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Investigating the effects of phytohormones on growth and β-carotene production in a naturally isolates stain of *Dunaliella salina*

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ABSTRACT

The algae of the genus *Dunaliella* especially *D. salina* is among the microalgae most studied for mass culture. This alga is the richest algal source of glycerol and β-carotene, which is grown as a food source in aquaculture. In this study the effect of growth regulators (kinetin, gibberellic acid, indole-3-acetic acid, 6γ, γ-dimethylallyl aminopurine, salicylic acid and benzyl aminopurine) on the growth and β-carotene production in *D. salina* (MCCS 001) was investigated. Results pointed out that the β-carotene content and cell growth of *D. salina* could achieve the highest rates when kinetin and indole-3-acetic acid were used at 1 µM. Besides, it was shown that almost all of plant hormones has a positive effect on cell growth and β-carotene production in the microalga *D. salina*.

INTRODUCTION

Algae constitute a vast part of many ecosystems such as sea, fresh water, desert sands, and snowy or icy regions. They provide more than 50% of total initiatory productivity at the base of world food chain (Ghasemi *et al.*, 2011a). The algae of the genus *Dunaliella* especially *D. salina* and *D. tertiolecta* are among the microalgae most studied for mass culture. *Dunaliella* spp. are grown as a food source in aquaculture and *D. salina* is the richest algal source of glycerol and β-carotene. β-carotene and other carotenoids are naturally occurring pigments that have important biological properties and nutritional (Emeish, 2012, Rasoul-Amini *et al.*, 2014). Microalgal culture is one of the modern concepts in biotechnology (Rasoul-Amini *et al.*, 2014, Venkatesan *et al.*, 2013, Yazdi *et al.*, 2005). Growing interest in carotenoid production by microalgae especially *D. salina* is due to the crucial commercial applications of these natural compounds and to the market demand of carotenoids, especially for nutritional and pharmaceutical applications (Guedes *et al.*, 2011). Carotenoids have conventionally been industrialized as food additives including antioxidants, colorants and vitamins (Ye *et al.*, 2008). Their protective capability against oxygen free radicals appears to be responsible for the therapeutic applications of carotenoids as anticancer agents, immune system stimulators and degenerative diseases preventives as claimed by several researchers (Salguero *et al.*, 2003). β-carotene plays an important role in the human body because of its pro-vitamin A activity. Carotenoids are also powerful antioxidants, scavenging potential hurtful oxy radicals, which are generally associated with the induction of particular cancers. Therefore, carotenoids, and mostly β-carotene are extensively used by pharmaceutical, food, and cosmetic industries.

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Rising demands for β-carotene, mainly natural β-carotene, has brought about growing interest in β-carotene extraction from diverse natural sources (Cardoso *et al.*, 2012). The halo-tolerant microalgae *Dunaliella salina*, produces the highest concentrations of β-carotene, obtaining levels of up to 100 gKg^{-1} on dry weight basis (Ben-Amotz *et al.*, 1988).

D. salina can fabricate and accumulate β-carotene compound in response to stress conditions (Lamers *et al.*, 2008). Industrial production of β-carotene from *D. salina* occurs in two steps. The first step involves cell growth, followed by a second step in which cells are stressed. Due to stress, β-carotene accumulates, then the cells are harvested and β-carotene extracted and purified (Hejazi *et al.*, 2004). Several methods have been suggested to improve the productivity of fermentation systems (Ghasemi *et al.*, 2012; Hejazi *et al.*, 2004) such as increasing salinity (Chen *et al.* 2009), light/dark cycles and temperature (Dipak and Lele, 2005).

Genetic elements, membrane, and enzymatic pathways inside the organism modulate the life conditions of a single cell organism. Regarding the evolution hypothesis about the eukaryotic cell derivation, the plastid apparatus is derived from a prokaryotic photosynthesizing organism. In addition, synthesis and function of phytohormones is related to plastids. Therefore, it is suggested that phytohormones could play their plant like modulatory roles in algae (Tarakhovskaya *et al.*, 2007). *D. salina* is not a plant but somehow it is similar to plant cells. Gibberellins (GAs) belong to a big group of plant hormones in throughout the life cycle of plants involved in different responses. The general role of GAs could be summarized in germination stimulation, flowering time regulation and cell expansion. They were isolated from *Giberella* fungus but found afterwards in various bacteria and many plant species including unicellular and multicellular algae (Sabovljević *et al.*, 2010).

IAA belongs to auxins, which exert a strong influence over processes such as cell growth expansion and initiation of cell division in plant cells. KN and BAP are the members of cytokinin class which stimulate cell division in plant cells. On the other hand, auxins affect DNA replication, whereas cytokines seem to exert some events leading to mitosis and cytokinesis. Gibberellic acid, belonging to the gibberellins class, increase the cell division in plant cells (Gaspar *et al.*, 1996, Gross, 1975). In one study, the relationship between abscisic acid (a plant growth regulator) production and β-carotene accumulation was inspected in salt-stressed cells of *D. salina* and a likely role of abscisic acid as a regulator of carotenogenesis in the cells of *D. salina* was evaluated (Cowan and Rose, 1991).

Therefore, it seems that some other plant growth regulators could be as a kind of stress. It was supposed these plant growth regulators could affect unicellular eukaryote *D. salina* in a way that they affect plant cells (Hejazi *et al.*, 2004). To our best knowledge, this study is one of the few attempts to disclose the role of BAP, Benzyl amino purine; 2, 4-D, 2,4-Dichlorophenoxy acetic acid; GA_3, Gibberellic acid; KN, Kinetin; SA, salicylic acid; DAP, 6γ, γ-Dimethylallyl aminopurine on β-carotene production by *D. salina*, which are able to enhance carotenoid production in that microalga.

MATERIALS AND METHODS

Organism and culture conditions

D. salina was isolated from water samples collected from Maharlu Salt Lake located 30 km southeast of Shiraz (latitude 29.26 N, longitude 52.48 E), Iran. Single colonies were derived from individual cells by repeated sub-culturing on agar plates as described elsewhere (Ghasemi *et al.*, 2008; Powtongsook *et al.*, 1995). Each colony was transferred to liquid nutrient medium. Purified *D. salina* was cultured in modified Johnson medium.

Identification of microalga

Identification of the isolated unicellular green alga was done using morphological studies and taxonomical approaches (Polle *et al.*, 2008). Besides, the 18S rRNA sequence of the isolated strain was studied.

18S ribosomal RNA sequencing

DNA content was first extracted from the microalga and then PCR was applied using two set primers. Sequences were amplified using the primers 5´-GTCAGAGGTGAAATTCTTG GATTTA-3´ and 5´-AGGGCAGGGACGTAATCAACG-3´, which amplify a ~700-bp region of the 18S rRNA gene. To extract of DNA from the *D. salina*, a fresh biomass was obtained by centrifuging at 12000 rpm. Cell lysis was done with incubation at 96º tor 5 min and centrifugation at 12000 rpm after washing of biomass two times with distilled water. The supernatant used as a template for PCR. The applied PCR condition has been described before (Ghasemi *et al.*, 2011b). PCR products were electrophoresed in a 1% (w/v) agarose gel using TBE buffer containing 1μg/mL ethidium bromide. A single ~700-bp band of DNA was cut and extracted from the gel using the Core Bio Gel Extraction Kit. The sequence was determined by the CinnaGen Company with the primers. Sequence similarity searches were done with BLAST through the NCBI database.

D. salina was cultivated in Johnson culture medium supplemented with different growth regulators with various concentrations (0.01, 0.1 and 1 μM) of each kinetin (KN), indole-3-acetic acid (IAA), benzyl amino purine (BAP), 6γ,γ-Dimethylallylaminopurine (DAP), salicylic acid (SA) and gibberellic acid (GA_3), respectively. All the media contained 12% NaCl and the pH of the medium was adjusted to 7.5 before autoclaving. Cells were grown in 100 mL of culture medium in 250 mL Erlenmeyer flasks at 25±2 °C on a rotary shaker (70 rpm) under continuous illumination of60 $\mu Em^{-2}\ s^{-1}$. Each Erlenmeyer flask was incubated with 1 mL of 3-week old pure culture containing 5×10^5 cells mL^{-1}.

The evolutionary history was inferred using the Minimum Evolution method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches

corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method is in the units of the number of base substitutions per site (Tamura *et al.*, 2004). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm [4] at a search level of 3. The Neighbor-joining algorithm (Tamura *et al.*, 2007) was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 333 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

Cell counting

The cell number was determined daily by direct counting, using a light microscope (magnification ×10) with a 0.1 mm deep counting chamber (Neubauer improved).

β-carotene extraction

Prior to β-carotene analysis, the sample preparation was carried out as follows. After 5, 10, 15, 20, 25 days of incubation, one mL sample was taken from the culture while being mixed thoroughly. After 5 min. centrifugation at 3000 rpm, the supernatant was discarded. Then, 3mL of n-hexane: ethanol (1:2) was added to the remaining pellet and each sample was mixed by vortex for 1-2 minutes to reach a complete extraction followed by adding 2 mL distilled water and 4 mL n-hexane respectively. The samples were centrifuged again for 5 minutes at 3000 rpm for separation of the remaining pellet colorless from the solvent phase. The extracted pigments were solved in n-hexane phase. Finally, the extracted pigments in the solvent phase were quantified by spectrophotometer (Eijckelhoff and Dekker, 1997).

β-carotene assay

Beta-carotene content was measured at 450 nm using a UV/Visible spectrophotometer (PG instrument Ltd.). The amount of β-carotene extracted in n-hexane was determined using spectrophotometry method using the following equation (1):

$$ß - \text{carotene } (\mu g/mL) = 25.2 \times A450 \quad \text{(Eq.1)}$$

Data analysis

The alignment tool of the MEGA software package was used for aligning 18S ribosomal RNA sequenceswith previously deposited sequences in the NCBI database. A dendrogram was constructed using then different methods integrated in the software, including Maximum Composite Likelihood method, maximum parsimony, and neighbor-joining procedures (Tamura *et al.*, 2007). Three independent experiment run in triplicate each and data were statistically analyzed using the SPSS version 16.0, Tukey method and 95% confidence and were reported as means ± SE (p<0.05 was considered as significant).

RESULTS

D. salina isolated from Maharlu Salt Lake in Shiraz, Iran was confirmed by morphological property and PCR amplification of 18S rRNA partial sequence. The partial sequence of the 18S rRNA was deposited in NCBI as EF682841.2 (Fig. 1).

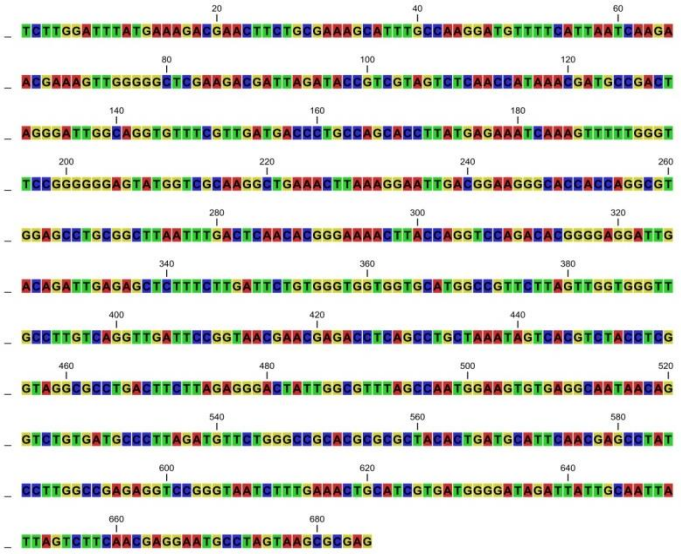

Fig. 1: Partial sequence of 18S rRNA from the naturally isolated strain of *D. salina* deposited in NCBI under EF682841.2 accession number.

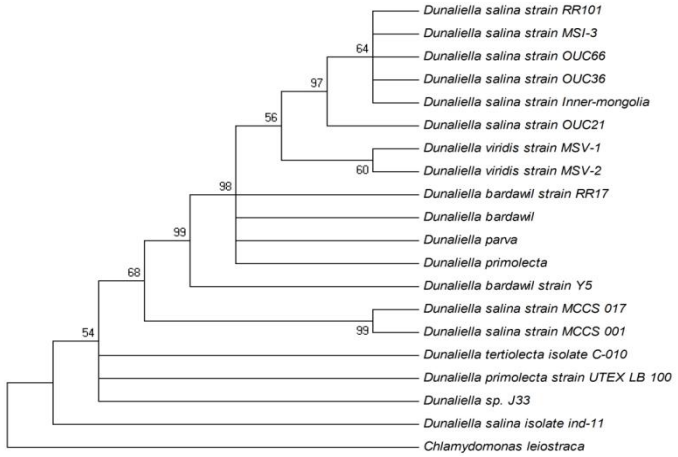

Fig. 2: Evolutionary relationships of 20 taxa (linearized). A phylogenic tree based on the entire 18S rRNA partial sequences of several species and strains of the genus *D. salina* and *Chlamydomonas leiostraca* (FR865590) asout-group was depicted by neighbor-joining analysis and maximum composite likelihood method. The indicator shows the *D. salina* strain MCC 001.

PCR amplification of partial sequence of 18S rRNA was resulted in a single band on electrophoretic gel with ~661 bp in length. The result of PCR blasted with other sequenced microalgae in NCBI showed 99% homology to the 18S small subunit rRNA of different strains of *D. salina*. A dendrogram based on the entire 18S rRNA partial sequences of several species and strains of the genus *D. salina* and *Chlamydomonas leiostraca* (FR865590) asout-group was depicted by neighbor-joining analysis and maximum composite likelihood method (Fig. 2). The tree shows

that the new isolated strain is part of a major clade containing several strains of *D. salina* and the closest relative of the isolated strain MCCS 001 was *D.salina* MCCS 017 with the accession number EU621363.1.

Spectrophotometric assay

Equation (1) shows that it is possible to give an estimation of the concentrations of β-carotene in n-hexane extract.

Effect of hormone treatment on cell number

The number of cells in all 5 groups (5, 10, 15, 20, 25 days of incubation) was determined within 25 days as shown in Table 1. All various concentrations of BAP, 2, 4-D, GA_3, KN, SA and DAP seems to increase the cell number as compared to control group ($p< 0.05$). Table 1 showed that both KN and IAA hormone had more effect on cell number than other hormones ($p< 0.05$) and also two times more than those in the control group. There were no significant differences between KN and IAA groups on increasing cell number. In contrast, BAP, GA_3 DAP and SA hormones had less effect on rising cell number. The statistical analysis using the Tukey method and 95% confidence for growth rate showed that the maximum rate of growth was achieved on day 20 and then declined in day 25.

Effect of hormone treatment on total β-carotene content

The β-carotene production of *D. salina* within 25 days was shown (Fig. 3). Concentrations of 0.01 to 1 µM of these plant hormones favored growth of *D. salina* with significant difference as compared to control group.

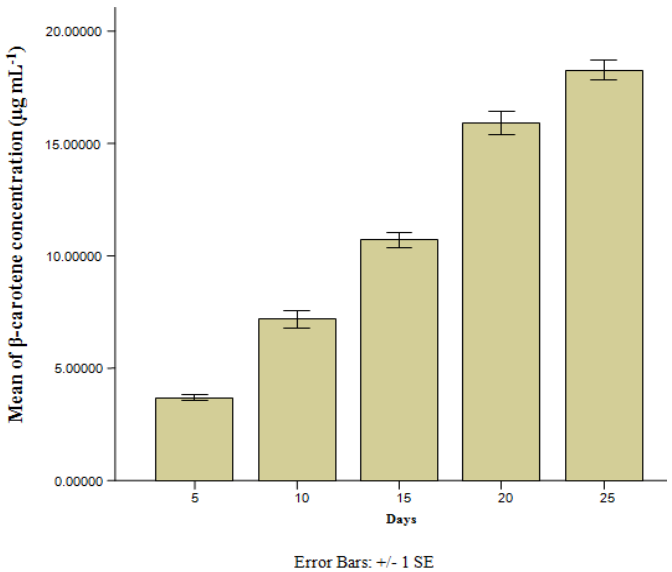

Fig. 3: The β-carotene production of *D. salina* within 25 days.The mean values of total β-carotene production in *D. salina* within 25 days of cultivation. The experiment was repeated three times, and the results presented are from a typical experiment. Bars represent the standard errors.

The effect of these plant hormone concentrations (KN, GA_3, BAP, IAA, 6-γ, γ- DAP and SA group) on total content of β-carotene within 25 days was depicted in Table 2 and Fig. 4. The results showed that the concentrations of total content of β-carotene in all days and in all groups were ($p< 0.05$) higher than that of the control group. KN and IAA had more effect on total content of β-carotene than other hormones ($p< 0.05$).

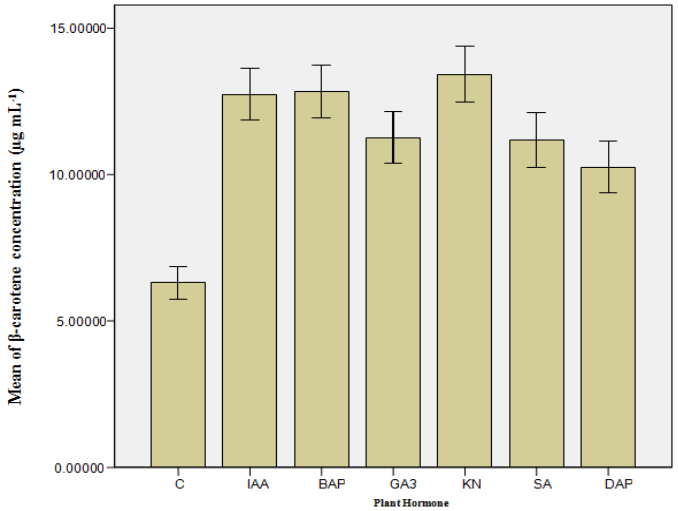

Fig. 4: The β-carotene production of *D. salina* in various hormone media (BAP, Benzyl amino purine; 2, 4-D, 2,4-Dichlorophenoxy acetic acid; GA_3, Gibberellic acid; KN, Kinetin; SA, salicylic acid; DAP, 6γ, γ-Dimethylallyl aminopurine) and in normal media within 25 days. The experiment was repeated three times, and the results presented are from a typical experiment. Bars represent the standard errors.

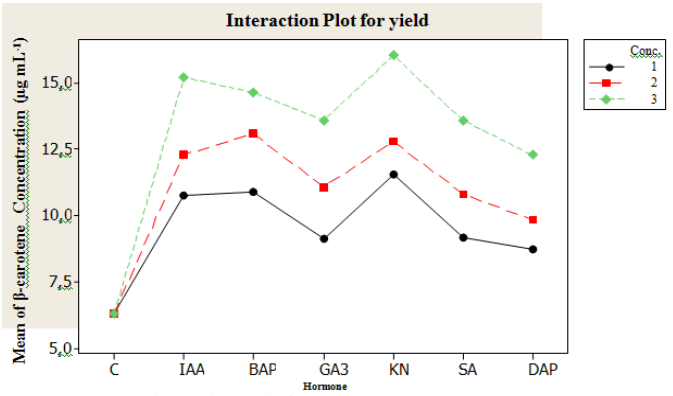

Fig. 5: Interaction plot of β-carotene production between hormones concentration. The experiment was repeated three times, and the results presented are from a typical experiment.

These amounts in KN groups were more than those in the control group and then IAA came after. The amount of total carotenoids observed on day 25 at hormone concentration of 1 µM was the highest level of carotenoids compared to all other growth conditions. The interaction plot of β-carotene production between hormones different concentrations is shown in Fig. 5. The statistical analysis using the Tukey method and 95% confidence for total content of beta-carotene showed that all hormones (at 1µM) had the most effect on beta-carotene content and there was a significant difference in interaction between time (days) and concentration on beta-carotene production. However, higher plant hormone concentration increased the total carotenoid and the highest level of carotenoid content (23.03 µg mL^{-1}) was gained at 1 µM KN hormone within 25 days.

Table 1: Effect of IAA, BAP, GA_3, KN, SA and DAP on *D. salina* cell number.

Time (Days)	Hormone concentration (µM)	Cell number Mean ± SD (IAA*)	Cell number Mean ± SD (BAP)	Cell number Mean ± SD (GA_3)`1	Cell number Mean ± SD (KN)	Cell number Mean ± SD (SA)	Cell number Mean ± SD (DAP)`1
5	C	60.58±1.04	60.58±1.04	60.58±1.04	60.58±1.04	60.58±1.04	60.58±1.04
	1	91.35±1.32	85.3±0.4	90±4.73	88.75±1.01	83.16±3.82	85.25±1.32
	0.1	83.33±1.04	78.83±3.55	79.41±2.02	85.16±2.55	70.16±1.01	74±0.09
	0.01	77.58±10.5	67.83±5.1	63.5±0.01	80.16±6	58.16±3.32	71.25±
10	C	128.7±4.8	128.7±4.8	128.7±4.8	128.7±4.8	128.7±4.8	128.7±4.8
	1	213.15±4.4	206.83±6.8	222.2±2.71	223.5±3.55	196.4±8.95	204.6±4.41
	0.1	188.25±2.83	186.2±1.07	185.8±2.6	194.3±3.61	163.9±0.72	17.08±3.32
	0.01	181.7±6.12	164.3±6.3	172.7±6.42	196.3±4.4	135.2±0.43	156.1±3.57
15	C	252.2±2.4	252.2±2.4	252.2±2.4	252.2±2.4	252.2±2.4	252.2±2.4
	1	457.4±9.2	365.9±4.91	3.88.9±1.89	484±6.67	274.3±7.63	290.5±4.67
	0.1	369.6±6.73	337.2±8.75	338.5±4.75	360.5±5.13	328.6±5.04	333.6±5.53
	0.01	330.5±5.44	317.4±3.76	324.4±0.26	333.9±7.28	304.3±9.7	307.5±5.81
20	C	430.4±3.76	430.4±3.76	430.4±3.76	430.4±3.76	430.4±3.76	430.4±3.76
	1	649.91±4.75	573.6±0.93	592.2±1.32	667±6.6	541.2±5.5	562.2±6.08
	0.1	466.9±2.87	437±2.5	454.2±4.25	608.9±2.9	431.3±4.1	441.6±0.62
	0.01	481.3±1.02	452.5±2.6	461.7±2.6	601.9±2.7	440.5±1.39	449±3.3
25	C	377.6±1.56	377.6±1.56	377.6±1.56	377.6±1.56	377.6±1.56	377.6±1.56
	1	502.3±1.84	446.2±2.3	596.6±3.4	662.5±1.94	538.9±4.3	559.3±4.4
	0.1	523±1.84	435.9±1.8	456.8±3.7	607±2.67	427.1±0.49	440.7±3.04
	0.01	479.3±1.15	455.6±2.26	465±1.4	648±0.28	442.3±4.47	451.5±4.75

* BAP, Benzyl amino purine; 2, 4-D, 2,4-Dichlorophenoxy acetic acid; GA_3, Gibberellic acid; KN, Kinetin; SA, salicylic acid; DAP, 6γ, γ-Dimethylallyl aminopurine. Each experiment was performed in triplicate and the mean values with standard deviations were presented.

Table 2: Effect of IAA, BAP, GA_3, KN, SA and DAP on β-carotene production in volume.

Time (Days)	Hormone concentration (µM)	β-carotene Content (µg mL^{-1}) Mean ± SD (IAA*)	β-carotene Content (µg mL^{-1}) Mean ± SD (BAP)	β-carotene Content (µg mL^{-1}) Mean ± SD (GA_3)	β-carotene Content (µg mL^{-1}) Mean ± SD (KN)	β-carotene Content (µg mL^{-1}) Mean ± SD (SA)	β-carotene Content (µg mL^{-1}) Mean ± SD (DAP)
5	C	1.52±0.040	1.52±0.040	1.52±0.040	1.52±0.040	1.52±0.040	1.52±0.040
	0.01	4.08±0.005	3.52±0.004	3.1±0.008	4.23±0.01	2.81±0.003	3.3±0.0085
	0.1	4.28±0.004	4.032±0.003	4.29±0.001	4.36±0.003	3.21±0.004	3.44±0.001
	1	4.53±0.03	4.89±0.017	4.62±0.006	4.83±0.028	4.51±0.026	4.17±0.0045
10	C	2.77±0.11	2.77±0.11	2.77±0.11	2.77±0.11	2.77±0.11	2.77±0.11
	0.01	7.73±0.008	8.27±0.56	4.29±0.005	7.96±0.004	3.33±0.0064	3.37±0.065
	0.1	8.55±0.002	9.01±0.024	6.81±0.011	8.96±0.001	4.93±0.006	5.58±0.0092
	1	12.34±0.005	10.7±0.011	9.93±0.004	13.02±0.002	8.87±0.0081	6.8±0.0015
15	C	6.67±0.036	6.67±0.036	6.67±0.036	6.67±0.036	6.67±0.036	6.67±0.036
	0.01	11.23±0.005	9.67±0.006	7.82±0.005	11.93±0.041	8.75±0.0021	7.36±0.0021
	0.1	12.02±0.013	13.13±0.062	9.14±0.011	12.02±0.011	11.54±0.002	13.1±0.0081
	1	13.91±0.045	14.81±0.035	13.65±0.004	13.91±0.003	14.2±0.0037	14.8±0.0049
20	C	8.64±0.03	8.64±0.03	8.64±0.03	8.64±0.03	8.64±0.03	8.64±0.03
	0.01	15.17±0.066	15.49±0.026	12.79±0.005	15.17±0.004	14.75±0.016	15.4±0.0028
	0.1	17.99±0.026	18.84±0.026	15.6±0.0011	17.99±0.001	15.54±0.002	18.8±0.0089
	1	22.02±0.019	21.36±0.041	18.45±0.004	22.02±0.002	19.2±0.017	21.3±0.0051
25	C	11.03±0.014	11.03±0.014	11.03±0.014	11.03±0.014	11.03±0.014	11.03±0.014
	0.01	16.07±0.13	17.06±0.002	16.83±0.058	16.07±0.016	16.88±0.061	17.06±0.047
	0.1	18.22±0.014	20.1±0.013	19.85±0.014	18.22±0.004	18.1±0.0067	20.1±0.049
	1	23.03±0.021	22.35±0.037	20.1±0.018	23.03±0.008	21.9±0.0031	22.35±0.064

* BAP, Benzyl amino purine; 2, 4-D, 2,4-Dichlorophenoxy acetic acid; GA_3, Gibberellic acid; KN, Kinetin; SA, salicylic acid; DAP, 6γ, γ-Dimethylallyl aminopurine. Each experiment was performed in triplicate and the mean values with standard deviations were presented.

Effect of hormonal treatment on β-carotene production per cell

β-carotene content per cell was measured by dividing total content of β-Carotene on cell number in concentrations of control (o) to 1 µM of these plant hormones. The effect of these plant hormone concentrations (KN, GA_3, BAP, IAA, 6-γ, γ- DAP and SA group) on β-carotene content per cell within 25 days was depicted in Table 3. The results showed that the concentrations of β-carotene content per cell in all days and in all groups were ($p< 0.05$) higher than that of the control group. SA in concentration of 0.01 µM has more effect on β-carotene content per cell than other hormones ($p< 0.05$). KN in concentration of 1 µM has less effect on β-carotene content per cell than other hormones ($p< 0.05$) on day 25.

Table 3: Effect of IAA, BAP, GA_3, KN, SA and DAP on β-carotene production per cell.

Time (Days)	Hormone concentration (μM)	β-carotene Content per cell (pg mL^{-1}) Mean ± SD (IAA*)	β-carotene Content per cell (pg mL^{-1}) Mean ± SD (BAP)	β-carotene Content per cell (pg mL^{-1}) Mean ± SD (GA_3)	β-carotene Content per cell (pg mL^{-1}) Mean ± SD (KN)	β-carotene Content per cell (pg mL^{-1}) Mean ± SD (SA)	β-carotene Content per cell (pg mL^{-1}) Mean ± SD (DAP)
5	C(0)	0.021±0.53	0.021±0.53	0.021±0.53	0.021±0.53	0.021±0.53	0.021±0.53
	0.01	0.0516±0.054	0.051±0.19	0.068±0.65	0.051±0.004	0.06±0.0087	0.033±0.0091
	0.1	0.052±0.6	0.051±0.38	0.06±0.76	0.05±0.003	0.057±0.031	0.044±0.058
	1	0.0509±0.55	0.049±0.19	0.035±0.965	0.046±0.009	0.077±0.009	0.048±0.076
10	C(0)	0.022±0.9	0.022±0.9	0.022±0.9	0.022±0.9	0.022±0.9	0.022±0.9
	0.01	0.045±0.043	0.045±1.24	0.063±0.09	0.057±0.0067	0.051±0.003	0.017±0.106
	0.1	0.045±0.976	0.044±0.86	0.04±0.030	0.04±0.076	0.048±0.006	0.026±0.009
	1	0.0596±0.47	0.049±0.047	0.023±0.765	0.055±0.032	0.064±0.079	0.042±0.032
15	C(0)	0.03±0.45	0.03±0.45	0.03±0.45	0.03±0.45	0.03±0.45	0.03±0.45
	0.01	0.036±.005	0.0311±0.52	0.042±0.12	0.033±0.18	0.03±0.54	0.026±0.072
	0.1	0.0314±0.32	0.026±0.95	0.025±0.099	0.032±0.61	0.037±0.070	0.031±0.051
	1	0.031±0.86	0.033±0.29	0.021±0.154	0.036±0.006	0.047±0.180	0.035±0.007
20	C(0)	0.02±0.76	0.02±0.76	0.02±0.76	0.02±0.76	0.02±0.76	0.02±0.76
	0.01	0.025±1.1	0.028±0.62	0.023±0.34	0.025±0.43	0.027±0.0029	0.033±0.006
	0.1	0.028±0.23	0.032±0.006	0.024±0.611	0.028±0.095	0.032±0.009	0.029±0.602
	1	0.0326±0.005	0.031±0.54	0.018±0.054	0.032±0.614	0.038±0.112	0.028±0.009
25	C(0)	0.023±1.01	0.023±1.01	0.023±1.01	0.023±1.01	0.023±1.01	0.023±1.01
	0.01	0.025±0.12	0.028±0.34	0.032±0.65	0.026±0.025	0.028±0.008	0.033±0.001
	0.1	0.027±.025	0.031±0.45	0.029±0.901	0.027±0.0761	0.033±0.094	0.031±0.008
	1	0.0322±.054	0.031±0.49	0.023±0.701	0.031±0.349	0.037±0.101	0.031±0.104

* BAP, Benzyl amino purine; 2, 4-D, 2,4-Dichlorophenoxy acetic acid; GA_3, Gibberellic acid; KN, Kinetin; SA, salicylic acid; DAP, 6γ, γ-Dimethylallyl aminopurine. Each experiment was performed in triplicate and the mean values with standard deviations were presented.

DISCUSSION

The amount of plant hormones has been identified in numerous higher order plants, and these diverse forms have been associated to different growth and developmental processes. As to our best knowledge, this study investigates the effect of plant growth regulators on the growth and production of β-carotene in *D. salina*. The studied microalga was cultured in 250 mL flasks with 12% salinity, because the optimum salinity for β-carotene production is 12% NaCl. In this study, six plant hormones were used: kinetin (KN), indole-3-acetic acid (IAA), benzyl adenine purine (BAP), and gibberellic acid (GA_3), 6γ, γ-Dimethylallyl amino purine (DAP), salicylic acid (SA). According to the results, KN and IAA had the most effect on cell growth. The cell number in the media with KN and IAA were about two times more than that in control groups (Table 3). DAP and SA had less effect on cell number of *D. salina* in the culture media than KN and IAA, but there was a significant difference between them and the control groups. These hormones may affect the number of cells or growth rate of *D. salina* through these mechanisms. The results illustrated that these hormones have positive effects on cell number, which are in accordance with those reported by Liangxia who studied the effect of six hormones on the growth rate of *D. salina* (Liangxia, 2007). Activation of downstream genes of hormone-regulated plant reaction depends on understanding plant hormones mechanisms which have been discussed for auxin, GA, cytokinins, ABA, ethylene, BR and JA. However, receptors for these hormones have been verified and different interesting biochemical natures of these hormone receptors along with some important downstream signaling parts of the receptors are still necessary to be discovered. More receptors for these hormones may have been known in the near future. However, recent studies indicated that the Auxin- Binding Protein 1 (ABP1) perceived auxin signal to mediate TIR1-independent auxin responses (Shan *et al.*, 2012).Very few reports are collected about the physiological role of cytokines in the growth and development of microalgae. In most reports, the role of exogenous cytokines instigates growth and cell division and increases dry weight, protein contents, and photosynthetic pigments. The results of this research are in contrary with Ördög *et al*. who reported that exogenous application of cytokines had little or no effect on micro algal cultures (Ördög *et al.*, 2004). By using hormones, more biomass will be obtained in a shorter period, which can be useful in producing tablets or capsules of *D. salina* or in production of single cell proteins. DAP had more effect on total content of β-carotene than other hormones ($p< 0.05$). The influence of KN (also IAA and to some extent BAP) on total β-carotene production of *D. salina* is really intriguing, because more biomass and more total content of β-carotene can be obtained, which is the purpose of many studies in a shorter time for β-carotene production. According to results, it seems that increasing in carotenoid content is dependent upon increasing the cell number. As mentioned above, carotenoid content in starvation and stress condition is increased. Arash Rad *et al.* have shown that growth rate has affected the carotenoid production, when growth rate reduces, the carotenogenesis rate increases (Arash Rad *et al*., 2011). Therefore, to correct investigation of hormone effect, it was needed to measure cell growth and β-carotene production per cell. In this experiment measurement of β-carotene production per cell of *D. salina* was carried out with dividing total content of β-carotene on

cell number for all samples. Result from measuring β-carotene production per cell of *D. salina* showed that β-carotene content (per cell) of *Dunaliella* sp. was decreased for all plant hormone compared whit control (media without plant hormone) during 25 days. These Result showed that total carotenoid content increase in alga under the influence of KN correlated with their cell proliferation. As shown in Table 1 and 3, plant hormones had stimulatory effects on algal growth, but they had reducing effect on carotenoid production. A simple and available spectrophotometric method has been developed in this study for quantitation of β-carotene. A notable advantage of this method over the previously reported spectrophotometric methods for β-carotene quantification was its considerable longer run times as well as availability and popularity, both of which being of great impact in practice, particularly when a high number of samples are to be analyzed. This method can provide a simple and easy way to assay β-carotene in such sources like food, drugs, etc. It was recently announced by the National Cancer Institute that β-carotene has anti carcinogenic effects. Other studies have found that β-carotene is effective in controlling cholesterol and in reducing the risk of heart disease. These new findings make β-carotene much more valuable and are likely to increase the demand for the product. Being fat soluble, the natural β-carotene is a much superior anti carcinogenic and anti-heart disease agent. Thus the new findings of these desirable medicinal properties are likely to increase even more the demand and desirability of natural β-carotene.The main challenges facing the algal β-carotene producer are the need to continue to improve the algal culture process. Therefore, there is a need for increasing the β-carotene productivity by escalating the growth rate and reliability and also by improving the β-carotene content of the cells. Thus, this requires improvements in the design of the production plants, a better understanding of *Dunaliella* physiology and finding new algal strains, which can be obtained by mutagenesis procedures and genetic engineering techniques (Borowitzka, 1999).

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