Proc. Indian Acad. Sci. (Plant Sci.), Vol. 99, No. 4, August 1989, pp. 319–325.

Effect of environment on pigment ratios in *Dunaliella* spp. from the salt pans of Gujarat

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MS received 31 October 1988

Abstract. The effect of varying salinity and pH of the medium on growth and pigment ratios of *Dunaliella* cells in culture was studied. Cell growth was found to be optimal at pH 8 and at intermediate salt concentrations of 1–2 M NaCl. Ninety per cent acetone extracts of cells grown in media containing up to 2 M NaCl showed two distinct absorption peaks with maxima at 433 and 663 nm due to carotenoids and chlorophylls. The ratio of carotene to chlorophyll appeared to be maximum for cells grown in 1 M NaCl and to increase with the age of the cells in culture.

Keywords. *Dunaliella*; salt pans; pigment ratios; chlorophyll; β-carotene.

1. Introduction

The unicellular phytoplankton alga *Dunaliella* (Chlorophyta, Volvocales) is widely distributed all over the world, in water masses containing sodium chloride at almost any concentration above zero (Ginzburg and Ginzburg 1985). Both in nature as well as under some laboratory conditions, certain species of this flagellate have been known to possess the unique ability to accumulate large amounts of β-carotene (Ben-Amotz and Avron 1983a). Although there is no agreement in the literature as regards the factors controlling β-carotene synthesis in *Dunaliella*, it has been shown that certain combinations of environmental stress conditions such as high salt concentration, extremes of temperature or pH, nutritional deficiency and high light intensity do cause β-carotene to accumulate within the cell (Ben-Amotz and Avron 1983a).

Studies have been carried out on species of *Dunaliella* obtained from waters of such varied compositions and geographical locations as the Dead sea, the English channel and the Great salt lake in Utah. The organism owes its halotolerance to the unique ability of the cell to photosynthetically produce high concentrations of intracellular glycerol and thereby maintain an osmotic balance with the extracellular salt concentration (Ben-Amotz *et al* 1982b). The feasibility of commercial cultivation of the alga is already being explored, with a view to economically produce the valuable products glycerol and β-carotene and the remaining high protein dry algal meal (Ben-Amotz and Avron 1980; Ben-Amotz and Avron 1983b).

There is very little literature available on studies on *Dunaliella* of Indian origin (Rao *et al* 1982). The present investigation reports preliminary studies in the laboratory on growth conditions and the possible production of β-carotene from *Dunaliella* spp. occurring in salt pans along the coast of Gujarat (W. India).

2. Materials and methods

Cells of *Dunaliella* spp. collected from the salt pans of Jamnagar, Gujarat, India were

grown in 300 ml of medium in 500 ml Erlenmeyer flasks. Unless otherwise stated, the growth medium consisted of natural seawater enriched as given by Parsons *et al* (1984). Desired salinity levels in the media were attained by addition of pure NaCl (analytical grade reagent, BDH). The cultures were grown at about 30°C and were illuminated with fluorescent lamps providing cool, white light for 8 h during the day. The cultures were unialgal and although the media used were autoclaved beforehand, no special precautions were taken to deter bacterial growth, for it is believed that the very composition of the medium would be unfavourable for the growth of most of the likely bacterial contaminants (Ginzburg and Ginzburg 1981).

Cells were counted with a model T A II Coulter counter using a 140 μ aperture tube. Sample volumes of 0·5–1 ml in a volume of 200–250 ml electrolyte (filtered seawater) were sufficient to produce counts in the appropriate range. For each determination, 2 ml of the suspension was drawn into the aperture tube. At least 4 determinations were performed on each sample and the mean value calculated. Background counts in the electrolyte medium were estimated prior to addition of every aliquot of the sample and then subtracted from the sample counts in each case.

Absorbance studies were performed on a Beckman DU-6 scanning spectrophotometer. Acetone extracts of samples were prepared as follows: 25 ml of culture was filtered through a Whatman GF/C filter paper which was then left overnight at 5°C in 10 ml of 90% acetone taken in blackened air-tight glass tubes. The paper was crushed and centrifuged down and the clear supernatant scanned for absorbance in the visible region.

3. Results

3.1 *Growth studies*

Dunaliella cells were grown in the standard culture medium containing sodium chloride at concentrations ranging from that of normal seawater (ca 0·5 M NaCl) to 4 M. In one set of experiments the initial pH of the medium was adjusted to 8 and in a second set to 9·5. At different time points of growth, aliquots were drawn from each tube, the number of cells estimated using the Coulter counter and growth curves plotted. A representative picture of the growth patterns of *Dunaliella* cells at various concentrations of NaCl at pH 8 is given in figure 1. The initial lag phase averaged 7 days in most of the cases. At a concentration of 2 M NaCl, the exponential phase appeared slightly earlier and the progress was comparatively rapid. At 1 M NaCl, although the growth rate was less, the final yield was higher than at 2 M. At both higher and lower concentrations of NaCl the growth curves consisted of an extended phase of slow growth followed by a faster one. The mean doubling time values (recorded during exponential growth) have been plotted against salt concentration and compared with the results from the second set of cultures maintained at an initial pH of 9·5 (figure 2). In both cases it was noticed that the doubling times were much lower in the concentration range of 1–3 M NaCl and that they exhibited large increases at the two extremes. The shortest doubling time observed was 37 h for cells grown at 2 M NaCl and pH 8.

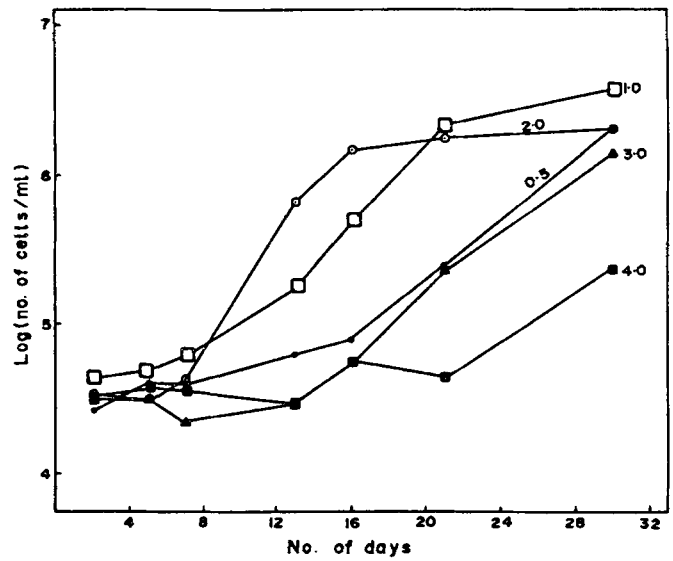

Figure 1. Growth curves of *Dunaliella* isolates at pH 8. The molar concentrations of NaCl in the growth medium are as indicated on each curve.

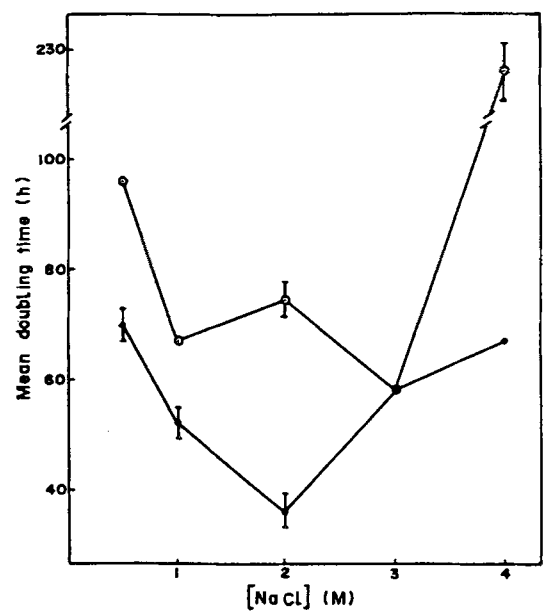

Figure 2. Doubling time of *Dunaliella* cells as a function of NaCl concentration, at initial pH of 8 (●) and 9·5 (○). The points indicate mean ± SD from 4 determinations.

3.2 *Spectral studies*

Cells grown in a medium at an initial pH of 8 and over a range of NaCl concentrations from 0·5–6 M were extracted with 90% acetone at different time points of

their growth. Absorption spectra of the acetone extracts were recorded against a blank of 90% acetone. For cells grown in NaCl concentrations from 0·5–2 M, two distinct peaks were observed at 433 nm (peak A) and 663 nm (peak B) whereas no specific peaks could be detected for higher salt concentrations (>2 M), probably because of the poor growth at the time points tested. Typical absorption spectra of acetone extracts of cells at two salt concentrations are given in figure 3. It was found that with an increase in the salt content of the medium, a shoulder appeared in association with peak A at about 455 nm, which was most pronounced at 2 M NaCl.

For any given time period during the exponential phase of growth, the increase in area under peak A was always greater than that under peak B for each population of cells. The ratios of the areas under peaks A and B (denoted as S_A/S_B) were calculated for cells at two different stages of growth in various salt concentrations and compared (figure 4). The ratios increased with the age of the cells in culture. For both the time points studied, the highest value of S_A/S_B was obtained for cells grown in 1 M NaCl, closely followed by those in 2 M.

4. Discussion

Under the conditions of growth in the present study, NaCl concentrations of 1–2 M

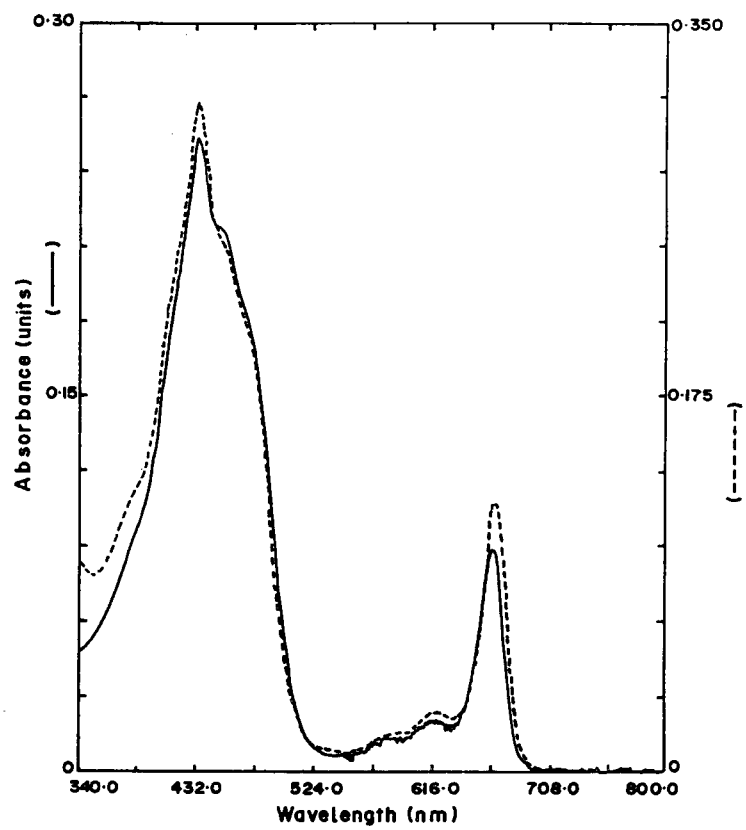

Figure 3. Absorption spectra of 90% acetone extracts of *Dunaliella* cells grown for 13 days in media containing NaCl at concentrations of 0·5 M (----) and 2 M (———).

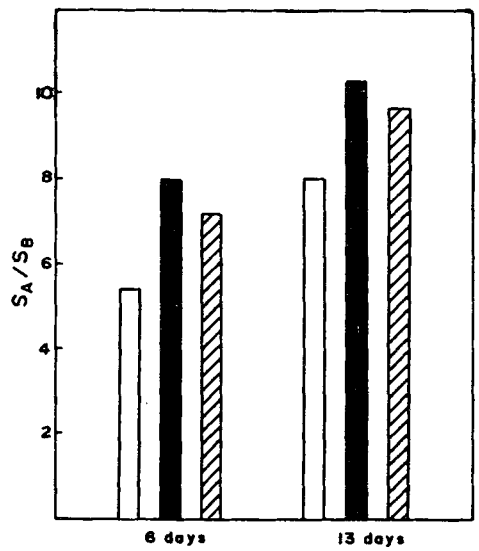

Figure 4. Ratio of areas covered by spectral peaks A and B of 90% acetone extracts of *Dunaliella* cells grown for 6 and 13 days in media containing NaCl at concentrations of 0·5 M (□), 1 M (■) and 2 M (▨).

were found to be optimum for growth of *Dunaliella*. Ginzburg and Ginzburg (1985) have also observed maximal yield at some intermediate concentrations of NaCl, for most of the *Dunaliella* isolates studied by them. In fact, they have reported that the most usual response of *Dunaliella* sp. to NaCl concentration is that the doubling time has a minimum value throughout a wide concentration range, with large increases at the two extremes. The results of growth studies on the species of *Dunaliella* used in the present study also follow a very similar pattern (figure 2). The growth response of halophilic and halotolerant cells to salt has been discussed at length (Ginzburg and Ginzburg 1985) but there have been no clear explanations to date as to why growth is optimal at intermediate salt concentrations.

The present investigation reports the preference of *Dunaliella* spp. to pH 8 than pH 9·5, for optimal growth. Rao *et al* (1982) have studied the effect of pH 6, 7 and 8 on the growth of *Dunaliella* sp. isolated from salt farm brines and a solar pond at Bhavnagar and found that while growth was optimum at pH 8, it was quite high at pH 6 and 7 also.

The results of the present study show the presence of a shoulder at around 455 nm (figure 3) for 90% acetone extracts of cells grown in 2 M NaCl, in addition to the major peak (A) at 433 nm. It is known that certain strains of *Dunaliella* tend to turn from green to red at higher salinity levels due to the accumulation of β-carotene (Loeblich 1982). Electron micrographs of *D. bardawil* cultured under optimal conditions have shown the presence of many β-carotene containing globules located in the interthylakoid space of the chloroplast (Ben-Amotz *et al* 1982a). The absorption spectrum of an 80% acetone extract of purified β-carotene globules from *D. bardawil* has been shown to have characteristic maxima at 452 and 478 nm (Ben-Amotz *et al* 1982a). A comparison of the spectra of acetone extracts of green and red cells indicates the appearance of a well-defined shoulder at 478 nm in the latter case (Ben-Amotz and Avron 1980). Thus the spectral pattern observed in

the present study agrees remarkably well with reports on known strains of *Dunaliella*. The blue shift of the entire spectrum in our studies as compared to earlier reported data could well be due to differences in the extraction procedures and solvents used for spectroscopic examination (Davies 1965). Besides, the ratio of different stereoisomers of β-carotene present in the sample could also contribute to spectral shifts (Weedon 1965).

While it cannot be disputed that peak B is due to the chlorophylls it has always been difficult to establish the exact location of the carotenoid bands *in vivo* because of their strong overlapping with the blue-violet bands of chlorophylls (Govindjee and Brown 1974). Nevertheless, the variations in the ratio of the area under peak A (expected position of β-carotene) to that under peak B, with growth of cells and with the salinity of the medium (figure 4) are interesting enough to merit a more thorough investigation. While it may be generalized that β-carotene accumulation is best in cells grown under high light intensity in media containing limiting nitrogen, high salt concentration or extreme growth temperatures, it must be pointed out that growth conditions which permit maximal specific growth rate of cell biomass reduce the β-carotene level in the alga (Ben-Amotz *et al* 1982a).

There have been several recent reports of experiments conducted to test the feasibility of large-scale outdoor cultivation and harvesting of *Dunaliella* rich in glycerol and β-carotene. It may be noted that pilot plants have already been constructed in Israel and Australia for this purpose (Ben-Amotz and Avron 1983b). The success of such ventures would pave the way for further studies on other biological products exploitable from the algal biomass. There is also the possibility of making good use of other microorganisms that may coexist in the same ecosystem. It is certain that locally available forms of the genus *Dunaliella* have almost if not the same potential for economically feasible large-scale cultivation and extraction of medicinally important compounds as those reported from other regions of the globe and it is hoped that the data from the present studies would form the basis of such ventures.

Acknowledgement

Financial assistance to one of the authors (UDM) from the Department of Ocean Development, New Delhi is gratefully acknowledged.

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