Research Article

Preliminary report on β-carotene production in the halotolerant microalga *Dunaliella bardawil*

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Summary: *Algae of the genus* Dunaliella *are among the most widely studied for mass culture and for use as a source of food and nutraceuticals. In this paper we review the information available on this important microalga, together with some of the results we have obtained in our laboratory.*

Keywords: β-carotene, *Dunaliella bardawil,* microalga

Introduction

The use of *Dunaliella* and other microalgae for biotechnology purposes is not new. Countries with hot, dry summers and mild winters such as Australia and Israel have long been exploiting the organism as a source of the pigment β-carotene. The Molecular Biotechnology section of the Laboratory of Molecular Genetics at the University of Malta has been involved in studies of this microorganism since 1998. In this preliminary report we summarise some of the key aspects of the biotechnological use of *Dunaliella.*

Dunaliella is a unicellular bi-flagellate green alga of the class Chlorophyceae. It inhabits a wide variety of habitats including fresh, euryhaline and hypersaline waters as well as saline soils. In most of these habitats, the organism is a fairly minor component but in brines two species, *Dunaliella salina* (Dunal) Teodoresco and *Dunaliella bardawil,* predominate. Both organisms show interesting and, in certain instances, unique mechanisms to withstand the salt fluctuations, dessication and intense solar radiation encountered in their ecological niche. Not surprisingly, these strains have been described as the dominant species in the hypersaline brines of the Dead Sea, Israel (Volcani, 1944), the Great Salt Lake, Utah (Brock, 1975) and the Pink Lake in Western Australia (Borowitzka and Borowitzka, 1988). Both strains are known to tolerate salt fluctuations between 3.5% and 35% (Borowitzka and Borowitzka, 1988), although in our laboratory tolerance to salinities as low as 0.6% was regularly recorded. This makes them one of the most halotolerant organisms known.

For many years, there was strong debate about whether *D. salina* (Dunal) Teodoresco and *D. bardawil* were in fact two different species. Using the Polymerase Chain Reaction (PCR) and Restriction Fragment Length Polymorphism (RFLP) analysis of DNA coding for the 18SrRNA, Olmos and co-workers, (2000) were able to demonstrate clearly that these are in fact two separate species.

Conditions in Malta, especially the hot dry summers, would be expected to provide ideal conditions for this organism to flourish. However, random samples taken from the Salina saltern in the summer of 1997 failed to yield any specimens, although a number of interesting halophiles such as the multicellular blue-green algae *Spirulina,* which is widely used as a dietary supplement, were identified. Local records of *Dunaliella salina* are in fact limited to a single sighting in a rock pool at Manoel Island (Lanfranco, 1974). The increase in the use of this area for recreational purposes, however, has probably contributed to the disappearance of the microalga from the site since then. To the best of our knowledge there have been no other recorded sightings since. As a result, stocks of the microalga had to be imported for this work. The strain used in our laboratory was *D. bardawil* and was purchased from the American Type Culture Collection (ATCC).

Uses of β-carotene

Dunaliella is best known as a source of the orange pigment β-carotene. The pigment is in wide use in the nutraceutical and pharmaceutical industries as a natural colourant and as a vitamin supplement in its own right (Mirasol, 1998). The world market for both the natural and the synthetic forms is worth in excess of 180 million dollars and is growing at a fast rate.

The uses of β-carotene in the human body are various. It is the precursor of vitamin A and it is important for cellular differentiation, vision, bone growth, erythropoeisis and the integrity of the immune system. It can improve the absorption of non-heme iron from rice, wheat and corn (Garcia-Casal *et al,* 1998). Epidemiological and oncological studies also indicate that normal to high levels of dietary β-carotene may protect against atherosclerosis (Tornwall *et al,* 2000) and may lower the risk for a number of cancers

including colo-rectal, adenocarcinoma, gastric and cardiac cancers (Ekstrom *et al,* 2000). Lung cancer is an exception, as raised levels of β-carotene are associated with an increased risk of illness (Goodman, 2000; Ratnasinghe *et al,* 2000).

β-carotene production

In the natural habitat, β-carotene acts as a sun shield, effectively protecting *Dunaliella* from the damaging light energy in the blue region of the spectrum. In fact, under conditions of high light intensity and high temperatures between 26-36°C, the pigment collects as oily globules within the interthylakoid spaces of the chloroplast giving the whole organism a distinctive orange colour. The pigment alone can account for more than 10% of the dry weight of each organism (Ben-Amotz *et al,* 1982), making *Dunaliella* the highest known natural producer of β-carotene. By comparison, the β-carotene content in a typical leaf or alga is only around 0.3% (Ben-Amotz and Avron, 1990). The β-carotene produced in *Dunaliella* is a mixture of two stereoisomers, the 9-cis and all-trans forms (Figure 1), although a minor amount of α-carotene is also present. Both the amount of the accumulated β-carotene and the 9-cis to all-trans ratio depend on the intensity of light incident on the cell. Higher light intensities induce the production of more cellular β-carotene and increase the 9-cis to all-trans ratio. The physico-chemical properties of 9-cis β-carotene differ from those of the all-trans. The cis form is much more soluble in hydrophobic solvents (Ben-Amotz and Avron, 1990). It is therefore more easily absorbed into tissue and consequently may account for the greater protective role suggested in various studies.

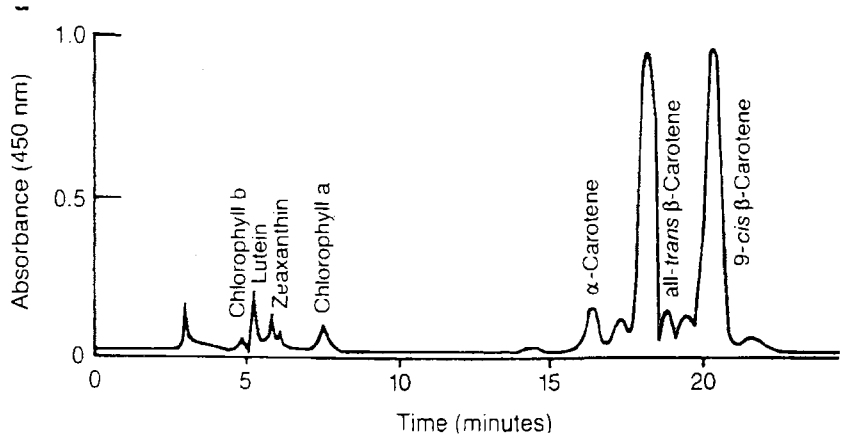

Figure 1. Typical high pressure liquid chromatogram of a total pigment extract from a Dunaliella bardawil strain containing high levels of b-carotene. Smaller amounts of a-carotene and the usual complement of chlorophylls found in green algae also occur (Source: Ben-Amotz and Avron, 1990)

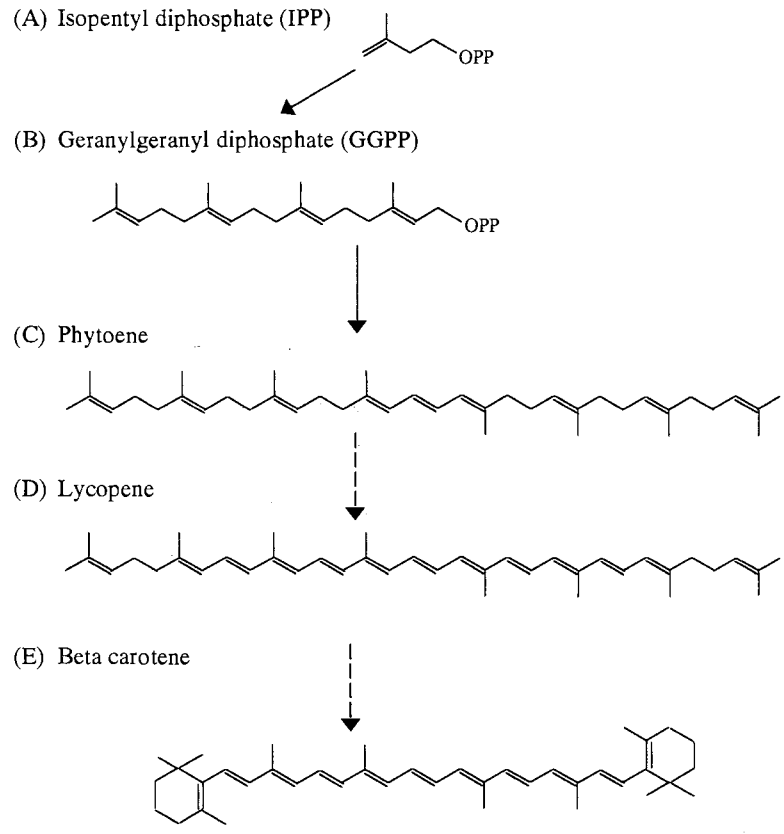

Figure 2. The β-carotene biosynthetic pathway.

The steps in the biosynthesis of β-carotene were first identified by Shaishi *et al,* (1992). They are similar to those existent in plants where carotenoids are derived from the general isoprenoid pathway (Figure 2).

The isoprenoids are built from a common precursor isopentenyl diphosphate (IPP), which in plastids is believed to be formed from pyruvate and glyceraldehyde-3-phosphate. The condensation of two molecules of geranyl geranyl diphosphate (GGPP) to produce phytoene is the first committed step in the synthesis of β-carotene. GGPP is then dehydrogenated to lycopene and finally lycopene is cyclized to form β-carotene (see Guerinot, 2000). Synthesis of β-carotene appears to be triggered by a number of stressors,

including high solute concentration, metals such as lead and copper (Pace *et al*, 1977) and nitrate deficiency (Ben-Amotz and Avron, 1983). The reason for the effect of each of these stressors on the pathway is as yet unexplained, but the effect of the heavy metals may reflect an activating effect on some of the key enzymes involved in the biosynthetic pathway.

Although β-carotene can be chemically synthesised by a patented process that starts with acetone and is owned by the chemical company Badische Anilin- & Soda-Fabrik AG (BASF), the synthetic form consists of mainly trans β-carotene and much lower levels of the healthier-perceived cis-form. This difference in composition between the natural and the synthetic forms is being exploited in a successful yet aggressive marketing campaign in favour of the natural form. In particular, as consumer demands for natural products in Europe and Japan continues to increase, the market should continue to expand (Mirasol, 1988). There is certainly room for further small to medium sized culture plants that can supply the European niche.

The real challenges in supplying this growing market actually lie in harnessing the biosynthetic β-carotene pathway. Unlike the *Penicillium* fungi where a variety of conventional mutagenic techniques have been repeatedly used to produce high producing antibiotic strains, there have been no such programmes for *Dunaliella*. To date, attempts to increase the levels of β-carotene in production have centred around searches for high producers already existent in nature. This has been carried out with efficiency by the Australian, American and Israeli research HHHcorporations involved this area. It is therefore logical to predict that the next step would be to carry out mutagenic studies to isolate higher producing β-carotene strains. Genomics assisted strain improvement programmes are another way by which carotene production could be improved, although the absence of basic molecular biology protocols for *Dunaliella* could hinder success in this area.

Dunaliella cultivation

The halophilic strains of *Dunaliella* are particularly attractive candidates for mass culture. Firstly, they flourish in poor quality water that is not of agricultural use. Secondly, they possess the ability to survive in high salinities. As a result, they can be cultivated outdoors with few potential predators or competitors. Even under extensive culture, the difficulties with sterility that are encountered with the usual organisms used in fermentation such as *Escherichia coli*, *Saccharomyces* or fungal hosts are low. Axenic cultures can be maintained fairly easily without the need of antibiotics, insecticides or pesticides. In any case, studies in our laboratory have shown that *Dunaliella* is insensitive to most antibiotics. This result was also seen to a lesser extent in studies with the closely related microalga *Chlamydamonas reinhardtii* (Harris[1], personal communication). It would appear that to survive in its environmental niche, *Dunaliella* has developed stringent methods for limiting the intake of material across its membranes. Cultivation is also comparatively cheap since it is based on autotrophic growth in media containing carbon dioxide and inorganic nutrients. Supplementation of media with organic substrates is unnecessary, as *Dunaliella* does not show heterotrophic use to any extent (Ben-Amotz and Avron, 1990).

Current methods of cultivation are geared towards maximising incident sunlight and high temperatures. An open pond extensive mode of culture is most often used in the majority of plants. The principal advantages of such systems include a relatively small capital investment and a free source of energy in the form of natural sunlight. Yields of around 10mg of β-carotene per square meter of culture per day have been recorded (Ben-Amotz, 1993). Simple improvement to this system by, for example, culturing the alga in long raceways and supplementing the system with aeration by means of slow moving paddles can improve yields of β-carotene 40-fold to about 400mg per square meter per day.

However, both extensive and intensive cultures suffer from the disadvantage that they are sensitive to any sudden adverse changes in weather. Production also decreases during the night and the cooler months of the year. Attempts are being made to overcome these disadvantages by experimenting with growing cultures in enclosed conditions. Most of these attempts utilise a variety of electric light and sunlight driven photobioreactors (Ben-Amotz, 1993). To date, the electric light driven models are capital intensive and bear high operating costs. The greater outlay could be justified by the reduction in cost of downstream processing and by the production of a sufficiently high value product. The genetic modification of *Dunaliella* to result in such a product may be one way in which the use of a photobioreactor may be justified. Currently no such bioengineered strain exists.

The second option is to use sunlight-driven photobioreactors. These offer the advantages of increased control and reduced costs of downstream processing. In our laboratory we have had some success using transparent tubular plastic bioreactors, where up to fifty litre culture bags and a two step salinity growth system were utilised. Cultures were initially grown at low salinities of around 12% to optimise biomass production and then salinity was rapidly increased to approximately 23% to induce β-carotene production. Throughout this process, maximum cell concentrations ranged from around 1.8×10^6 cells/ml to 4.0×10^6 cells/ml in the lower salinities to about 30 to 80×10^4 cells/ml for the higher salinities. These results were comparable to the readings recorded by Moulton and Burford (1990)

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using the related species *Dunaliella viridis* in open ponds. Although further work needs to be carried out to improve the separation technique, more than a 10-fold increase in total β-carotene was recorded with the bioreactor derived cells compared to an equal amount of packed cells derived from a non induced culture.

It is unlikely that much higher quantities of β-carotene could be induced from larger volumes based on the same bioreactor design, as the level of sunlight incident on the inner core of the culture would be expected to become a limiting factor. However, these preliminary results indicate that such sunlight-driven photobioreactors could have a good potential in areas where land is at a premium, to generate seed cultures prior to inoculating in larger ponds. This method could also be used to ensure axenic cultures during the initial stages of culture when biomass generation is a priority.

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