

Carotenoid production from microalga, *Dunaliella salina*

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Carotenoid content of microalga, *Dunaliella salina* was increased using different stress parameters, such as cell division inhibition (vinblastine), nitrogen starvation, high salinity and high irradiation with high temperature. Vinblastine had very little favourable effect. Salinity above 3 M significantly decreased the cell growth rate due to cell death but increased the per cell carotenoid content. With nitrogen starvation, β -carotene content increased from 1.65 pg/cell to 7.05 pg/cell. However, the maximum β -carotene (8.28 pg/cell) was obtained with high irradiation along with high temperature.

Keywords: β -carotene, carotenoids, *Dunaliella salina*, microalgae

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Introduction

Microalgae are among the fastest growing autotrophs on the earth, which utilize commonly available material for growth. India is a tropical country with abundant coastal area and superfluous solar energy that offer favourable environment for algal cultivation. Spirulina, a microalga promoted as nutraceutical for human consumption, is commercially grown in a few countries and India is one of them. Microalgae produce vast array of natural products including proteins, enzymes, bioactive compounds and carotenoids^{1,2}. Carotenoids are a large group of more than 600 different biochemicals. Some of these molecules are provitamin A and have a range of diverse biological functions and actions, especially in relation to human health. The worldwide carotenoid market is estimated to be \$786 million in 1999. With an estimated average annual growth rate of 2.9%, this market is expected to reach \$935 million by 2005 (www.bccresearch.com). Carotenoids are known to be crucial for normal vision and have been associated with reducing the risk of several degenerative diseases including cancer^{1,3}. There are more than 400 carotenoids found in nature and β -carotene is perhaps the most important one. β -Carotene is used in food

industry as colouring additive, antioxidant and as provitamin A. It also has anticancer, antiaging, immunomodulatory properties^{4,5}. β -Carotene is commercially synthesized as well as recovered by extraction from natural sources. Carrot and algae (*Dunaliella salina*, *D. bardawil*) are "classical" natural sources of β -carotene among vegetables, fruits and other natural sources that have been exploited commercially⁶.

The unicellular microalga, *Dunaliella salina* accumulates massive amounts of β -carotene. The β -carotene synthesis increases with the unbalanced physiological conditions of cell, created due to various stress factors. A normal cell has a condition of physiological balance. Under stress conditions, this balance is disturbed, such as formation of excessive free radicals. In order to protect and continue growth, the cells are known to generate additional β -carotene under stressed conditions⁷. The pigment has antioxidant property that quenches excessive free radicals, restoring the physiological balance⁸. In comparison to others, *Dunaliella* has the following advantages: a) Disruption of cells is much easier than that in other algae because of its wall-less nature. b) Continuous culture in laboratory is easy and the growth rate is relatively high. c) Resistance to various environmental conditions is higher than in other algae⁹. Hence, a number of research reports are published on it relating to extraction of β -carotene, isomer composition and stability¹⁰⁻¹⁴. Since light

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intensity is a major contributing factor for the pigment production, a variety of light arrangements, such as white fluorescent lamps, light emitting diodes, xenon lamps and metal halide lamps, have been used by different research workers^{15,16}.

The present study deals with the use of *D. salina* for production of β -carotene. The primary objective of this work has been to increase the β -carotene content of *D. salina* using different stress conditions, like retarded cell growth by vinblastin, nitrogen depletion, salinity, high irradiation and high irradiation with high temperature.

Materials and Methods

Materials and Chemicals

D. salina 19.3 was procured from Sammlung von Algenkulturen Gottingen, Germany. All other chemicals used were of analytical grade and purchased from local suppliers (S.D. Fine Chemicals Ltd., Merck India Ltd., Mumbai, India).

Growth Conditions

Alga was cultivated in growth medium containing 2 M NaCl, 5 mM MgSO₄, 0.75 mM KNO₃, 0.2 mM KH₂PO₄, 0.3 mM CaCl₂, 7 μ M MnCl₂, 5 μ M EDTA, 2 μ M FeCl₃, 1 μ M CuCl₂, 1 μ M COCl₂, 1 μ M (NH₄)₆Mo₇O₂₄, 1 μ M ZnCl₂, 50 mM NaHCO₃, and 40 mM Tris-HCl (pH-7.5)¹⁷. The media were sterilized at 121°C for 20 min. Phosphates and carbon sources were autoclaved separately.

Pigment Extraction and Analysis

Cell counts were carried out using a Neubauer Haemocytometer. Each count was repeated four times and average value was considered. An aliquot (5 mL) of *D. salina* cell suspension was centrifuged at 3000 \times g for 15 min. The pellet obtained was washed with distilled water and after removal of water by centrifugation again suspended in acetone and vortex mixed until a white precipitate appeared, which generally require 1 min. The cell membrane gets ruptured because of organic solvent (acetone), thus extracting β -carotene along with chlorophyll. Acetone extract separated from cell debris by centrifuging it at 3000 \times g for 10 min. Total carotenoid and chlorophyll levels were determined by UV/visible spectroscopy split beam spectrophotometer of samples in 80% acetone by using the equations of Lichtenthaler¹⁸. β -Carotene was assayed according to Ben-Amotz and Avron¹⁹. E1% 1 cm of 2273 at 480 nm has been used to calculate β -carotene concentration.

Stress Parameters

Algae were cultivated in an optimized medium and pre-grown cells after sufficient growth were inoculated under control and stress conditions. As it is a biological process and the long cultivation period of algae make it more prone to contamination, separate set of experiments were carried out in order to decrease the error. For inoculation, 10 mL of algae (5×10^5 cells mL⁻¹) were transferred into a 250 mL Erlenmeyer flasks containing 100 mL fresh medium. These flasks were incubated at 25°C with occasional shaking in a cabinet equipped with cool white fluorescent lamps, giving (total 1200 lux) for 16 days. The samples were withdrawn after every 48 h and assayed for chlorophyll, β -carotene content and biomass (cell number). In case of vinblastine stress, when cells enter into log phase, vinblastine sulphate (0.5 to 2 mM) was added into the flasks. For nitrogen starvation, two different media with (0.75 mM) and without nitrogen were used. For high salinity stress, when cells enter into log phase, NaCl ranging from (1.5 to 5 M) was added into the flasks. For high irradiation experiment, when cells enter into log phase, all flasks except control were subjected to the high light intensity of about 2300 lux with the help of metal halide lamps. For high irradiance with high temperature, light irradiance of 6000 lux was employed using metal halide lamps and temperature of 35°C was achieved due to self-heating.

Results and Discussion

Effect of Vinblastine

Boussiba and Vonshank⁷ studied the effect of vinblastine on production of astaxanthin in *Haemato-coccus pluvialis*. It was observed that alga produces carotenoids under the stress conditions in which cell division is retarded^{7,20}. In the present study also, increase in the concentration of vinblastin (0 to 2 mM) in the culture medium gradually decreased the cell mass and increased the pigment contents, including β -carotene, carotenoids and corresponding chlorophyll, in the cells of *D. salina* (Table 1). Nearly 45% reduction in the cell mass and 2.18 ± 0.08 pg/cell of β -carotene (2-fold increase from the normal value) was observed at 2 mM concentration of vinblastine. The corresponding chlorophyll content was also increased from 3.53 ± 0.11 to 5.85 ± 0.21 pg/cell (~66% increase). Vinblastin is known to stop the cell growth by arresting microtubules in the metaphase of mitosis. Thus, a stress condition is developed which results in

Table 1—Effect of vinblastine on growth and pigment production in *D. salina*

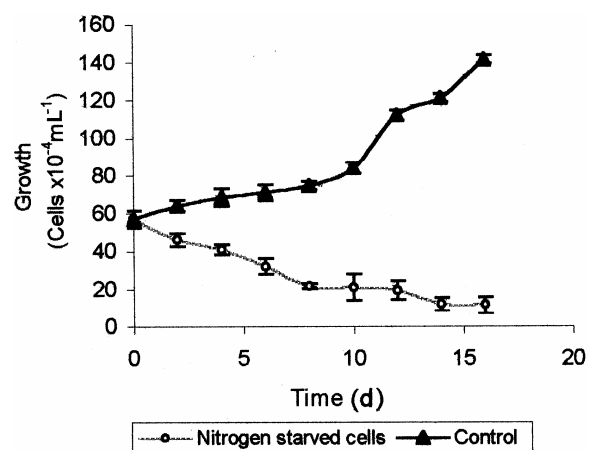
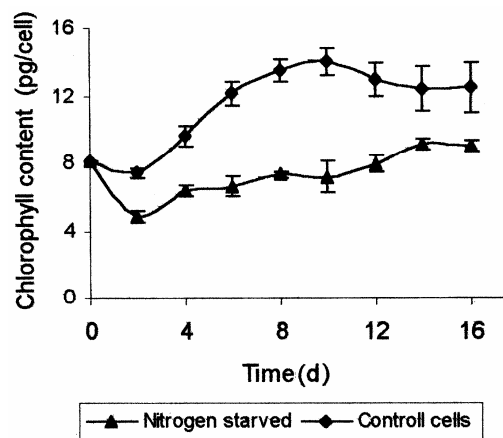
Vinblastine. mM	Cell number Organisms/ml	Carotenoids (pg/cell)	Chlorophyll (pg/cell)	β -Carotene (pg/cell)	Carotenoids/ Chlorophyll	β -Carotene/ Chlorophyll
0	$2.56 \times 10^6 \pm 9.41$	1.10 ± 0.11	3.53 ± 0.11	1.03 ± 0.03	0.310	0.290
0.5	$2.36 \times 10^6 \pm 8.71$	1.21 ± 0.10	3.96 ± 0.10	1.13 ± 0.03	0.300	0.286
1	$1.94 \times 10^6 \pm 4.39$	1.50 ± 0.08	4.61 ± 0.08	1.43 ± 0.02	0.328	0.311
1.5	$1.66 \times 10^6 \pm 5.09$	2.03 ± 0.07	5.61 ± 0.07	1.80 ± 0.02	0.360	0.320
2	$1.44 \times 10^6 \pm 7.02$	2.80 ± 0.21	5.85 ± 0.21	2.18 ± 0.08	0.470	0.370

enhancement of pigment production. However, vinblastine is an expensive chemical and also increases the chlorophyll content that may make the downstream processing less economical. Hence, this stress was not further studied.

Effect of Nitrogen Starvation

Nitrogen is one of the primary requirements of growth media for any cell. Absence of nitrogen or the starvation condition is considered as stress by the organisms. In the present study, *D. salina* was grown in normal media either with 0.75 mM of nitrates¹⁷ or without nitrates (nitrogen starvation stress). Figs 1A to D illustrate the effect of nitrate depletion on growth and pigment content of *D. salina* cultivated at 1200 lux. The algae ceased to divide when nitrogen was not supplied in the growth medium as nitrogen is the primary requirement for all the metabolic activities of the cell (Fig. 1A). Further, β -carotene content increased markedly to 7.05 ± 1.01 pg/cell from 1.65 ± 0.13 pg/cell (4-fold increase from the normal value). The increase in β -carotene content of nitrogen-starved cells may be attributed to excessive formation of free radicals under the stress. β -carotene has antioxidant properties that quench excessive free radicals, restoring the physiological balance⁸. Additional β -carotene is produced in order to protect the cells and to continue their growth⁷. Hence, the β -carotene production is markedly increased under nitrogen starvation.

Fig. 1B shows amount of chlorophyll per cell under nitrogen starvation. Chlorophyll decreased with the decrease in availability of nitrate. This shows that chlorophyll synthesis gets adversely affected, whereas β -carotene increases²¹. The significant decrease in chlorophyll during nitrogen starvation may be because chlorophyll molecule contains four nitrogen atoms in its structure and, therefore, it becomes very difficult for the cell organelles to synthesize chlorophyll in the absence of nitrogen. As a result,

Fig. 1A—Effect of nitrogen depletion on cell growth of *D. salina*.Fig. 1B—Effect of nitrogen starvation on chlorophyll production by *D. salina*.

β -carotene/chlorophyll ratio was significantly higher in starved cells than in the control.

Effect of Salinity

In contrast to other green algae, *Dunaliella* do not contain rigid cell wall. As a result, cells respond rapidly (by alteration in cell volume) to the changes in osmotic pressure. β -Carotene accumulation in

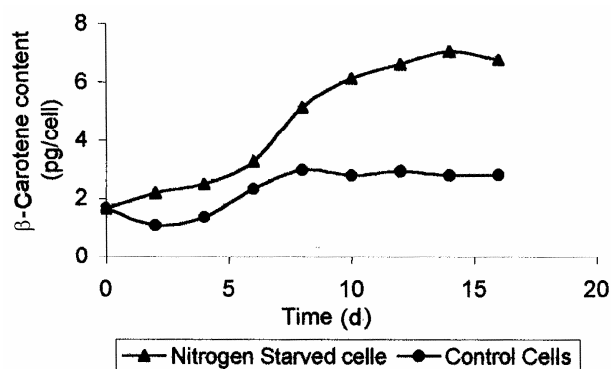


Fig. 1C—Effect of nitrogen starvation on β -carotene production by *D. salina*.

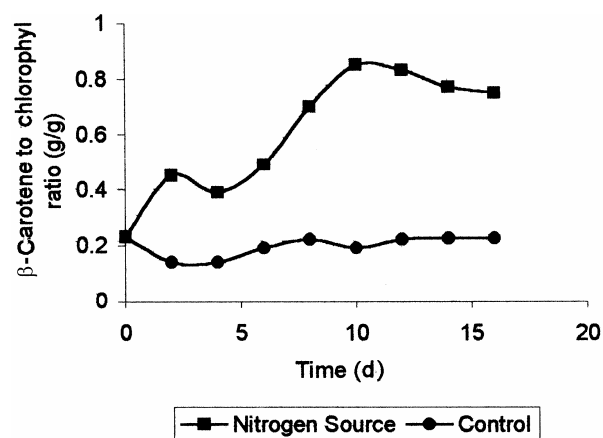


Fig. 1D—Effect of nitrogen starvation on β -carotene/chlorophyll ratio by *D. salina*.

D. salina at different salinities from 1.5 to 5 M was studied. The cell density decreased from $3.7 \times 10^5 \pm 2.94$ to $2.3 \times 10^5 \pm 2.16$ cells/mL at 4 M NaCl, while the flasks containing 5 M NaCl turned completely white within 2 days after addition of salt. This may be because when a microalga is grown in saline environment, osmosis plays a role. If very high salinity is used in the medium, the external environment of the cell contains the hypertonic solution, i.e. higher concentration of the solute (NaCl) and lower the concentration of the water, than that present inside the cell. In such conditions, there is a net flux of water molecules leaving the cell. This results in cell shrinkage and subsequent destruction of the cell and cell components (plasmolysis). Since *Dunaliella* does not have rigid walls unlike other green algae, it exhibits some ability to adapt to salinity as seen from the behaviour at 4 M salinity. In this study, it was observed that the cells started growing after 6 days

(Fig. 2A). In the medium with 1.5 M salinity, the external environment has low salinity and forms a hypotonic solution. In such condition, there is a net flux of water molecules entering the cell. Thus, causing swelling and eventually bursting of the cells. However, 2 M salinity showed maximum biomass of $2.51 \times 10^6 \pm 6.21$ cells/mL (Fig. 2A) and, hence, it can be said that 2 M is the optimum concentration for the growth of *Dunaliella* cells.

Effect of salinity on chlorophyll content of the cell is shown in Fig. 2B. When a cell is depleted of water due to high salinity, photosynthesis also gets adversely affected. Hence, the chlorophyll content was decreased with the increase in salinity. β -carotene is a secondary metabolite and these molecules are produced by the cells in stress condition as cell protecting mechanism. When salinity is increased it acts as stress that enhances β -carotene production. As a result, in the present study also, chlorophyll content decreased and β -carotene increased at intermediate salinity level (Fig. 2C). Since there was increase in β -carotene content and decrease in chlorophyll content, β -carotene/chlorophyll ratio is maximum at 4 M salinity (Fig. 2D). However, cells adopt to the high salinity due to presence of glycerol as it has ability to balance the extracellular osmotic stress. It has been reported that the intracellular glycerol concentrations exceeds nearly 50% and is sufficient to develop adequate osmotic pressure necessary to balance the extracellular osmolarity²².

Effect of High Irradiation with High Temperature

Light intensity plays an important role in controlling the β -carotene accumulation in algal cells. Preliminary experiments for cultivation were carried out with 800 lux continuous irradiation. However, continuous irradiation of 2300 lux was used during light stress. The effect of light intensity on growth and pigment content of *D. salina* under the continuous irradiation of 2300 lux is illustrated in Table 2. The chlorophyll content per cell showed the inverse dependence on light intensity, being low at the high light intensity. The β -carotene content per cell, on other hand, increased sharply with light intensity. It indicates that β -carotene can be greatly enhanced at higher irradiation. Hence, it was thought desirable to explore the possibility of using higher light stress for carotenogenesis.

It is considered that the β -carotene accumulation is related to the protecting effect of β -carotene against

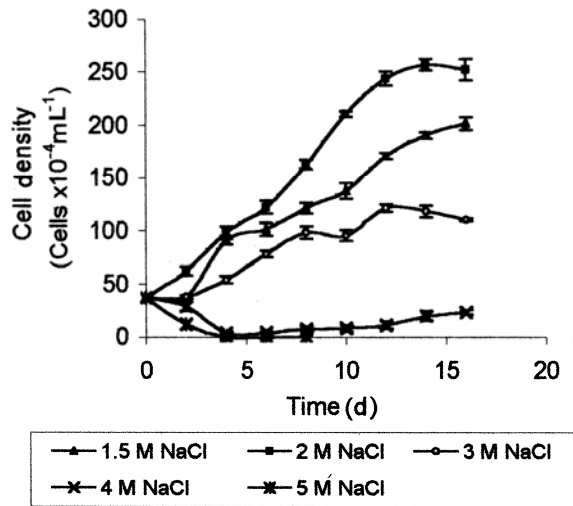


Fig. 2A—Effect of high salinity on cell growth of *D. salina*.

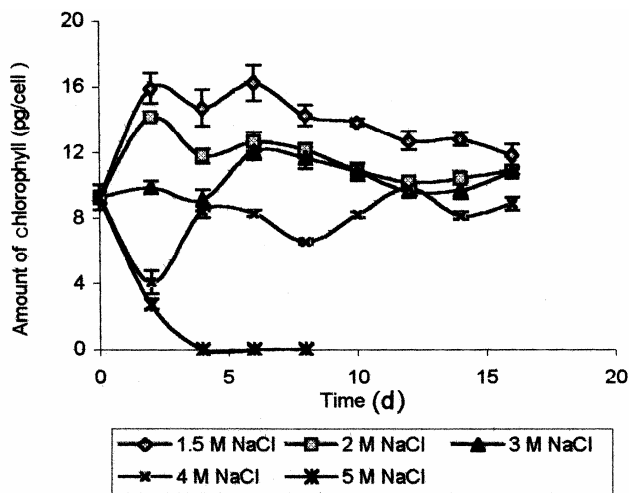


Fig. 2B—Effect of high salinity on chlorophyll production by *D. salina*.

chlorophyll catalyzed singlet oxygen production under high intensity. However, Ben Amotz²³ in *D. bardawil* showed that it cannot be considered as true because the electron micrographs taken by him showed the massive accumulation of β -carotene globules located in intrathylacoid space of the chloroplast, which was in disagreement with the existing hypothesis as the large distance between the β -carotene globules and the thylakoid located chlorophyll would not allow efficient quenching of singlet oxygen or any other chlorophyll generated product. Hence, the exact reason explained is that the β -carotene globules protect the cell against injury by high intensity irradiation under limited growth conditions by acting as a screen to absorb excess

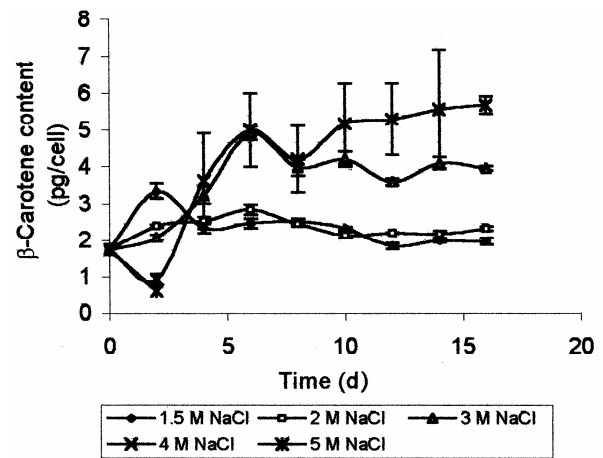


Fig. 2C—Effect of high salinity on β -carotene production by *D. salina*.

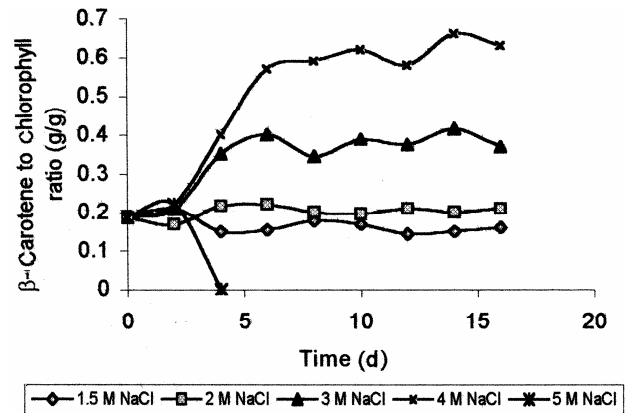


Fig. 2D—Effect of high salinity on β -carotene/chlorophyll ratio by *D. salina*.

irradiation. This hypothesis is further supported by other studies^{17,19,23}.

In the present study, metal halide lamps (Phillips BLV 70W, 1200 lux,) were used to produce high irradiation with low power consumption. It was observed that there was natural heating and external cooling was necessary to conduct isothermal growth experiments. If a microalga is to be grown at commercial scale, solar pond is the cheapest feasible option. Some natural heating of the aqueous medium is expected. Further, higher temperature may have faster growth kinetics (within acceptable temperature range for a given microalga) and will also generate a temperature stress effect that may increase the carotenoid production. Maintaining constant temperature at commercial scale will be unattractive due to additional power requirements. Hence, effect of high irradiation along with high temperature on growth and pigment content was also studied. Light at 6000 lux

Table 2—Effect of high light intensity on *D. salina*

Light intensity (lux)	Cell density	Chlorophyll (pg/cell)	β -Carotene (pg/cell)	β -Carotene/Chlorophyll ($\text{g}\cdot\text{g}^{-1}$)
800	2.31×10^6 ± 5.71	10.25 ± 0.30	1.51 ± 0.04	0.14
2300	1.91×10^6 ± 7.87	6.81 ± 0.17	2.72 ± 0.07	0.41

Note: Data given is after 10 days of cultivation

and temperature of 35°C was employed. It can be seen from Fig. 3A that the cell count decreased from 5.9×10^5 to 2.6×10^5 cells/mL within 8 days. It is reported¹² that *D. bardawil* and some variants of *D. salina* strain can survive and grow at light intensity upto 40000 lux. The decrease in cell count and reduction in growth rate may be due to higher temperature. However, growth rate was found to increase in the next 8 days (5.9×10^5 cells/mL). This suggests that cells could adapt to high light intensity and temperature after a sufficient time.

At 6000 lux and 35°C, the chlorophyll content per cell decreased. The decrease in chlorophyll content was prominent for the first two days, which might be due to sudden exposure of cells to high irradiation. However, there was slight increase in chlorophyll content after two days but the chlorophyll content of control was still higher than the stressed cells (Fig. 3B). Further, β -carotene/cell increased from 1.65 to 8.28 pg/cell, which suggests a 5-fold increase in the carotene content (Fig. 3C). The β -carotene/chlorophyll ratio also increased from 0.181 to 0.725 (Fig. 3D), which is indication of preferable increase in the production of carotenoids than chlorophyll. Thus, overall results show that, although, the biomass of *D. salina* contains 1.6 pg/cell β -carotene under normal cultivation conditions (1200 lux, 25°C), this value can be increased by a factor of 5 (8.28 pg/cell) using high irradiation (6000 lux) along with high temperature (35°C) (Fig. 4). Orset and Young¹² have also reported a value 6.38 pg/cell with similar light intensity.

Conclusion

Dunaliella salina 19.3 was cultivated at laboratory scale to increase the β -carotene content of the biomass by using different stress conditions. The typical β -carotene content was 1.65 pg/cell (1200 lux and 25°C). Vinblastine, a growth inhibitor, increased the

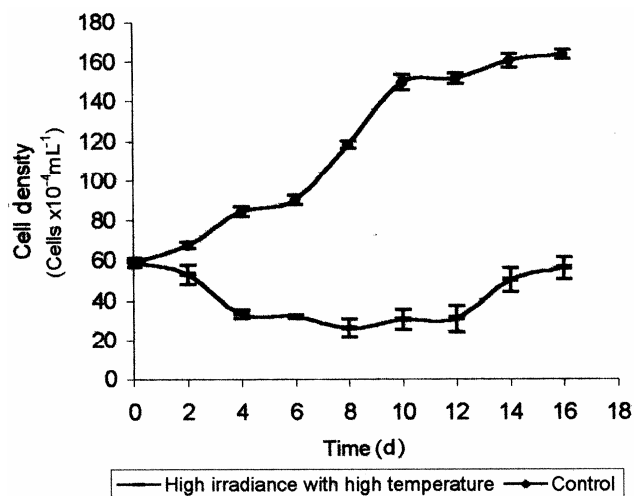


Fig. 3A—Effect of high irradiation with high temperature on cell growth of *D. salina*.

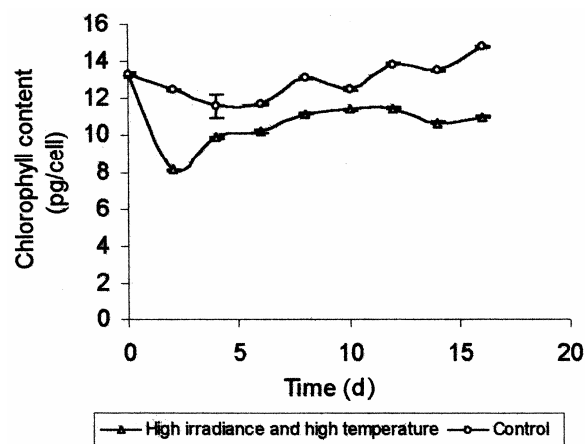


Fig. 3B—Effect of high irradiation with high temperature on chlorophyll production by *D. salina*.

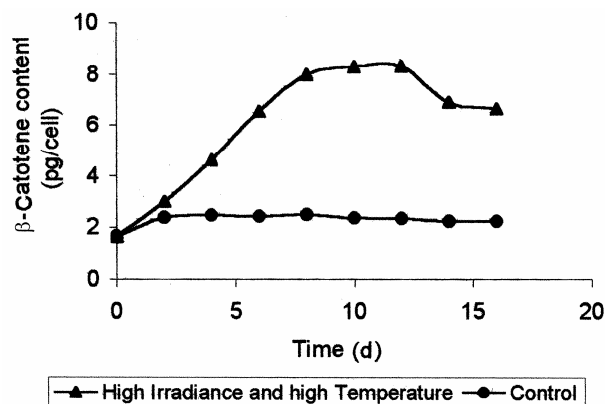


Fig. 3C—Effect of high irradiation with high temperature on β -carotene production by *D. salina*.

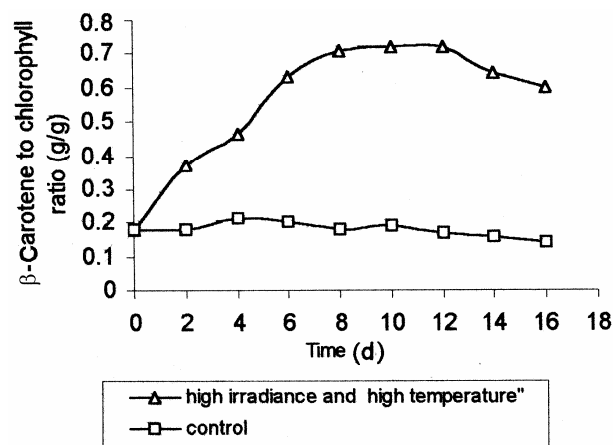


Fig. 3D—Effect of high irradiation with high temperature on β -carotene/chlorophyll ratio by *D. salina*.

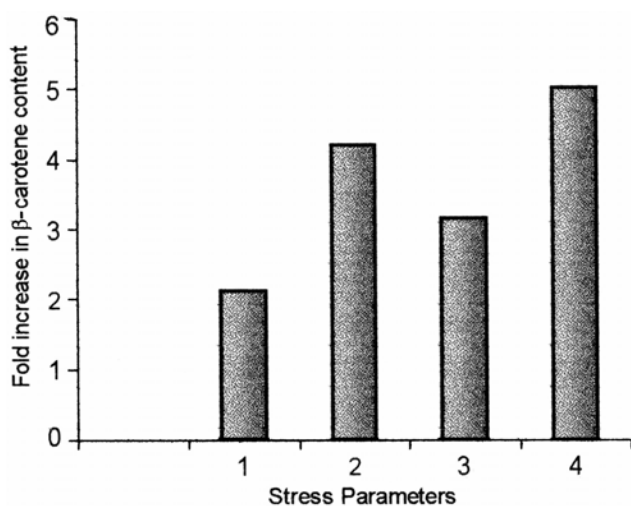


Fig. 4—Fold increase in β -carotene content in *D. salina*.

β -carotene content by 2-fold as compared to the normal value. However, vinblastine is expensive and also increases the chlorophyll content, which is not desirable. Hence, it is not recommendable for commercial use. In nitrogen stress β -carotene content increased markedly to 7.05 pg/cell. Optimum concentration of salinity for carotenoid production was found to be 3 M, whereas 2 M NaCl supported maximum growth. High irradiation with high temperature stress studies showed that the β -carotene accumulation increased from 1.77 ± 0.082 pg/cell to 8.28 ± 0.61 pg/cell. Thus, there was a 5-fold increase in the pigment production. To summarize, enhanced production of carotenoids from *D. salina* can be obtained by maintaining 3 M salinity, nitrogen deficient media and high irradiation with high

temperature. Since the results are encouraging, it is felt that, considering the consumers preference for natural products, β -carotene production based on a process of *D. salina* will have a potential in India.

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