Light intensity effects on some molecular and biochemical characteristics of *Dunaliella salina*

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Abstract

To gain a better understanding of molecular and biochemical events involved in light intensity adaptations of *Dunaliella salina,* we studied the expression of phytoen synthase (*psy*) gene; pigments, carbohydrates, proteins and lipids accumulation under two light intensities. The cells were pre-cultured under 50 µmol photon $m^{-2}s^{-1}$ light intensity and then transferred to two different light intensities of 200 and 1000 µmol photon $m^{-2}s^{-1}$. The *psy* gene expression in high light (1000 µmol photon $m^{-2}s^{-1}$) decreased and remained fairly constant until 48 h however, a more decrease was appeared under the low light (200 µmol photon $m^{-2}s^{-1}$). After 2 weeks cultivation, the high light led to a considerable decrease of Chl *a* and Chl *b* contents, and an increase in carotenoids/total chlorophylls and Chl *a*/Chl *b* ratios. Total, soluble and non-soluble (starch) carbohydrates; proteins and lipids contents of cells were increased under the high light conditions. The results suggest that *D. salina* cells can have light intensity dependent adjustments in gene expressions, photosynthesis apparatus function, and structure as well as biochemical cell composition.

Keywords: Dunaliella salina; light, pigments; protein; *psy* gene expression

Abbreviations:
Car: carotenoid; Chl: chlorophyll; *D. salina*: *Dunaliella salina*; *psy*: phytoen synthase; ROS: reactive oxygen species

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Introduction

Dunaliella species are flagellated unicellular algae that lack a rigid cell wall. *D. salina* is a hyper-halotolerant green microalga especially found in environments with high salt concentration. Cells contain one single cup-shaped chloroplast with one central pyrenoid (Borowitzka and Borowitzka, 1988). To survive in harsh saline conditions, these organisms evolved some molecular and biochemical mechanisms. Accumulation of high amount of β-carotene and glycerol protect cells against light and osmotic stresses, respectively. Therefore, this combination

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proffers an opportunity in biotechnological applications for the scope of commercial bio-products of these metabolites (Schlipalius, 1991). *β*-carotene contributes to the antioxidant activity of *D. salina* and it is used in cosmetics and dietary supplements for human and animal as a source of vitamin A (Shariati and Hadi, 2011). However, due to the low productivity of β-carotene, commercial uses of *D. salina* are limited. In order to maximize β-carotene production, *D. salina* should be grown under given light intensity, salt concentration, etc.

Light is the ultimate energy source for photosynthesis and light intensity is one of the most important environmental factors for all photosynthetic organisms (Lamers et al., 2008). Non-optimal light intensity can adversely affect metabolism and excess light absorption creates an over excitation of the photosynthetic apparatus, which can induce photoinhibiton, photodamage and degradation of photosynthetic proteins in the cells (Aro et al., 1993). When photosynthetic cells absorb more energy than is used in photosynthesis, the excess electrons react with the abundantly present oxygen. Under these conditions, the chlorophyll molecules coupled with the electron transport system are the primary source for creation of singlet oxygen (Ramel et al., 2013). High irradiance, also leading to over reduction of photosynthetic electron transport chain, and hence, increases production of several free radicals, referred as reactive oxygen species (ROS). If the ROS are not controlled, they can widely attack different cell biomolecules and organelles and lead to cell death (Imlay and Linn, 1988). Biological systems respond to elevated ROS levels by activating a number of anti-oxidative defense mechanisms. Because of the central role of photosynthesis, photosynthetic organisms have developed many strategies to acclimate to a broad range of environmental conditions such as high light by modulating their metabolism. In this way, they have to maintain photosynthetic efficiency at the highest level possible (Pfannschmidt, 2003). To avoid stress, several short-term and long-term quenching processes enhance the efficiency of photosynthesis (Chow et al., 1990). The escape of photoinhibition depends on the ability to safely dissipate excess light energy by photoprotective pigments. Carotenoids play an important role in the photoprotection of photosynthetic cells against over excitation in excess light and quenching singlet oxygen by physical and chemical mechanisms (Ramel et al., 2013). In addition, ROS activate or inactivate the expression of chloroplast and nuclear genes (Carvalho et al., 2004; Pfannschmidt, 2003). Changes in the biosynthetic pathways of carbohydrates, proteins, lipid, and pigments could regulate cell adaptation power and viability. Further, crosstalk is necessary between metabolic pathways such as β-carotene and lipid (Rabbani et al., 1998) or chlorophyll and carotenoid biosynthesis (Al-Hasan et al., 1987; Giordano, 2001). On the other hand, it is well documented that de novo protein biosynthesis is required for physiological and biochemical changes. Light stress seems to be an important factor in the transcriptional regulation of pigments biosynthesis pathway (Pfannschmidt, 2003). Phytoen synthase appears to be a crucial enzyme for carotenoids and chlorophyll biosynthesis in *D. salina* (Qin et al., 2007).

D. salina has remarkable tolerance upon exposure to various abiotic stresses and the physiology of this alga has been investigated in many studies. Hence, *D. salina* is a good model organism to study the effects of stress factors in alga and understanding molecular and biochemical responses of higher plants (Hicks et al., 2001; Ramos et al., 2011). It will be interesting to compare *D. salina* physiological responses under different light intensities. Consequently, the purpose of the present study was investigation of the growth and biochemical responses with the pattern of *psy* gene, a β-carotene key gene, expression in *D. salina*, when exposed to different light intensities.

Materials and Methods

Algal strain and growth conditions

D. salina CCAP 19/18 was obtained from the Agricultural Biotechnology Research Institute of Iran, Northwest and West region [Tabriz]. Cells were grown in modified Johnson medium (Hejazi and Wijffels, 2003). Medium pH was adjusted to 7.5 using Tris-buffer. All stock solutions were sterilized (in 121 °C) separately and pooled aseptically to avoid precipitation of certain compounds. Culture salinity was 3 M NaCl and the

Table 1
Properties of selected primers used in experiment

Gene	Coding product	Ac.no		Primer sequence	MW	GC content	Tm	Amplicon length
18S	18S rRNA	EF682843.1	F	TGCATGGCCGTTCTTAGTTG	6130	50%	64	76
18S	18S rRNA		R	ATTTAGCAGGCTGAGGTCTCG	6477.3	52%	64	76
psy	phytoene synthase	AY601075.1	F	ACTTCCAGGAGGCTGAGGATG	6511.3	57%	64	75
psy	phytoene synthase		R	AGATGAGCGCAGACCACAC	5815.8	58%	64	75

temperature was kept at 34±2 °C which seems to be the appropriate temperature for *D. salina* growth (Garcia et al., 2007). Light conditions were applied by putting cultures under 200 and 1000 µmol photon $m^{-2}s^{-1}$ light intensity. Continuous illumination was used for cell growth to prevent superimposed diurnal variations in the level of *psy* mRNA. All experiments were done in 3 replicate in 250 ml Erlenmeyer flasks, containing 150 ml of fresh medium and cultures placed in an incubator for 2 weeks with shaking at 120 rpm.

Cell number counts

At first, cells were immobilized and stained by addition of 10 µl of Lugol´s solution (1 g iodine and 0.5 g potassium iodide in 100 ml H_2O) to 190 µl aliquots of the algal cultures. Then cell counts were determined using a 1% mm deep counting chamber and light microscope (magnification × 10) (Hejazi and Wijffels, 2003).

RT-PCR analysis of psy gene expression

RNA was extracted using RNX- plus reagents (cat no: RN7713C) according to the manufacturer's instructions. Quality of total RNA was checked spectrophotometrically and gel electrophoresis. Quantity of total RNA was calculated using A_{260}. Samples containing (5-10 µg) of RNA were used for complementary DNA (cDNA) synthesis, using 2-steps RT-PCR kit (product code: RTPL12) according to the manufacturer's instructions. Primer sequences of 19-21 nucleotides in length were designed using primer 3 web-based software. Quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) analysis was performed with a CFX96 1000 instrument (Bio Rad) using the Quanti-Tect SYBR Green PCR kit(ABI). All quantifications were normalized to the amount of 18S rRNA as internal standard by the $\Delta\Delta C_T$ method. All reactions were set up in duplicate for each sub replicate. The sequences and other properties of the primers are listed in Table 1. The following standard thermal profiles were used for PCR reactions: Initial denaturation: 94 °C for 10 min, (Denaturation: 94 °C for 1min, Annealing: 64 °C for 30 sec. and Elongation: 72 °C for 15 sec) ×50, Final extension: 72 °C for 10 min.

Pigment Analysis

Chlorophylls and carotenoids were measured according to (Lichtenthaler and Buschmann, 2001). Pigments were extracted from algal pellets in 80% acetone after removal of cell debris by centrifugation at 8000 rpm for 5 min. Supernatant absorbency was measured at 663.2, 646.8 & 470 nm with a spectrophotometer (Perkin Elmer precisely-Lambda 35-UV/Vis). Pigment concentration was measured per 1 ml of medium and converted for each *D. salina* cell (pg/cell).

Measurement of carbohydrates, total proteins and lipid contents

Carbohydrates were analyzed according to the phenol sulphuric acid method (Dubois et al., 1956). According to this method two mL of 1 N HCl was added to 2×10^7 cells. The mixture was boiled for 20 min, cooled, and centrifuged (5 min, 2000 rpm). Then, 0.5 ml of samples was mixed with 0.5 ml of 5% phenol and 2.5 ml of H_2SO_4. Then, optical density was determined at 488 nm and compared with calibration standards. Total, soluble, and

Table 2
Analysis of variance (ANOVA) for evaluated parameters of *D. salina*

Dependent Variable	Sum of squares	df	Mean square	F	Sig.
Cell number	1.27E+12	1	1.27E+12	16.9	0.015
Chl *a*	9.494	1	9.494	3.92E+03	0.000
Chl *b*	30.381	1	30.381	1.81E+03	0.000
Total carotenoids	0.283	1	0.283	205.959	0.000
Total carbohydrates	1228.821	1	1228.821	13.655	0.021
Soluble carbohydrates	18.627	1	18.627	33.987	0.004
Starch	944.863	1	944.863	11.691	0.027
Proteins	29.004	1	29.004	8.364	0.044
Lipids	37.002	1	37.00	46.44	0.002

non-soluble carbohydrate contents were represented in µg per 10^6 cells.

Total protein contents were measured according to the Bradford method (Bradford, 1976). One ml algal culture cells was separated from the supernatant after centrifugation in 5000 rpm. Using deionized water on ice, cells were destroyed. Then, Bradford reagent (l00 mg Coomassie Brilliant Blue G-250 dissolved in 50 ml 95% v/v ethanol) added and absorbance of mixture at 595 nm was used for final calculation (µg per 10^6 cells).

Lipid measurement was accomplished using an optimized gravimetric method using petroleum benzene. For this purpose, algal plates were dried in oven (70 ˚C) and then shaken overnight in petroleum benzene. After removing the organic solvent, plates were weighed and lipid content was calculated in percent of dry weight.

Statistical Analysis

All experiments were done with three replicates and data were shown as mean ± standard error. Mean values ($p \leq 0.05$) were determined using SPSS software. One-way analysis of variance was applied to the data to evaluate the light effect on each individual parameter.

Results

Cell number

Light intensity had a marked effect on *D. salina* cell number per ml after 14 day exposure to

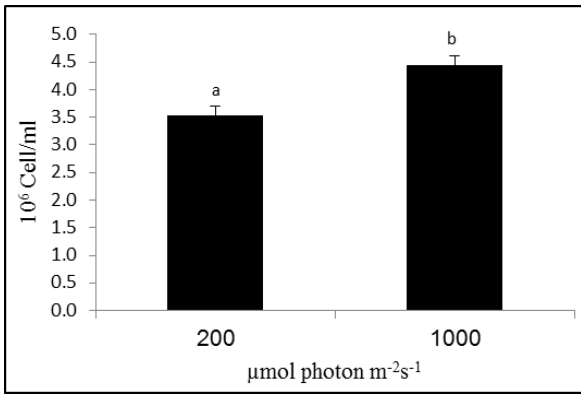

Fig. I. Effect of light intensity on *D. salina* cell numbers

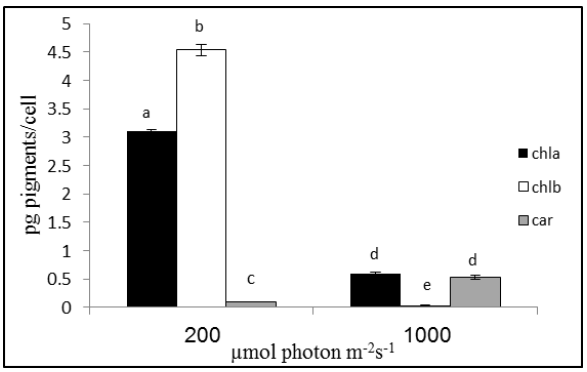

Fig. II. Effect of light intensity on *D. salina* pigments content

200 and 1000 µmol photon $m^{-2}s^{-1}$ (Fig. I). The high light was proper for growth and cell division because cell population under 1000 µmol photon $m^{-2}s^{-1}$ increased significantly (Table 2).

Expression of psy Gene

The steady-state mRNA levels of *psy* gene, encoding phytoene synthase, in *D. salina* were compared, as a time course study for 48 h, when cells were transferred from 50 to 200 or 1000 µmol photon $m^{-2}s^{-1}$. The *psy* gene expression (number of transcripts) at high light (1000 µmol photon $m^{-2}s^{-1}$) decreased and remained fairly constant until 48 h. However, a more decrease in mRNA level was appeared after 48 h under low light (200 µmol photon $m^{-2}s^{-1}$) (Fig. II).

Pigments

Photosynthetic pigments showed relevant changes between low (200 µmol photon $m^{-2}s^{-1}$) and high light (1000 µmol photon $m^{-2}s^{-1}$) light treatments (Fig. III). The results also showed that at high light intensity Chl *a* and Chl *b* contents decreased and carotenoid contents increased, significantly (Table 2). As a result of the change in pigment contents, both carotenoids/total chlorophylls (Car/total Chl) and Chl *a*/Chl *b* ratios increased considerably (Table 3).

Carbohydrates

Total, soluble, and non-soluble carbohydrate productions of *D. salina* were significantly affected by light intensity (Table 2). Maximum contents were observed for all kinds of carbohydrates under high light intensity (Fig. IV).

Proteins

Protein content per cell was significantly affected by light intensity (Table 2). An increase in light intensity was associated with a significant increase of growth rate and protein content (Fig. V).

Lipids

Results yielded from this study showed that high light intensity induce significantly lipid formation within algal cells (Fig. VI), without the drop in biomass productivity (Fig. I).

Discussion

Table 3
Effect of light intensity on pigments special ratios of *D. salin*

Pigment ratio	Light intensity (µmol photon $m^{-2}s^{-1}$)	
	200	1000
Car/total Chl	0.013	0.851
Chl *a*/Chl *b*	0.685	16.588
Car + Chl *b*/Chl *a*	1.492	0.963

Cell number

Cell number indicator is helpful to clarify the role of biochemical mechanisms underlying the responses observed. Our results indicate that growth and division of *D. salina* increased significantly under 1000 µmol photon $m^{-2}s^{-1}$ (Fig. I and Table 2). This may relate to adequate and effective cell adaptation (Coesel et al., 2008). In *Chlorella vulgaris*, increase in light intensity led to an increase in cell number (Seyfabadi et al., 2011).

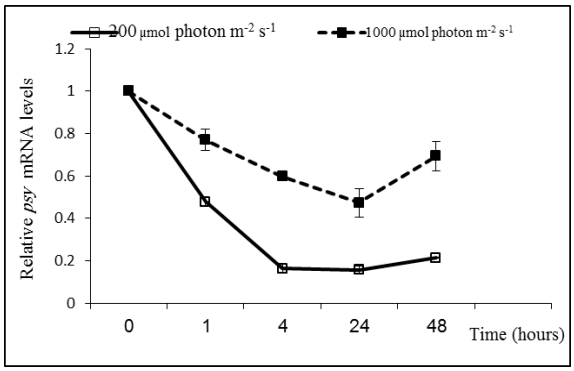

Fig. III. Effect of light intensity on relative *psy* mRNA levels of *D. salina*

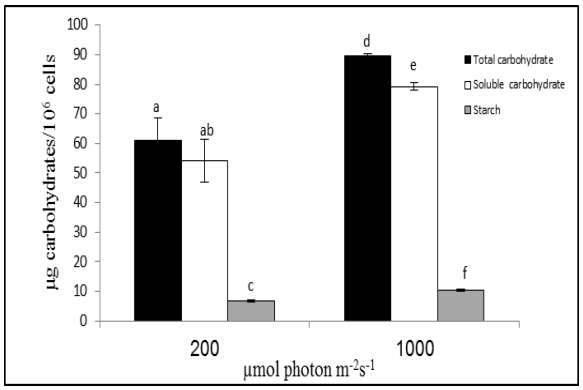

Fig. IV. Effect of light intensity on carbohydrates content of *D. salina*

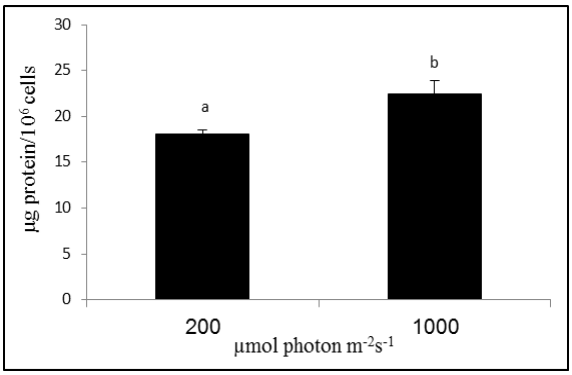

Fig. V. Effect of light intensity on total proteins content of *D. salina*

Cell doubling time of *D. salina* under 2000 µmol photon $m^{-2}s^{-1}$ did not differ from cell grown in 100 µmol photon $m^{-2}s^{-1}$ (Melis et al., 1998). Growth rate of *D. viridis* was extremely affected by light intensity, increasing from darkness to 700 µmol photon m-2s-1 but decreasing at the 1500 µmol photon $m^{-2}s^{-1}$ (Gordillo et al., 1998).

Expression of psy Gene

To understand the mechanism of light intensity dependent adjustments in photosynthetic pigmentation of *D. Salina*, the *psy* gene expression were quantified by qRT-PCR at high (1000 µmol photon $m^{-2}s^{-1}$) and low (200 µmol photon $m^{-2}s^{-1}$) light conditions. After transferring cells form 50 µmol photon $m^{-2}s^{-1}$ to both light intensities, the *psy* gene expression was reduced. However, low light had an obvious effect on *psy* gene expression compared with high light (Fig. II). A constant *psy* mRNA expression was reported in response to high light irradiance in *D. bardawil* (Rabbani et al., 1998). As shown in Fig. (III), carotenoid accumulation was stimulated by high light irradiance. Because carotenoid accumulating cells maintained elevated steady-state *psy* mRNA levels, it is suggested that gene regulation may be an important control step of carotenogenesis in *D. salina* (Ramos et al., 2011). Although we found that high light is an effective factor for carotenoid accumulation in long run, according to our results, the increase in the steady-state level of *psy* mRNA may occur relatively slowly. On the other hand, the accumulation of carotenoid seems to be dependent not only on the expression of carotenogenesis genes but also on other factors such as development of photosynthetic apparatus, destruction of pigments by photooxidation and the biosynthesis of chlorophylls (Bohne and Linden, 2002). Environmental factors influence the patterns of gene expressions (Coesel et al., 2008). In *D. salina* when nitrogen was sufficient in growth medium, *psy* had no significant changes (Sanchez-Estudillo et al., 2006). In *Haematococcus pluvialis*, a unicellular green alga, the regulation of carotenogenesis genes was found to be under photosynthetic redox control during light acclimation (Steinbrenner and Linden, 2003). The redox state of the plastoquinone pool in the chloroplast thylakoids may serve in the signal transduction pathway (Masuda et al., 2003).

Pigments

Changing in pigment contents led to increasing in carotenoids/total chlorophylls (Car/total Chl) and Chl *a*/Chl *b* ratios (Table 3). The green to the orange transformation of *D. salina* cell during cultivation under high light was accompanied by 65-fold in Car/total Chl ratio. Under high light, increase in Car/total Chl ratio was as a result of a decrease in total Chl and the increase in carotenoids contents. As appeared in

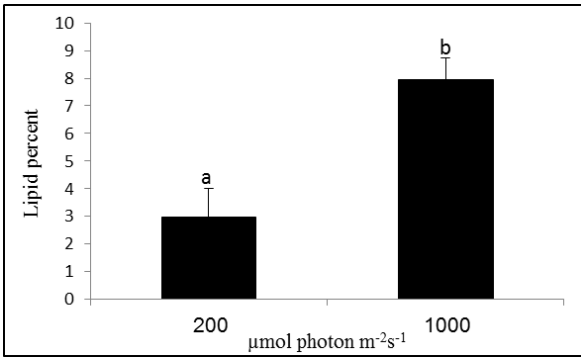

Fig. VI. Effect of light intensity on lipids percent form total dry weight of *D. salina*

Fig. (III) the high light affected chlorophylls more than carotenoids. Fazeli and his coworkers (2006) showed increasing in carotenoids production and Car/total Chl ratio by *D. salina* when light intensity changes from 50 to 150 µmol photon $m^{-2}s^{-1}$. Under high light intensity, secondary carotenoids accumulate in special structures such as plastoglobulin of plastids or cytoplasmic lipid bodies, and serve as photoprotective agents in

preventing excess light energy from reaching the photosynthetic machinery (Solovchenko et al., 2011). Generally, carotenoid accumulation may result from the change in carbon and nitrogen balance under unfavorable conditions (Hu, 2004). We find that the ratio of Chl *a*/Chl *b* enhanced 24 fold under high light, considerably at the expense of Chl *b* (Fig. III). The same result was reported in another work (Webb and Melis, 1995). Evidence in the literature (Anderson, 1986; Melis, 1991; Alavi et al. 2014, Ghorbanli et al. 2013) suggests that variations in the chlorophyll content and Chl a/Chl b ratio occur naturally in higher plants and green algae in response to changes in irradiance. It has been demonstrated that the chlorophyll antenna size in *D. salina* becomes smaller in response to high irradiance (Smith et al., 1990). The dropping in the ratio of carotenoids plus Chl *b*/Chl *a* (Table 3) is another index for reduction in light harvesting antennae size during high light irradiance (Gordillo et al., 2001).

Changes of light intensity during plant and algal growth results in reversible structural and functional adjustments in the photosynthetic apparatus (Anderson, 1986). Photosynthetic organisms acclimate to the level of irradiance by adjusting the size of the chlorophyll antenna associated with photosystems (Melis, 1991). When plants are grown under high light intensity, photosystems contains small antennas and relatively low amounts of Chl *b* in comparison with low light intensity (Larsson et al., 1987; Leong and Anderson, 1984). Thus, the mechanism of chloroplast photoacclimation might involve regulation of Chl *b* biosynthesis and changing in chlorophyll antenna size is a dynamic irradiance dependent recovery response (Masuda et al., 2003).

Carbohydrates

In the present study, an increase was observed in the production of carbohydrates under high light (Fig. II). The results of soluble and non-soluble (starch) carbohydrates productions of *D. salina* are in agreement with other studies. According to Friedman et al. (1991), growth condition was more suitable for polysaccharide production in *Porphyridium aerugineum* when light intensity increased from 75 to 300 µmol photon $m^{-2}s^{-1}$. It has been observed that an increase in the net carbohydrate accumulation can result from the promotion of the photosynthetic rate. *D. salina* shows the typical biochemical composition: 30-50% proteins, 20-40% carbohydrate and 8-15% of lipids under favorable environmental conditions; however, under unfavorable environmental conditions it can accumulate up to 80% of hydrocarbons, on the basis of the dry weight (Hu, 2004). So, environmental factors influence the pattern, pathway and activity of cellular metabolism and thus dynamic cell composition (Mishra et al., 2008; Said, 2009). In the case of each environmental factor, for maximum production of carbohydrates, we can find an optimum value (Khalil et al., 2010). There is a competition for the reducing power between carbon and nitrogen assimilation in photosynthetic organisms for the production of carbohydrates and proteins. As concluding remarks, the 1000 µmol photon $m^{-2}s^{-1}$ was suitable for *D. salina* cell growth without any serious competition between accumulation of carbohydrates and proteins (Figs. IV and V).

Proteins

Our results indicate that protein content of *D. salina* cells is light level-dependent (Fig. I). The requirement of *de novo* protein biosynthesis for an irradiance-dependent rapid change in different light conditions led to deep change in total protein content. Changing in the biochemical composition of cells may be regarded as a general response to light conditions. This is in accordance with a report that the increase in irradiance caused an increase in protein concentration in *Chlorella vulgaris* (Seyfabadi et al., 2011). Although the response to stresses varies between species, different stressors may be affecting the protein biosynthesis by different patterns (Khalil et al., 2010; Ordog et al., 2012; Said, 2009). In a strain of *D. salina*, total protein was decreased in 3.0 M NaCl in comparison to 0.5 M NaCl; however, a significant increase was observed at 5.5 M. This suggests that some proteins were hyper salt responsive and expressed in high salt concentration (Mishra et al., 2008). Nowadays, beside carotenoids the protein contents in algal cultures are regarded as an important economic

criterion. The protein content per cell is considered as one of the major factors determining nutritional values of microalgae. The dried algal meal, after extracting glycerol and β-carotene, because of the high amount of proteins is introduced as an excellent poultry and aquaculture feed (Hosseini Tafreshi and Shariati, 2009).

Lipids

In our study under high light intensity, *D. salina* cells had higher amount of lipids (Fig. VI). Stress conditions have been shown to bring differences in biochemical composition of microalgae. *D. salina* can produce high amounts of lipids in terms of dry weight (El-Baky et al., 2004; Weldy and Huesemann, 2007). For many microalgae, excess solar energy captured by photosynthesis is primarily stored as lipids, often with high levels of triacylglycerols (Chen et al., 2011). Some algae possess photoprotective mechanisms based on coordinated syntheses of extraplastidic secondary carotenoids and storage lipids (Solovchenko et al., 2011). Our results are in accordance with (Rabbani et al., 1998) who noted that increase in irradiance caused an increase in lipid contents in *D. bardawil*. Also, lipid accumulation can be induced by high salinity in *D. salina* (Hadi et al., 2008). Gordillo et al., (1998) reported a decrease of total lipids and an increase of triacylglycerols (main lipid reservoir) in the *D. viridis* when passing from darkness to 1500 µmol photon $m^{-2}s^{-1}$. Light intensity has a major role in determining the quantity and quality of lipids produced by microalgae (Gordillo et al., 1998). Seyfabadi et al., (2011) mentioned that the increase in irradiance was associated with increased saturated fatty acids and decreased unsaturated fatty acids in *Chlorella vulgaris*. The same results were reported for *D. salina* during high light intensity (Yokthongwattana et al., 2010). Accumulation of saturated fatty acids prevents photooxidative damage of cells by dissipation of excess light energy. The alteration in fatty acid composition is referred to the adaptive response to varying irradiance (Seyfabadi et al., 2011). This study has shown that *D. salina* achieves relatively high productivities in high light conditions, which is vital for producing an outdoor monoculture with low chances of contamination because of saline culture. However, most microalgal species produce large amounts of lipid only under stress conditions (Chaffin et al., 2012). Since stress conditions generally lead to lower growth rates, lipid increase is negated overall by the drop in biomass productivity. As mentioned above, the growth and lipid content of *D. salina* increased under high light irradiance. Biodiesel is produced by the transesterification of triacylglycerols with an alcohol in the presence of an appropriate catalyst, with glycerol as a byproduct of *D. salina*. As the ideal culture system is a low energy input open culture system, *D. salina* is one of the most suitable organism known as a biofuel producer on a large scale (Griffiths and Harrison, 2009).

Conclusion

This study revealed that *D. salina* could grow in a high light intensity (1000 µmol photon $m^{-2}s^{-1}$) and has an ability to produce large percentage content of lipids including β-carotene, proteins, and carbohydrates. This irradince-dependent adjustment ability makes them suitable candidates for open system cultivation.

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