In the name of God
The compassion & the merciful

Faculty of Pharmacy,
Tehran University of Medical Sciences,
Tehran, Iran.

Title of Thesis:

Effect of environmental stresses such as light intensity, salinity and temperature on production of vitamin E and β-carotene in *Dunaliella tertiolecta* DCCBC26 isolated from the Urmia hypersaline lake

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By:
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Academic year: 2006-2007 **No. of Thesis: 4640**

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This thesis is dedicated to my parents....

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Abstract

Carotenoids are widely used for production of neutraceutical and cosmetics as additives, colorants and antioxidants. Dunaliella is the main source for natural carotenoids which accumulates massive amount of carotenoids. This study examined the effects of different salt concentrations (0.05 to 3 M of NaCl), light intensities (50 to 600 μmol photons m^{-2} s^{-1}) and temperatures (18 °C, 25 °C, 32 °C) on the kinetics of growth, total carotenoids, β-carotene (all-trans and 9-Cis) and vitamin E (α-tocopherol) accumulated in *Dunaliella tertiolecta* DCCBC26, a microalgae strain isolated from the Urmia hypersaline lake, northwest of Iran. Results indicated that the highest amount of carotenoids detected (11.73 mg/l) was in the salinity of 0.5 M NaCl during the stationary growth phase. The percentage of the all-trans and 9-Cis β-carotene in the exponential phase were 92% and 32% in salinities of 3M and 0.5M respectively. However, only 23% of the β-carotene was detected in the stationary growth phase of the microalgae in 0.5M and was 9-Cis isomer. Also, increasing temperature to 32 °C or decreasing to 18 °C had a negative effect on β-carotene and α-tocopherol. Likewise, light quality and intensity are known to affect metabolite production by microalgae. Production of α-tocopherol (α-T) by *D. tertiolecta* DCCBC26 was investigated under fluorescent and halogen light sources. Under illuminance of fluorescent light the maximum α-tocopherol

accumulated by the microalgae was 135 μg g^{-1}dw which was achieved at salinity of 0.5M after 28 days incubation at 25°C. α-Tocopherol content of the cells decreased with increasing light intensity from 217 μg g^{-1}dw at light intensity of 150 μmol photons m^{-2} s^{-1} to 80 μg g^{-1}dw at 600 μmol photons m^{-2} s^{-1} when halogen lamps were used as light source. Although the level of dry weight obtained (0.965 g/l) after 28 days of cultivation using halogen lamp source was not much different from that achieved under illuminance of fluorescent lamp (1.045 g/l) but cell counts were higher with fluorescent lamp (26.3 × 10^6 cell/ml) compared to those of halogen light (15.25 ×10^6 cell/ml). As fluorescent and halogen lamps produce different spectrum of light, both morphological and metabolite accumulation by the microalgae seems to be affected by light quality.

1. Introduction

A century has passed since the description of the genus Dunaliella, the unicellular green alga which is responsible for most of the primary production in hypersaline environments worldwide. First sighted in 1838 in saltern evaporation ponds in the south of France by Michel Felix Dunal, it was named after its discoverer by Teodoresco in 1905 (1). In the century that has elapsed since its formal description, Dunaliella has become a convenient model organism for the study of salt adaptation in algae. The massive accumulation of β-carotene by some strains under suitable growth conditions has led to interesting biotechnological applications. The green unicellular flagellate *Dunaliella salina* and *Dunaliella bardawil* are the richest natural source of the β-carotene (2, 3). The halophilic species of Dunaliella also accumulate a great amount of glycerol (4). A common feature of most of the algal species currently produced commercially (i.e. Chlorella, Spirulina and Dunaliella) is that they grow in highly selective environments which means that they can be grown in open air cultures and still of contamination by other algae and protozoa (Fig. 1).

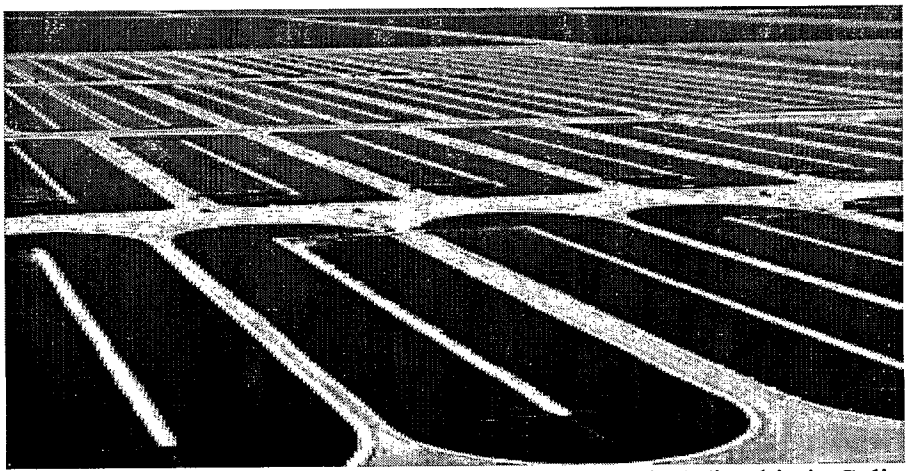

Fig. 1 Raceway Ponds used for the culture of *Spirulina platensis* by Microbio in Calipatria, California.

β-Carotene from Dunaliella is now being produced on a commercial scale in some countries e.g. the USA, Australia, (Fig. 2) and pilot-scale projects are under way in China, and Kuwait. More recently *Dunaliella viridis* has also been proposed as a source of oxygenated carotenoid (5). Therefore, a historical survey of research on Dunaliella, from the early work in the 19th century to the thorough taxonomic studies has been described by Teodoresco, Hamburger, Lerche and others from the beginning of the 20th century onwards.

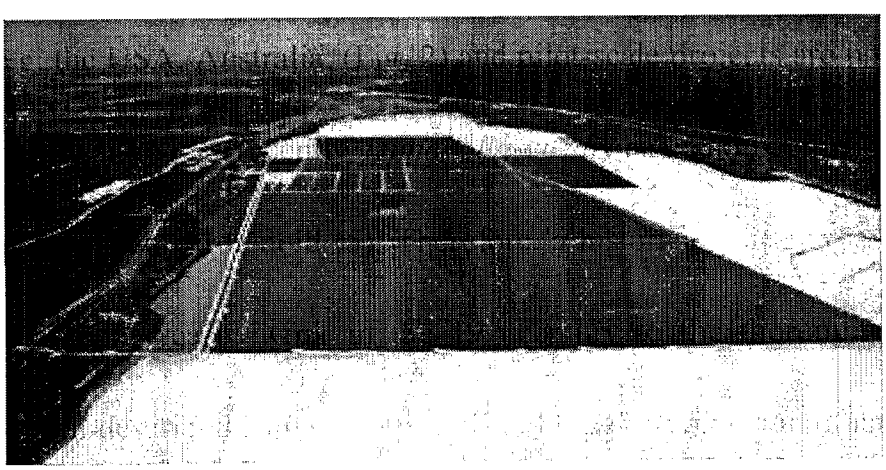

Fig.2 The large open ponds used for the culture of *D. salina* at Hutt Lagoon, Western Australia.

1.1. Genus Dunaliella

1.1.1. History of Dunaliella

The first description of a unicellular biflagellate red colored alga living in concentrated brines was given in 1838 by Dunal (1), who reported occurrence of the organism we know today as *D. salina* in the salterns of Montpellier, in the Mediterranean coast of France. He named the organisms observed *Haematococcus salinus* and *Protococcus*. The discovery of these algae was made in the framework of an investigation, invited by the Académie des Sciences, Paris, of the cause of the red coloration of saltern brines. At the time it was widely assumed that chemical and physical parameters are responsible for the coloration of these brines. Dunal refuted an earlier claim that the color is due to the brine shrimp *Artemia salina*. The Académie then appointed a committee to reexamine the matter, and this committee confirmed Dunal's finding (1). Nowadays it is clear that, although β-carotene-rich *D. salina* are indeed present in the saltern ponds, most of the coloration of the crystallizer brine is caused not by the algae but by red halophilic Archaea instead (6).

1.1.2. Taxonomy, life-history and morphology of the genus Dunaliella

Based on a number of biochemical and cellular differences, two major groups of green microalgae are recognized: the Chlorophyta and the Conjugaphyta. Although the latter group is almost five times larger than the Chlorophyta, none of the Conjugaphyta has yet been employed for biotechnological applications. The Chlorophyta are subdivided into four groups. Most species inhabit marine and brackish environments, while others prefer freshwater (7). The Chlorophyceae represent the largest group, with about 2,500 species in 350 genera. Most species are unicellular or filamentous freshwater forms. The best known algae, such as Chlorella, Chlamydomonas, Dunaliella and Haematococcus, belong to this group. Some species accumulate high concentrations of carotenoids under certain culture conditions. Dunaliella is a unicellular, bi-flagellate, naked green alga (Chlorophyta, Chlorophyceae). The genus was first described by Teodoresco (1) with the type of species being *D. salina*, and at present a total of 29 species, as well as a number of varieties and forms, are recognized (8). Dunaliella is morphologically similar to Chlamydomonas, with the main difference being the absence of a cell wall in Dunaliella. Dunaliella has two flagella of equal length and a single, cup-shaped chloroplast, which in the marine and halophilic species has a central pyrenoid. Cell

shape in this genus is very variable, being oval, spherical, cylindrical, ellipsoidal, egg, pear or spindle-shaped with radial, bilateral or dorsoventral symmetry or being asymmetrical. Cells in any given species may change shape with changing conditions, often becoming spherical under unfavorable conditions. Cell size also varies with growth conditions and light intensity. The extraction of β-carotene from *D. salina* has already reached large-scale production (9). Another promising carotenoid is astaxanthin, a high-value pigmentation source in aquaculture, especially for trout and salmon. Efforts have been made to produce astaxanthin cost-efficiently from *Haematococcus pluvialis*, which accumulates up to 3% astaxanthin (dry weight) (10). We now know that not all Dunaliella species produce massive amounts of carotene, and those that can, do so only under suitable conditions (exposure to high light intensities, nutrient limitation, etc.) thus under suitable conditions all red clones became green, but after several weeks they turned olive to yellow green and after several months they were red again (11). Additional species were later added to the genus, especially thanks to the in-depth studies by Lerche and Butcher (Table 1) (1). Some are typically marine organisms that were never reported to occur in hypersaline environments. An in-depth taxonomic treatment of the genus was given in Massyuk's 1973 monograph. She divided the genus into two subgenera, Dunaliella (23 species) and Pascheria (5 species), the

latter consisting of freshwater species only. Molecular phylogeny techniques have been applied to the taxonomic study of Dunaliella from 1999 onwards. These studies have encompassed the 18S rRNA genes and the internal transcribed spacer regions, and have been based on gene sequence comparisons as well as on restriction fragment length polymorphism studies. Little correlation was found between the molecular data and the morphological-physiological attributes used in older studies to delineate species within the genus (12, 13). On the basis of 18S rRNA gene sequences, Olmos et al. (14) could differentiate between *D. salina*, *D. parva* and *D. bardawil* as species containing one, two and three introns, respectively, within the 18S rRNA gene. The molecular studies have made it clear that many culture collection strains are probably misnamed, and that some unnecessary species names may have been proposed in the past.

Table 1 Selected Dunaliella species. (1)

Name	Author, year
D. salina	Teodoresco, 1905, 1906
D. viridis	„
D. parva	Lerche, 1937
D. tertiolecta	Butcher, 1959
D. primolecta	„