was faster. As the light intensity increased, *D. salina* CCAP 19/18 cells were elongated and *D. bardawil* cells became larger. Both strains showed decrease of the maximum quantum yield of PSII (Fv/Fm) and election transport rate (ETR) under HL growth condition and *D. salina* CCAP 19/18 was less liable to the light stress. Both strains had about 1.8 and 5 times difference in the O₂ evolution rate at LL and HL conditions, respectively. The β-carotene and zeaxanthin production were increased as the light intensity increased in both strains. *D. bardawil* was more sensitive to light intensity than *D. salina* CCAP 19/18. The possible application of *D. salina* CCAP 19/18 as a carotenogenic strain will be discussed.

**Key Words:** β-carotene; *Dunaliella*; election transport rate; Fv/Fm; growth; light intensity; O₂ evolution; zeaxanthin

**INTRODUCTION**

Microalgae have been studied and exploited for the production of biomass or high value compounds such as glycerol, carotenoids and a variety of fine chemicals (Spolaore et al. 2006). Among such microalgae, *Dunaliella* are halotolerant green algae, which can grow in hypersaline aquatic environments having varied salinities from 0.5 to 5.5 M (Karni and Avron 1988, Chen and Jiang 2009, Polle et al. 2009). Some of *Dunaliella* strains are well known for the high production of carotenoids, especially β-carotene (Jin et al. 2003, Jin and Polle 2009). This alga produces large amount of β-carotene under stress conditions such as high light (HL) (Vorst et al. 1994, Coesel et al. 2008, Lamers et al. 2010), high salinity (Vorst et al. 1994, Hadi et al. 2008), nutrient limitation (Marín et al. 1998, Coesel et al. 2008), and nitrogen starvation (Sánchez-Estudillo et al. 2006, Lamers et al. 2012). Ability of *Dunaliella* to thrive under extreme salinities gives a selective advantage that inhibits growth of other algae and its predators.

β-Carotene is a red-orange pigment that belongs to isoprenoid compounds and is present in plants and algae. β-Carotene has various roles in the biological system such as precursor of provitamin A (Tanumihardjo 2002), absorption of light energy as an accessory pigment (Edwards and Walker 1983, Ben-Amotz et al. 1987), quenching of triplet state chlorophyll and singlet oxygen (Ben-Amotz et al. 1996, Ramel et al. 2012). β-Carotene is used in
food (Dufossé et al. 2005), pharmaceutical industries as antioxidants (Chidambara Murthy et al. 2005), anti-tumor agent (Raja et al. 2007a, Theodosiou et al. 2010) and heart disease preventive (Törnwall et al. 2004). Because of the beneficial effects of β-carotene, the demand of β-carotene is increasing. However, about 90% of β-carotene is produced synthetically (Raja et al. 2007b).

*D. bardawil* has been used as commercial strain for β-carotene production. To test if the *D. salina* CCAP 19/18 can be used as carotenogenic strain for commercial application, the effect of light intensity on growth, morphology, photosynthetic efficiency and carotenoid content of both strains were investigated and compared. This study shows that *D. salina* CCAP 19/18 can be used as a carotenogenic strain for the mass production of β-carotene under a certain light regime.

**MATERIALS AND METHODS**

**Dunaliella strains and growth condition**

*D. salina* CCAP 19/18 and *D. bardawil* LB 2538 were grown in 200 mL of artificial seawater (Pick et al. 1986) containing 1.5 M NaCl in a 500-mL Erlenmeyer flask. Cells were grown under various intensity of continuous cool white fluorescent light at 25 ± 2°C. The cultures were manually shaken occasionally. Density of the cell was measured by counting the number of cells under microscope using Neubauer hemacytometer (Superior, Bad Mergentheim, Germany). Morphology of cell was observed under Olympus microscope (BX 53; Olympus, Tokyo, Japan) and pictures were taken using CCD camera attached to the microscope (DMCe 310 plus; INS, Seoul, Korea).

**Chlorophyll fluorescence**

Chlorophyll fluorescence was measured using FMS2 pulse-amplitude-modulation (PAM) fluorometer (Hansatech Instruments Ltd., Norfolk, UK). *Dunaliella* cells were dark-adapted for 10 min prior to measurement, then subjected to a 0.7 s flash of saturating white light (14,000 μmol photons m⁻² s⁻¹) to measure *Fₐ*. To measure *Fₐ′*, actinic light (150 μmol photons m⁻² s⁻¹), was applied and the saturating pulses were applied at 20 s interval.

**Oxygen evolution measurement**

Oxygen evolution of the culture was measured using Clark type O₂ probe (Hansatech Instruments Ltd., Norfolk, UK) described in Jin et al. (2001) with slight modification. The chamber containing 2 mL of cell suspension adjusted to 2 μM chlorophyll was illuminated with a halogen lamp. The oxygen evolution was measured at 50, 85, 145, 450, 700, and 1,200 μmol photons m⁻² s⁻¹ intensity irradiated for 2 min at each light intensity. The rate of oxygen evolution at each light intensity step was recorded for 2 min.

**Pigment analysis**

Pigments were extracted by addition of 80% acetone with vigorous vortexing and centrifuged at 14,000 x g for 4 min. The extract was filtered through 0.2 μm nylon filter and the filtrate was immediately subjected to Shimadzu Prominence HPLC model LC-20A (Shimadzu, Kyoto, Japan) equipped with a Waters Spherisorb SS ODS1 4.6 x 250 mm cartridge column (Waters, Milford, MA, USA). The pigments were separated using a solvent mixture of 0.1 M Tris-HCl pH 8.0, acetonitrile, methanol, and ethylacetate. During the run, the solvent concentrations were 14% 0.1 M Tris-HCl, 84% acetonitrile, and 2% methanol from 0 to 15 min. From 15 to 19 min, the solvent mixture was consisted of 68% methanol and 32% acetonitrile. A post-run was performed for 6 min with the initial solvent mixture. The flow rate was constant at 1.2 mL per min. Pigments were detected at 445 nm. Concentration of the individual pigment was determined from the HPLC profiles calibrated with standard pigments of chlorophyll and carotenoids (14C Centralen; DHI, Hørsholm, Denmark).

**RESULTS**

**Growth under low and high light condition**

To investigate the effect of light intensity on the growth of *D. salina* CCAP 19/18 and *D. bardawil*, low light (LL) (40 and 80 μmol photons m⁻² s⁻¹) and high light (HL) (200 and 400 μmol photons m⁻² s⁻¹) were tested. The intensity of light significantly affected both cell density and doubling time of the culture (Fig. 1). Under LL growth conditions, growth of *D. bardawil* under 80 μmol photons m⁻² s⁻¹ of light was lower than that under 40 μmol photons m⁻² s⁻¹ of light (Fig. 1A). There was no difference in growth of *D. salina* CCAP 19/18 under both LL light conditions, except on day 5 (120 h) when the growth under 80 μmol photons m⁻² s⁻¹ of light was higher than that under 40 μmol photons m⁻² s⁻¹ of light (Fig. 1A). Both strains showed satura-
Under 18 and 40 μmol photons m\(^{-2}\) s\(^{-1}\) of light, both *D. bardawil* and *D. salina* CCAP 19/18 had ovoid shape (Fig. 2B). Under 80 μmol photons m\(^{-2}\) s\(^{-1}\) of light, *D. salina* CCAP 19/18 cells were elongated and *D. bardawil* cells became larger. From 80 to 400 μmol photons m\(^{-2}\) s\(^{-1}\), there were changes in morphology under various light intensities. Under 18 and 40 μmol photons m\(^{-2}\) s\(^{-1}\) of light, both *D. bardawil* and *D. salina* CCAP 19/18 had ovoid shape (Fig. 2B). Under 80 μmol photons m\(^{-2}\) s\(^{-1}\) of light, *D. salina* CCAP 19/18 cells were elongated and *D. bardawil* cells became larger. From 80 to 400 μmol photons m\(^{-2}\) s\(^{-1}\),
both strains started to show yellowish orange pigments in the cell and the color was intensified (Fig. 2B). Under 400 μmol photons m\(^{-2}\) s\(^{-1}\) of light, *D. bardawil* cell was almost round and *D. salina* CCAP 19/18 had an elongated shape than that of *D. bardawil* (Fig. 2B).

**Maximum quantum yield of photosystem II and electron transport rate under various light intensities**

To investigate the difference in photochemical conversion efficiency of photosystem II (PSII) between *D. bardawil* and *D. salina* CCAP 19/18 under various light conditions, the maximum quantum yield of PSII ($F_{v}/F_{m}$) and the electron transport rate (ETR) were measured. Decrease of the $F_{v}/F_{m}$ value indicates the extent of photo-inhibition in *Dunaliella* (Hofstraat et al. 1994, Gordillo et al. 2001). In both strains, there was little change in $F_{v}/F_{m}$ from 40 to 200 μmol photons m\(^{-2}\) s\(^{-1}\) light treatment (Fig. 3A). However, under 400 μmol photons m\(^{-2}\) s\(^{-1}\) of light, there was a small but significant decrease in $F_{v}/F_{m}$ in both strains (Fig. 3A). $F_{v}/F_{m}$ of *D. salina* CCAP 19/18 was slightly higher than that of *D. bardawil* under 400 μmol photons m\(^{-2}\) s\(^{-1}\) of light (Fig. 3A). ETR is one of an indicator of stress effects and correlates with gross photosynthetic rate. ETR was decreased in both strains when the light intensity increased (Fig. 3B). Interestingly, the ETR of *D. salina* CCAP 19/18 was higher than that of *D. bardawil* above 80 μmol photons m\(^{-2}\) s\(^{-1}\) of light (Fig. 3B). Under 400 μmol photons m\(^{-2}\) s\(^{-1}\) of light, ETR of *D. bardawil* was 50% of that of *D. salina* CCAP 19/18 (Fig. 3B). Therefore, decline in ETR of *D. bardawil* reflects the reduction in photosynthetic ability under the HL condition compared to *D. salina* CCAP 19/18.

**O\(_2\) evolution**

The efficiency of photosynthesis (photosynthetic capacity) was assessed to analyze the light saturation curve of photosynthesis in the two strains. For this purpose, the rate of O\(_2\) evolution was measured and plotted as a function of incident light intensity. The photosynthetic capacity on the cell basis was also saturated at 900 μmol photons m\(^{-2}\) s\(^{-1}\) of light (Fig. 4A). Under the same LL growth condition, *D. bardawil* showed about 1.5 times higher rate of O\(_2\) evolution on the cell basis than the *D. salina* CCAP 19/18 at 1,200 μmol photons m\(^{-2}\) s\(^{-1}\) of light (Fig. 4A). Under the HL growth conditions, the rate of O\(_2\) evolution on the cell basis was lower than that of under the LL growth condition (Fig. 4A & B). In case of *D. bardawil*, the rate of O\(_2\) evolution on the cell basis under the HL growth conditions (200 and 400 μmol photons m\(^{-2}\) s\(^{-1}\) was 2 times lower than that of under the LL condition at 1,200 μmol photons m\(^{-2}\) s\(^{-1}\) of light irradiance (Fig. 4A & B). However, in case of *D. salina* CCAP 19/18, the rate of O\(_2\) evolution on the cell basis under the HL growth conditions at 1,200 μmol photons m\(^{-2}\) s\(^{-1}\) of light irradiance was about 4.4-5.2 times lower than that under the LL growth condition at 1,200 μmol photons m\(^{-2}\) s\(^{-1}\) of light irradiance (Fig. 4A & B).

**β-Carotene and zeaxanthin contents**

One of the parameters of irradiance stress response in green algae is the de-epoxidase state of xanthophylls (Niyogi 1999, Jin et al. 2001). The xanthophyll cycle plays a significant role in the non-photochemical quenching (NPQ) of excitation and the photoprotection of the photosynthetic apparatus (Yamamoto 1979, Müller et al. 2001). In general, under the LL condition, cells accumulate violaxanthin and have low level of zeaxanthin. Under the HL stress condition, cells de-epoxidize violaxanthin to zeaxanthin by violaxanthin de-epoxidase (Müller et al. 2001). Zeaxanthin is known to quench the excited Chl*
The β-carotene content of both strains increased when the light intensity increased (Fig. 5A). The β-carotene content of *D. bardawil* was higher than that of *D. salina CCAP 19/18* under all light intensities and the largest difference between the two strains occurred at 80 μmol photons m⁻² s⁻¹ (2.37 times difference) (Fig. 5A). Under 400 μmol photons m⁻² s⁻¹ of light, the β-carotene content of *D. salina CCAP 19/18* was about 14% less than that of *D. salina CCAP 19/18*. Therefore, analyzing the components in the xanthophyll cycle is to understand the photoacclimation and photoinhibition process in algae (Baroli and Melis 1996). We addressed a question whether the difference in the de-epoxidation state exists between the two strains under various light conditions. The content of β-carotene and zeaxanthin was estimated on a per chlorophyll a basis. The β-carotene content of both strains increased when the light intensity increased (Fig. 5A). The β-carotene content of *D. bardawil* was higher than that of *D. salina CCAP 19/18* under all light intensities and the largest difference between the two strains occurred at 80 μmol photons m⁻² s⁻¹ (2.37 times difference) (Fig. 5A). Under 400 μmol photons m⁻² s⁻¹ of light, the β-carotene content of *D. salina CCAP 19/18* was about 14% less than
that of *D. bardawil*. Because the actual yield of β-carotene in *D. salina* CCAP 19/18 was much higher than that of *D. bardawil* due to much higher growth rate of *D. salina* CCAP 19/18, the β-carotene yield on the dried mass basis of the cells and the culture volume basis grown under HL was measured (Fig. 5B & C). There was little difference in the β-carotene content on the dry weight basis between the two species under 200 μmol photons m⁻² s⁻¹ of light. However, the β-carotene content on the dry weight basis of *D. salina* CCAP 19/18 was 2.4 times higher than that of *D. bardawil* under 400 μmol photons m⁻² s⁻¹ of light (Fig. 5B). On the culture volume basis, the β-carotene content of *D. salina* CCAP 19/18 was 3.8 and 2.5 times higher than that of *D. bardawil* under 200 and 400 μmol photons m⁻² s⁻¹ of light, respectively (Fig. 5C). Zeaxanthin content also showed increase in amount in response to the increasing light intensity in both species, and the amount of zeaxanthin content of *D. bardawil* was about 2 times higher than that of *D. salina* CCAP 19/18 except at 400 μmol photons m⁻² s⁻¹ of light (Fig. 5D). The largest difference in amount of zeaxanthin content occurred at 200 μmol photons m⁻² s⁻¹ of light (1.97 times difference between two strains).

**DISCUSSION**

In this report, we compared the growth kinetics, morphological change, parameters of photosynthetic rate (rate of *O₂* evolution, *F₅/F₇₅* and ETR) and the production of carotenoids in response to various intensity of light between two *Dunaliella* strains, *D. salina* 19/18 and *D. bardawil*.

In both strains, the growth was decreased as the light intensity increased (Fig. 1). The two strains showed different growth kinetics under HL growth conditions, but similar growth kinetics under LL growth condition (Fig. 1A & B). There were changes in morphology in response to light intensity and both strains showed difference. On one hand, *D. salina* CCAP 19/18 became more elongated as the light intensity increased and it was accompanied with the increase in cell number under HL growth condition (Figs 1B & 2B). On the other hand, *D. bardawil* cell seemed to be increased in volume as the light intensity increased, and the cell division was less than that of *D. salina* CCAP 19/18 (Figs 1B & 2B). This difference in the cell division rate and volume change might result in the difference in the *O₂* evolution rate on per chlorophyll basis between both strains. Little difference in the *O₂* evolution rate on per chlorophyll basis between the two strains implies that the degradation rate of chlorophyll of both strains might be similar in the range of light intensity investigated. The equiproportional increase in the production rate of cell volume and β-carotene was observed (Lamers et al. 2010). Also, β-carotene level can be regulated by the total amount of irradiation perceived during the cell division cycle (Ben-Amotz and Avron 1983, Lers et al. 1990).

According to the result of *F₅/F₇₅* (Fig. 3A) and ETR (Fig. 3B), *D. salina* CCAP 19/18 might be less liable to photoinhibition than *D. bardawil* under HL growth conditions. *D. bardawil* had about 1.5 and 5 times higher oxygen evolution rate than *D. salina* CCAP 19/18 under LL condition (Fig. 4A) and HL conditions (Fig. 4B), respectively. This implies that *D. bardawil* is more effective in photosynthesis than *D. salina* CCAP 19/18. Both strains accumulate large amount of β-carotene under HL growth condition and there is little difference between the two strains on per chlorophyll basis (data not shown). The increase of β-carotene content of *D. salina* CCAP 19/18 by HL matches the result in Lamers et al. (2010). The zeaxanthin production of *D. bardawil* was saturated at 200 μmol photons m⁻² s⁻¹ of light and that of *D. salina* CCAP 19/18 was not (Fig. 5B). The amount of zeaxanthin in the cell is known to have close relationship with the NPQ, and NPQ is one of parameters of photoprotection (Demmg-Adams and Adams 1992, Müller et al. 2001).

Previously, similar works were performed and reported (Ben-Amotz and Avron 1983, Vorst et al. 1994, Gómez and González 2005). However, in Ben-Amotz and Avron (1983) and Vorst et al. (1994), the *D. salina* strain they used was not able to accumulate β-carotene. In Gómez and González (2005), they used the same strain as we did. However, the HL they used was 110 μmol photons m⁻² s⁻¹, which was an intermediate intensity compared to that we used. The β-carotene production of *D. salina* CCAP 19/18 and *D. bardawil* was not different under 40 and 110 μmol photons m⁻² s⁻¹ of light, which was different from our result (Gómez and González 2005). This might be due to the difference in culture condition. Recently, the effect of light intensity on β-carotene production was performed with *D. salina* CCAP 19/18 and they also showed the increase in β-carotene content by HL (Lamers et al. 2010).

Development in biotechnology and bioengineering encouraged invention of many systems required to facilitate cultivation of *Dunaliella* and harvesting β-carotene in an industrial scale. Efforts for effective production of β-carotene include isolation of carotenogenic strain (Markovits et al. 1993), cloning of genes involved in carotenogenesis (Zhu et al. 2008), transformation of these genes into microalgae (León-Bañares et al. 2004, Walker et al. 2005), searching for optimum environmental stimuli
in β-carotene accumulation (Ben-Amotz and Avron 1983, Sánchez-Estudillo et al. 2006). These efforts, however, did not surpass the systematic technical development such as two-phase bioreactors (Hejazi et al. 2004). Because environmental stress such as high irradiance inhibits Dunaliella growth, even causes photo-bleaching, two separate steps of culture is required i.e., one for growth and one for β-carotene accumulation (Hejazi et al. 2004). Also, there is an inverse relationship between β-carotene content and the growth rate (Ben-Amotz et al. 1982). In this study, D. salina CCAP 19/18 has higher growth rate in D. bardawil at 200 μmol photons m⁻² s⁻¹ of light (Fig. 1B) and the same strain showed higher β-carotene content even under 2,000 μmol photons m⁻² s⁻¹ of light than LL growth condition (Lamers et al. 2010). Setting the light condition for the maximal growth rate and for the overproduction of β-carotene is indispensable for scaling up to the industrial scale both in the batch culture and turbidostat culture. In this sense, D. salina CCAP 19/18 is more beneficial to the carotenogenic D. bardawil because of its higher growth rate under HL condition and less liability to the light stress than D. bardawil. The growth of D. salina CCAP 19/18 was 3 times higher than that of D. bardawil and also 16% higher in Fv/Fm (Fig. 1B & 3A). Furthermore, D. salina CCAP 19/18 is more ‘milkable’ than any other Dunaliella strain including D. bardawil (Kleinegris et al. 2010). Lamers et al. (2010) also showed that β-carotene productivity of D. salina CCAP 19/18 was much higher than the average productivity by commercial production facility and that the β-carotene production was closely linked to the accumulation of C16:0 and C18:1 fatty acids. We propose that there is a possibility of improvement of the β-carotene production if the beneficial characteristics of D. salina CCAP 19/18 over D. bardawil are taken advantage of under certain light condition. D. bardawil has advantage in the production of β-carotene in an open pond, whereas D. salina CCAP 19/18 might be better than D. bardawil in the bioreactor at optimized condition.

It is well known that HL induces the overproduction of β-carotene on the transcriptional and translational level (Lers et al. 1990). However, the genes and enzymes for carotenogenesis and its regulatory mechanisms are not well understood (Jin and Polle 2009, Ramos et al. 2011). Genetic modification followed by screening of the Dunaliella strains have been explored for finding novel genes for the mass production of β-carotene (Jin and Melis 2003, Ramos et al. 2011). Applying high through-put molecular genetics technique to this precious microalga D. salina CCAP 19/18 will improve the output of carotenogenesis.

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