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OPTIMIZATION STUDY IN *DUNALIELLA SALINA* EU5891200 ISOLATED IN SALT PANS OF TAMIL NADU, SOUTH INDIA

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Abstract

This study examined the effect of different pH, light intensity and different concentrations of NaNO₃, NaH₂PO₄ and NaCl on the kinetics of growth, total carotenoids accumulated in *Dunaliella salina* EU5891200, a micro algal strain isolated from the salt pans of Tamil Nadu, South India. Results indicated that the highest amount of carotenoids detected grown at pH 8, 40 μEm⁻²s⁻¹ light intensity had maximum production of total carotenoids. Lower salinity of 1.0 M NaCl favored high accumulation of total carotenoids. The amounts of total carotenoids recorded at 1.0 mM NaNO₃ for *D. salina* had 20% more than the control. Phosphate was found to greatly influence the accumulation of total carotenoids (120.0 μg/mL) in the isolate.

Keywords: Total carotenoids, Chlorophyceae, *Dunaliella salina*, Specific primers

Introduction

The "green algae" comprise of more than 7000 species growing in a variety of habitats. Development of commercial-scale microalgal culturing techniques is being carried out globally for the production of bioactive compounds, aquaculture feed, fine chemicals, and renewable fuels.

Dunaliella is a unicellular, biflagellate, naked, green alga belonging to the family Polyblepharidaceae and the class Chlorophyceae which is originally described by Dunal in 1838 and later it was discovered by Teodoresco in 1905. In 1973, Massyuk divided the genus into subgenera: *Dunaliella* (23 species) and *Pascheria* (5 species). Nearly 1000 carotenoids are present in nature and only a few of them occur in abundance in fruits and vegetables. They include carotene (carrots), lycopene (tomatoes) and lutein (spinach). *Dunaliella* is common in hypersaline environments (Borowitzka and Borowitzka, 1988) and can yield three major valuable products namely glycerol, β-carotene, high protein and a variety of carotenoids such as α-carotene, xanthophylls like zeaxanthin, cryptoxanthin and lutein. Carotenoids are located in the form of oily droplets between the honeycomb structures of chloroplast which is responsible for the bright red orange colour of *Dunaliella salina*, often designated in the older literature as 'hematochrome'. Carotenoids are commercially significant since they are widely used as colouring agents in nutraceuticals, pharmaceuticals, cosmetics, and foods. The high demand for β-carotene led to the development of its synthetic alternative.

Natural β-carotene consists of two isomers: all-trans and 9-cis and is present only in natural environments (Gonzalez, 2005) whereas synthetic β-carotene contains only the all-trans isomer, which has lower liposolubility and lower antioxidant property than 9-cis isomer. *Dunaliella salina* is the best commercial source for natural β-carotene in the world (Borowitzka, 1995). This alga accumulates large amounts of β-carotene up to 14% of its dry weight in the presence of cofactors such as high light intensity, increased temperature, high salinity and nutrient deficiency (Ben-Amotz and Avron, 1983; Shaish and Ben-Amotz, 1992). In recent years, it is mainly cultivated for extraction of carotenoids. *Dunaliella salina* under ideal conditions can yield ~400 mg β-carotene/m² of cultivation area (Finney *et al.*, 1984). The first pilot plant for *Dunaliella* cultivation for β-carotene production was established in USSR in the year 1966.

Carotenoids have beneficial roles as dietaries in cataract and also in age-related macular degeneration. Epidemiological evidences have shown that β-carotene can prevent cancer of various organs like lungs, stomach, cervix, pancreas, colon, rectum, breast, prostate and ovary by means of its antioxidant activity (Poppel and Goldbohm, 1995). Other than antioxidant property, it influences intracellular communication (Sies and Stahl, 1997), immune responses (Hughes *et al.*, 1997), neoplastic transformation and control of growth (Bertram and Bortkiewicz, 1995). Further, the ketones, aldehydes and epoxides of the algae can influence the biochemical pathways (William *et al.*, 2000; Yeum and Russel, 2002).

Ambiguity always exists in the identification of *Dunaliella* when morphological characteristics

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(Massyuk, 1965) or physiological characteristics alone are considered since the alga is known to exhibit diverse morphological and physiological characteristics depending on the conditions of growth. Thus molecular identification is a useful tool to distinguish between inter- and intra-specific species with similar morphology (Olmos *et al.*, 2000; 2002) and mixed populations as well (Olsen *et al.*, 1986). Recently, the phylogenetic species concept was used in the classification of the genus *Dunaliella* by molecular analysis of the nuclear rDNA internal transcribe spacer region ITS1 and ITS2 (Coleman, 1997, 1998; Gonzalez *et al.*, 1999).

Materials and Methods

Microalgae isolation, identification and growth condition

Salt pan water sample were collected in sterile plastic vials from Vedaranyam of Tamil Nadu, South India for the isolation and screened for *Dunaliella* under compound microscope. The sample contained *Dunaliella* was serially diluted up to 10^{-4} and 0.1 mL spread on 2 % De Walne's agar medium. Distinct colonies developed on the plate was made axenic (Droop, 1967) and transferred to De Walne's medium (Orset and Young, 1999) and kept at 24 ± 1 °C in thermostatically controlled room, illuminated with cool fluorescent lamps at irradiance of $30 \mu\text{Em}^{-2}\text{s}^{-1}$, under 12 h/12 h light/dark photo period. Identification of the isolated microalgae was done using both microscopic observations as well as comparing the 18S rDNA sequence region of the isolate (submitted to Gen bank, NCBI with the accession number (EU5891200) to that stocked in Algal Culture Collection, CAS in Botany, University of Madras. Amplification of the 18S rDNA region and sequencing PCR amplification was performed on cells taken directly from plates. For PCR, The genomic DNA of the *D. salina* was isolated according to Sambrook *et al.* (1989), subjected to amplification with the oligonucleotides, MA1 [5' CGG GAT CCG TAG TCA TAT GCT TGT CTC 3'] MA3 [5' GGA ATT CCG GAA ACC TTG TTA CGAC 3'] was performed as described by Olmos *et al.* (2000). The amplified product of the 18S rDNA was partially sequenced using Applied Bio system Instrument (ABI) Prism 310 Genetic. Analytical grade chemicals were used for this purpose.

Optimization parameters and growth conditions for total carotenoids synthesis

Dunaliella salina EU5891200 grown at different initial pH (5.0 to 12.0). Light intensities (2, 4, 10, 20, 30 (control) and $40 \mu\text{Em}^{-2} \text{s}^{-1}$). Different concentrations of NaNO_3 (0.1, 0.25, 0.5, 0.8, 1.0, 1.18 (control), 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 and 10 mM), NaH_2PO_4 (0.13 (control), 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50) and NaCl (0.5, 1.0, 1.5, 2.14 (control), 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 M) amended in the De Walne's

medium with out the respective ingredients were sterilized. Ten mL of optimally grown cultures of *Dunaliella* were inoculated in 90 mL of basal De Walne's medium and kept under the laboratory conditions. This experiment was conducted for a period of 30 days. At every 3 days interval the following parameters viz., i) Cell number (Neubauer haemocytometer) (Cn) and ii) concentrations of pigments viz., Chlorophyll a (Chl a), Chlorophyll b (Chl b) and Total carotenoids (Tc) ($\mu\text{g/mL}$) were recorded (Lichtenthaler, 1987). In addition, division rates were calculated during the exponential phase (Guillard, 1973). Growth curves were plotted against days and \log_{10} of cell number. All the experiments were carried out in triplicates; the mean values are represented in the graph.

Results

Identification of the isolate was confirmed by comparing the sequence of the 18S rDNA region of the isolate with that of other sequences available from the Genebank database. The sequence of the isolate was 99% identical to the consensus sequence of *D. salina* (accession number EF473749). The microalga was deposited at the Algal Culture Collection, CAS in Botany, University of Madras.

Optimization of total carotenoids production

Effect of different initial pH

Dunaliella salina survived in all the different initial pH chosen for growth in the De Walne's medium. Maximum growth rates of 5.847 \log_{10} of cell numbers/mL were recorded in *D. salina* at pH 9.5 on 24th day (Fig.1). The increase in cell numbers was 21% more than that of control (pH 8.0). Maximum concentrations of Chl a i.e. $6.395 \mu\text{g/mL}$ and Chl b i.e. $4.8 \mu\text{g/mL}$ were recorded at pH 8.0 (control) and pH 7.5, respectively, on 21st day while maximum total carotenoids of $78.23 \mu\text{g/mL}$ was recorded at pH 8.0 (control) on 30th day (Figs. 2, 3, 4).

Fig. 1: Growth curve of *D. salina* at different initial pH

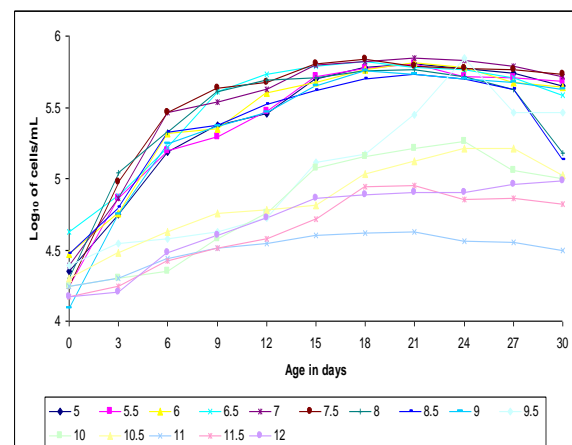


Fig. 2: Chl a content of *D. salina* at different initial pH

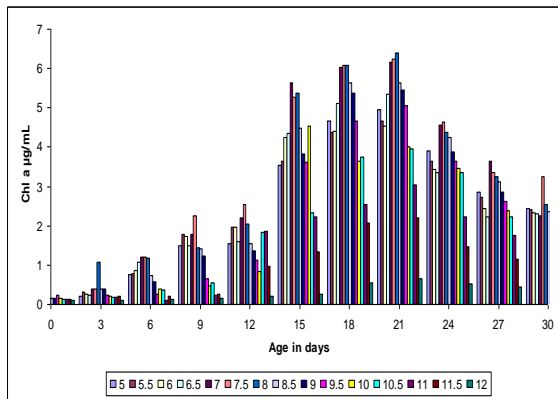


Fig.3 Chl b content of *D. salina* at different initial pH

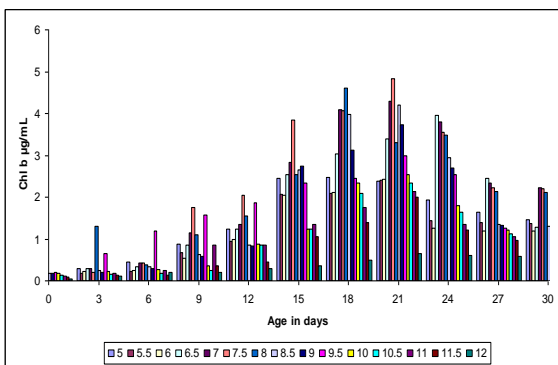
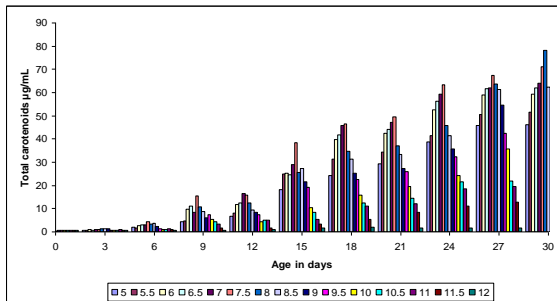


Fig. 4: Total carotenoids content of *D. salina* at different initial pH



Effect of different light intensities

Dunaliella kept responded to different light intensities in the following manner. *D. salina* showed maximum growth with 6.195 log₁₀ of cell number/mL at 40 µEm⁻² s⁻¹, on 24th day which was 35% more than that of control (Fig. 5). Maximum concentration of Chl a i.e. 8.23 µg/mL was observed in cells grown at a light intensity of 40 µEm⁻² s⁻¹ while the maximum level of Chl b (8.55 µg/mL) on 12th and 15th day, respectively. Total Carotenoids registered a maximum amount of 98.6 µg/mL on 30th day at 40 µEm⁻² s⁻¹ which was nearly 40% more than that of control (30 µEm⁻² s⁻¹) (Figs.6, 7 and 8).

Fig. 5: Growth curve of *D. salina* under different light intensities

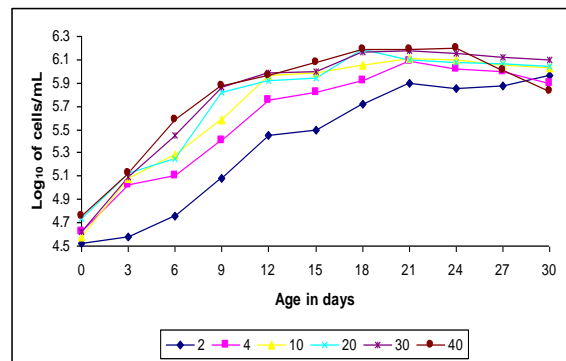


Fig. 6: Chl a content of *D. salina* under different light intensities

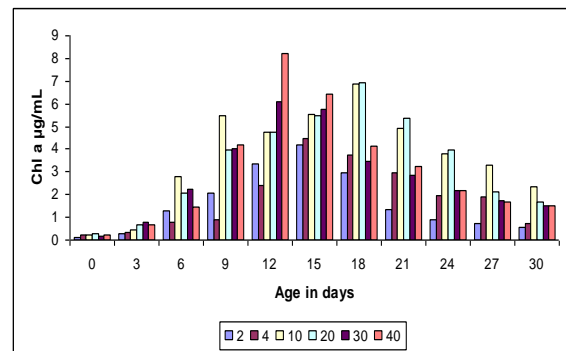


Fig. 7: Chl b content of *D. salina* under different light intensities

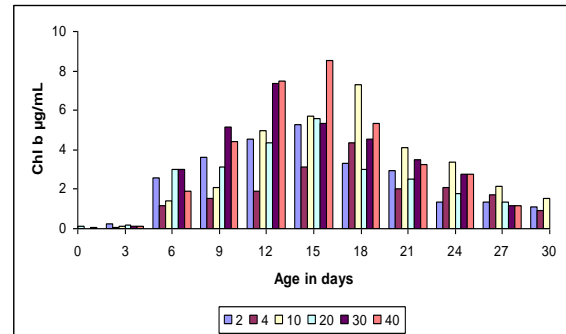
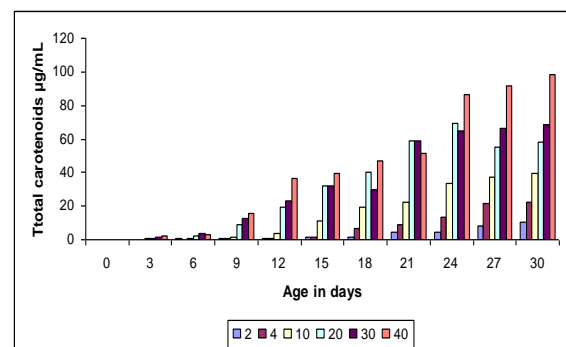


Fig. 8: Total carotenoids content of *D. salina* under different light intensities



Effect of different concentrations of NaNO₃

Dunaliella salina showed a maximum of 6.891 log₁₀ of cell number/mL at 4.5 mM NaNO₃ on 30th day (Fig. 9). The increment in growth by cell number for the isolate was more than 87% to that of control. Maximum concentrations of Chl a (14.7 µg/mL) and Chl b (11.241 µg/mL) were recorded in *Dunaliella salina* MUAP 302 at 10 mM NaNO₃ on 30th day. The increments of the above pigments were more than 100% and 115% when compared to control, respectively. Maximum amount of total carotenoids i.e. 78.5 µg/mL for the isolate was recorded on 30th day at 1 mM NaNO₃ and the observed level was more than 20% to that of control (1.18 mM NaNO₃) (Figs. 10,11 and12).

Fig. 9: Growth curve of *D. salina* at different concentrations of NaNO₃ (mM)

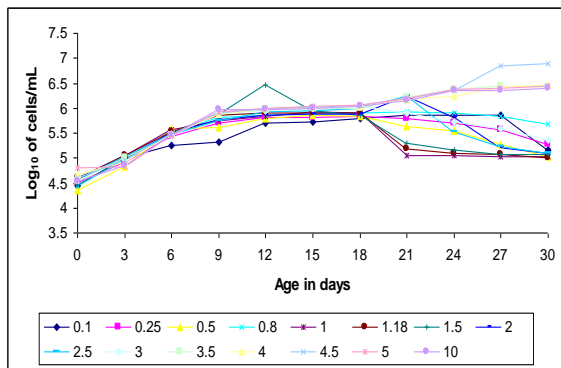


Fig. 10: Chl a content of *D. salina* at different concentrations of NaNO₃ (mM)

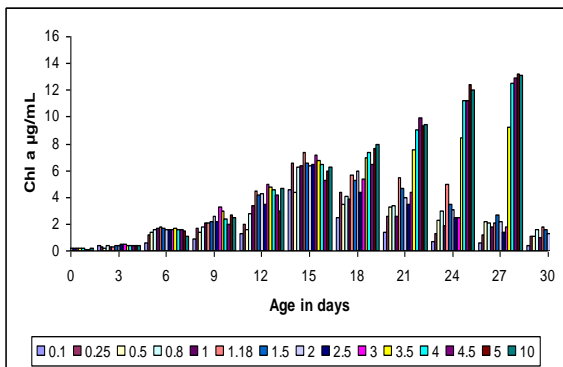


Fig. 11: Chl b content of *D. salina* at different at different concentrations of NaNO₃ (mM)

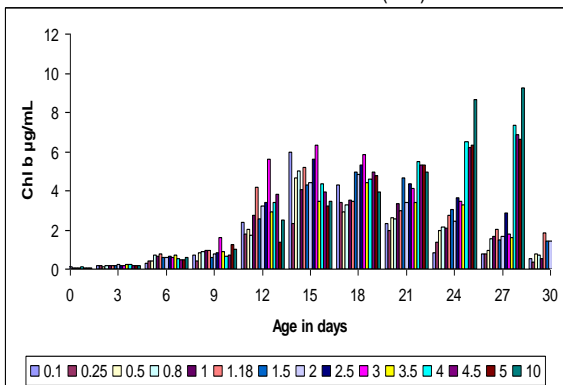
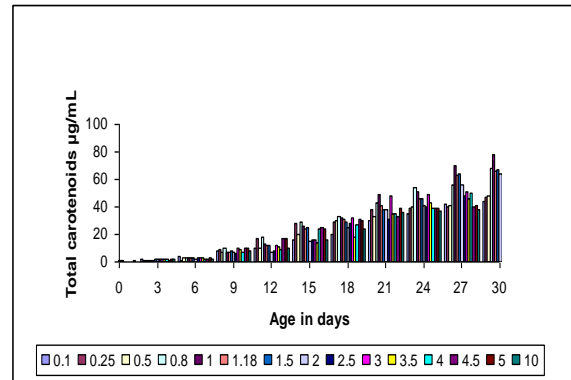


Fig. 12: Total carotenoids content of *D. salina* at different concentrations of NaNO₃ (mM)



Effect of different concentrations of NaH₂PO₄

The two isolates of *Dunaliella salina* grew well in the medium amended with different concentrations of NaH₂PO₄. The cell number in the presence of added phosphate increased steadily up to 24th day. The maximum number of cells recorded in *D. salina* was 7.154 log₁₀ of cell number/mL on 30th day at 1.0 mM NaH₂PO₄. The increments in cell number of *D. salina* 410% more when compared to control (Fig.13). Maximum concentrations of Chl a and Chl b of 7.154 µg/mL and 8.365 µg/mL were recorded in *Dunaliella salina* at 1mM NaH₂PO₄ on 30th and 18th day, respectively. The concentrations of Chl a and Chl b were more than 8.0%, 75.0% to that of control. The total carotenoids in the isolate at 0.45 mM NaH₂PO₄ on 30th day i.e. 120.0 µg/mL was the maximum and was 90% more than that of control (Figs. 14, 15 and 16).

Fig. 13: Growth curve of *D. salina* at different concentrations of NaH₂PO₄ (mM)

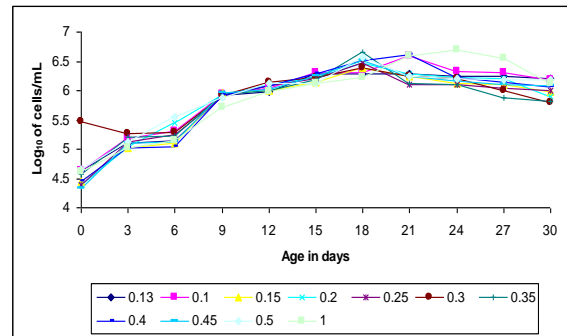


Fig. 14: Chl a content of *D. salina* at different concentrations of NaH₂PO₄ (mM)

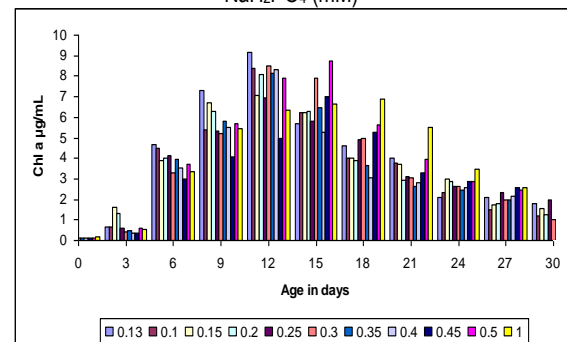


Fig. 15: Chl b content of *D. salina* at different concentrations of NaH₂PO₄ (mM)

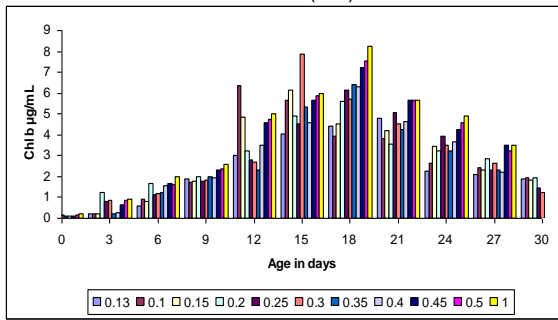


Fig. 16: Total carotenoids content of *D. salina* at different concentrations of NaH₂PO₄ (mM)

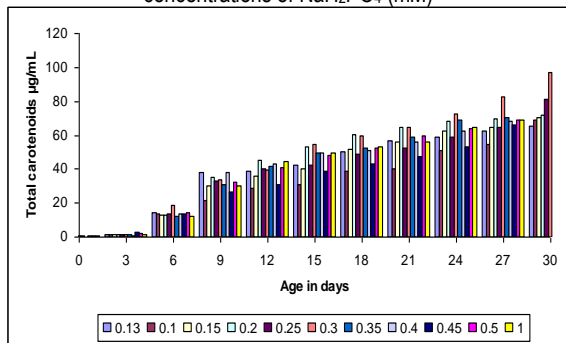


Fig. 18: Chl a content of *D. salina* at different concentrations of NaCl (M)

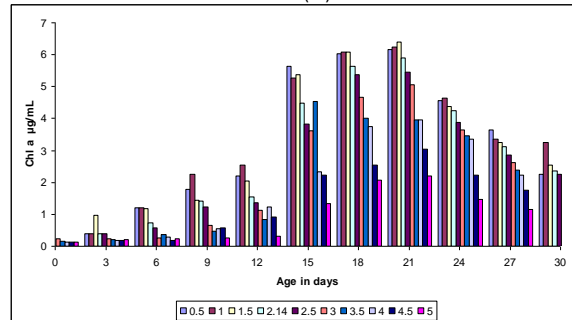
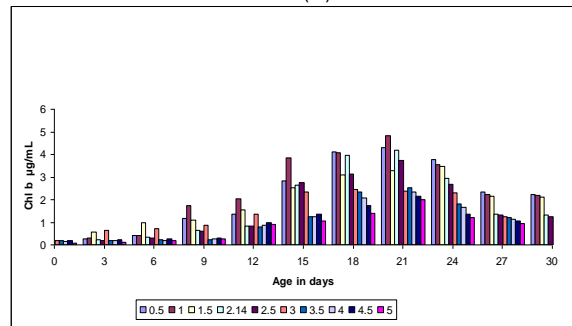


Fig. 19: Chl b content of *D. salina* at different concentrations of NaCl (M)



Effect of different concentrations of NaCl

The two isolates of *Dunaliella* survived in basal medium amended with all the different concentrations of NaCl tried. The algal cell numbers increased steadily up to 24th day, a maximum of 6.075 log₁₀ of cell number/mL at 1.0 M NaCl was registered in an isolate. The increments in cell numbers of *D. salina* were more than 225% when compared to the control (2.14 M) (Fig. 17). *Dunaliella salina* grown at 1.5 M NaCl contained maximum concentrations of Chl a (6.395 µg/mL) and Chl b (4.821 µg/mL) on the 21st day and the observed quantities of the two pigments were more than 10% and 15%, respectively, to that of control. Total carotenoids in the isolate registered a maximum of 86.5 µg/mL at 1.0 M NaCl on 30th day which was more than 40% to that of control (2.14 M NaCl) (Figs. 18,19 and 20).

Fig. 17. Growth curve of *D. salina* at different concentrations of NaCl (M)

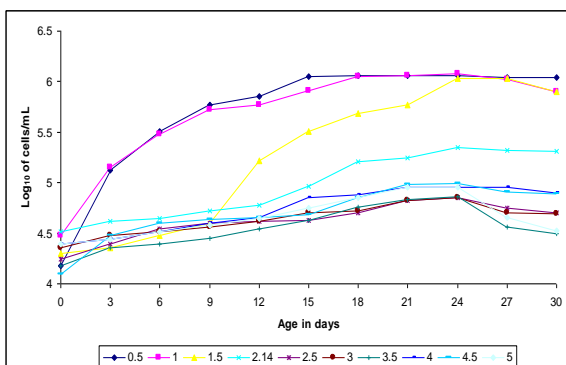
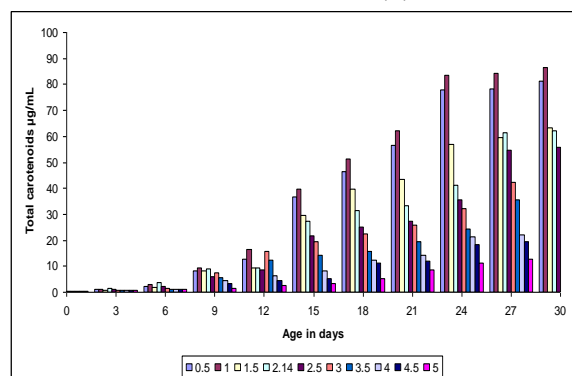


Fig. 20: Total carotenoids content of *D. salina* at different concentrations of NaCl (M)



Discussion

An attempt was made in the present study to enhance the production of β-carotene in the isolate *D. salina* since they accumulate high amounts of β-carotene. Species of *Dunaliella* have been reported to occur in environment with a wide range of pH. For example, *D. acidophila* isolated from Czech Republic was grown optimally at pH 0.5 – 2.0 (Kalina, 1965; Albertano et al., 1981; Gimmler and Weis, 1992). Shariati (2003) reported that the optimum pH for *D. salina*, *D. pseudosalina* and *D. parva* isolated from Iran was between 7.0 and 8.0. In the present study, *D. salina* preferred a pH of 9.5, respectively for their maximum growth by cell numbers whereas the accumulation of total carotenoids in both isolates was

optimal at pH 8.0. The isolate grown at pH 10 showed poor growth and less accumulation of total carotenoids but survived in all the different initial pH chosen for growth in the De Walne's medium.

Light is the main factor controlling the rate of synthesis of β -carotene in *Dunaliella* under favorable growth conditions (Harding and Shorshire, 1980; Ben Amotz, 1987). The cells of *Dunaliella* swell in light and shrink in dark thus, showing a variation in the surface area contributing to photosynthesis (Riisgard, 1981). A high ratio of β -Carotene: chlorophyll indicating the accumulation β -Carotene coupled with Chlorophyll depletion occurred in *D. bardawil* when exposed to high light intensity (Ben-Amotz and Avron, 1983).

In the present study, the cell numbers, and concentrations of Chl a and Chl b of isolate was found to be high under a light intensity of $40 \mu\text{Em}^{-2} \text{s}^{-1}$ when compared to lower light intensities such as $2 \mu\text{Em}^{-2}\text{s}^{-1}$, $4 \mu\text{Em}^{-2}\text{s}^{-1}$, $10 \mu\text{Em}^{-2}\text{s}^{-1}$ and $20 \mu\text{Em}^{-2}\text{s}^{-1}$. However, over a period of growth, normally after 21 days, the carotenoid levels increased while the levels of Chl a and Chl b decreased. Therefore, it is confirmed that exposure of *Dunaliella* isolates to high light intensity over a period of time induced the total carotenoid synthesis. It has been shown that a change in the amount of light per amount of cells would influence the synthesis of carotenoids in cells (Hejazi and Wijffels, 2003). The cells which receive more light produce more β -Carotene and a specific relationship is known to exist between β -Carotene content of cells and volumetric β -Carotene production (Ben-Amotz et al., 1993).

The best source of nitrogen for maximum growth of *D. salina*, *D. tertiolecta* and *D. viridis* was NaNO_3 (Mil'ko, 1962). The ratio of accessory photoprotective pigments (α and β -carotene) to chlorophyll normally increases under nitrogen limiting conditions (Geider et al., 1998). Likewise in the present study, NaNO_3 was used as a nitrogen source and its effect on the isolate are as follows. The growth in terms of cell numbers was maximum for the two isolates of *Dunaliella* at high concentrations of NaNO_3 (10.0 mM). Levels of the pigments, Chl a and Chl b also increased at the above concentration of N. Surprisingly, the growth and concentration of Chl a increased up to 2 folds as compared to other parameters considered in this investigation. But the accumulation of total carotenoids was maximum at lower concentrations of N i.e. 1.0 mM NaNO_3 for *D. salina*. Mojaat et al. (2008) also have recorded a similar observation on the accumulation of carotenoids at low levels of N. Mojaat et al. (2008) observed the accumulation of high quantities of β -carotene (35 pg/cell) in the nitrate-free culture. These values drastically dropped to 24 and 12 pg/cell, at the initial nitrate concentration of 1.2 and 6 mM, respectively. High levels of N in the medium would place a demand on carbon skeletons or

photosynthates for assimilation of the reduced nitrogen and this could result in a competition for carbon between carotenoid synthesis and amino acid biosynthesis. This may be the reason for reduced levels of carotenoids at high levels of N in the medium and for the accumulation of huge quantities of carotenoids under N-free conditions.

Phosphate is an essential macronutrient for all living organisms. Besides being a structural element in nucleic acids and phospholipids it plays crucial roles in various biological functions such as energy transformations, activation of metabolic intermediates, signal transduction cascades and regulation of enzymes (Daram et al., 1998). Phosphate plays an important role in cell energetics through high-energy phosphodiester bonds (ATP, sugar-phosphates) and in intracellular signaling (phosphorylation and dephosphorylation of proteins) (Geider et al., 1998).

Dunaliella blooms occur in the Dead Sea only during unusually wet winters when the upper water layers of the lake become sufficiently diluted and phosphate, the limiting nutrient, is available (Oren et al., 2005). The optimum phosphate concentration for the growth of *D. salina* and *D. viridis* was 0.002 - 0.025 gL^{-1} of K_2HPO_4 (Gibor, 1956; Mil'ko, 1962) and high concentrations ($>5\text{gL}^{-1}$) inhibited their growth. Replacement of phosphate by glycerophosphate significantly reduced the growth of *D. tertiolecta*. The ratio of accessory phosphoprotective pigments (α and β -carotene) to Chlorophyll a increased under limiting levels of nitrogen (Geider et al., 1998) and phosphate (Krom and Brenner, 1991).

The investigation made on the effect of NaH_2PO_4 on *D. salina* showed that their cell numbers and levels of total carotenoids attained maximum on 30th day when they were grown in basal medium amended with NaH_2PO_4 at 0.45 mM concentration. However, the concentrations of Chl a, Chl b, were at their maximum at 1.0 mM NaH_2PO_4 . Phosphate as NaH_2PO_4 greatly influenced the synthesis of total carotenoids in the two strains of *Dunaliella* than the other parameters such as pH, Light intensity, NaCl, and NaNO_3 , considered in this investigation.

Salinity also plays a major role in the accumulation of massive amounts of β -carotene in *Dunaliella*. It is the most widely studied model alga for its remarkable ability to adapt to a wide range of salt concentrations (Cowan et al., 1992; Giordano et al., 2000). *D. salina* survived in all the NaCl concentrations chosen for growth (0.5 M – 5.0 M). Fazeli et al. (2006) observed highest amount of total carotenoids (11.72 mg/L) in the stationary phase of the culture containing 0.5 M NaCl, which was about 4.8 times more than that in the exponential growth phase. They also found that productivity of total carotenoids on cellular basis to be very high at extreme salt concentrations (3.0 M NaCl).

However, Brock (1975) noted that natural populations of *Dunaliella* from the saturated brine to show optimum growth at salinity levels lower than its habitat. In general, most of the workers conducted experiments on increasing levels of salinities while the present study considered lower as well as higher range of salinities to find out its effect on the productivity of the two isolates. Cell numbers, Chl a, Chl b and the total carotenoids were maximum at the lower concentrations of NaCl ranging between 0.5 M and 1.5 M. *D. salina* grown at 1.0 M NaCl had maximum total carotenoids that amounted 86.370 µg/mL, on 30th day. This revealed the preferential salinity regime of the isolates for enhanced production of carotenoids.

It has been already pointed out that the accumulation of β-carotene in *Dunaliella* depends upon the factors which include high light intensity (Massyuk and Radchenko, 1970; Semenko and Abdullayev, 1980), extreme temperatures (Henley et al., 2002), high salinity (Evans et al., 1982; Mortain-Bertrand et al., 1994; Henley et al., 2002) and deprivation of mineral nutrients including nitrate, sulphate and possibly phosphate (Ben-Amotz et al., 1987; Borowitzka and Borowitzka, 1989; Vorst et al., 1994; Shelly et al., 2002). In the present attempt, enrichment of nutrients, enhanced the growth by cell number and concentrations of Chl a and Chl b on *Dunaliella* isolates. Nutrients are generally depleted due to utilization and assimilation by algae. Maximum accumulation of β-carotene was recorded on 30th day indicated that the decrements of nutrients enhanced the high accumulation of the pigments. Among the parameters chosen in the present attempt phosphate greatly influenced for maximum production of β-carotene content than other parameters. This study evidently reported that the isolate *D. salina* (EU5891200) was the richest β-carotene producing alga.

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