CHAPTER I

INTRODUCTION

Dunaliella salina (Dunal) Teodoresco is a green alga which can accumulate high β-carotene in its cell when it is in the extreme environment such as high light intensity, very high salinity and nutrient deficiency. Among all species of unicellular species, D. salina has the highest cell content of β-carotene, with the maximum reported concentrations of up to 20% dryweight (Yamaoka et al., 1992). This concentration is high enough for commercial cultivation.

Dunaliella has become a new species for algal biotechnology as source of fine chemical especially for β-carotene. Marketing of others algae such as Chlorella and Spirulina were seemed to decline (Vonshak, 1990). In addition, not merely β-carotene but also glycerol and protein concentrate can be extracted from Dunaliella (Gudin, 1988). β-carotene has a high coloring index, with this reason, it can provide both color and nutritive values such as vitamin A. β-carotene is now widely used for coloring soft-drinks, margarine, fruit juices, breads, cream-cakes, fishes products, ice-cream, etc., as well as being used as a nutritional supplement (Xihai, 1990). For example, dried Dunaliella from NBT company in Israel (incorporated with Japanese company) which contain 3-5% β-carotene is packed in capsules or tablets and sell as a health-food under the label 'natural β-carotene' (Ben-Amotz and Avron, 1990).

For commercially mass culture, Israel company (NBT) and two companies in U.S.A. (Microbio in California and Cyanotech in Hawaii) use paddle-wheel "raceway" pond for Dunaliella production. The intensive culture system such as in Israel

provides very high productivity up to 50-75 metric tons biomass a year in 5 Ha pond (Vonshak, 1990). Besides, two companies in Australia, Western Biotechnology and Betatene, operate on extensive pond system without stirrers. The large culture pond produce lower algal biomass than raceway pond. However, the production cost is much lower than the raceway system (Borowitzka and Borowitzka, 1990).

Thailand is in a tropical zone. Climate, temperature and water supply are proper for algal culture (Becker, 1983). Algal biotechnology in Thailand today is related to aquaculture and feed. Chaetoceros calcitrans, Skeletonema costatum, Tetraselmis sp. and Isocrysis galbana are commonly species for shrimp and mollusc culture (Kongkeo, 1991). Spirulina, however, is the only species being commercially mass cultured in Thailand now a day. Two companies, Siam Algae (Japanese company) and Neotech (Thai company), produce Spirulina more than 200 tons a year for health food and animal feed. Nevertheless, Spirulina requires complicate cultivation system and expensive culture medium. Dunaliella, on the other hand, need simple culture medium (Ben-Amotz and Avron, 1989a).

Salt production ponds in Thailand are located in the coastal area of Chon Buri, Samut Songkhram and some provinces around the Gulf of Thailand. However, salt price is only 1.6-3.5 baht per kilogram (farmer interviewed in 1992), therefore, Dunaliella cultivation may be a new prospect of algal biotechnology in Thailand for the high value β-carotene product and may be considered as an additional venture in the salt pond areas in the future.

Objectives

The objectives of this research are:

1. To isolate strains of Dunaliella spp. from salt ponds in Thailand.
2. To select Dunaliella salina strain that yields high β-carotene.
3. To study the effects of salinity, light intensity, nutrient and pH of culture medium on growth and carotenoid content of the selected strain.
4. To determine growth and β-carotene productivity of D. salina in the outdoor mass culture.

Expected Result

1. The locally isolated strain of D. salina could be cultivated for β-carotene production.
2. Obtaining the information of D. salina culture condition for β-carotene production.
3. Feasibility of local strain D. salina mass culture in Thailand.

LITERATURE REVIEW

Taxonomy

Division	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	Polyblepharidaceae
Genus	Dunaliella
	Dunaliella salina (Dunal) Teodoresco

Dunaliella is a motile unicellular green algae. Two flagella, elastic cell wall and ovoid to sub-globular shape are certain characteristics of all species. Cells dimension are between 8-25 μm length and 5-15 μm width. The species of this genus are closely resemble in Chlamydomonas but Dunaliella is lacking in rigid cell wall. A detailed study of Dunaliella ultrastructure was done by Hoshaw and Maluf (1981). They suggested that most internal cell features of D. tertiolecta, D. bioculata, D. primolecta and D. salina are chlamydomonad in structure and arrangement. Some ultrastructural differences were seen between the stationary and logarithmic growth phases of the culture.

The genus Dunaliella was first described by Teodoresco (1905) with the named in honor of M.F. Dunal in the year 1837, who was the first to recognized that the red color of hypersaline reservoirs was caused by an alga (Borowitzka and Borowitzka, 1988a). After that, Butcher (1959) revised the taxonomy of genus

Dunaliella and proposed the key to identified species as follows:

Genus Dunaliella

Key to species according to Butcher (1959):

1. Cells fusiform, both ends acute..... MINUTA
1. Cells ovoid to ellipsoid, both ends obtusely rounded..... MEDIA
1. Cells obovoid with acute posterior and truncate anterior..... 2
1. Cells ovoid, pyriform or cylindrical with rounded posterior and sub-acute to acute anterior. 4
   2. Stigma anterior, chromatophore smooth..... 3
   2. Stigma median, chromatophore with plastids in the region of the pyrenoid..... MINOR
3. Cells over 12 μ long, narrowly obovoid..... PIERCII
3. Cell under 12 μ long, broadly obovoid..... EUCHLORA
   4. Cells broadly ovoid, over 15 μ long..... SALINA
   4. Cells ovoid to ellipsoid, under 15 μ long... 5
5. Cells without refractive granules or, if present, few and scattered irregularly..... 6
5. Cell filled with refractive granules as a linear or U-shaped girdle or zone..... 8
   6. Cells 10-15 μ; stigma irregular, linear often two..... BIOCULATA
   6. Cells under 10 μ; stigma diffuse, orbicular. 7
7. Pyrenoid with a sheath of several starch gains. PARVA
7. Pyrenoid with a continuous sheath..... TERTIOLECTA

8. Granules 4-20, arranged as a simple median girdle..... PRIMOLECTA

8. Granules usually vary many, massed in the anterior zone..... 9

9. Pyrenoid globose with a continuous starch sheath ..... QUARTOLECTA

9. Pyrenoid irregular..... POLYMORPHA

Dunaliella salina is the most halotolerant alga. It can grow in media containing 0.2% to saturated salt (about 35% NaCl) (Ben-Amotz and Avron, 1989a). D. salina is also very tolerant of high temperatures and high light intensity (Borowitzka and Borowitzka, 1990). Cells become dominantly in high salinity water bodies such as salt lakes or salt ponds. In general, D. salina is not a dominant species of the native fresh and sea water. When salinity rise up, D. salina becomes major organism, then water color will turn to red.

Some Dunaliella spp. such as D. parva and D. viridis are usually found naturally associated with D. salina (Moulton et al., 1987a). D. viridis is predominant species in high saline lake but do not contain high amounts of β-carotene at high salinity. Both species are present in salt lakes around the world, though red D. salina cells usually predominate, cause the high saline lake become red (Borowitzka, 1981). The green species D. minuta Lerche, D. parva Lerche and D. euchlora Lerche were also reported from Lake Eyre in South Australia (Baas-Becking and Kaplan, 1956 cited by Borowitzka, 1981).

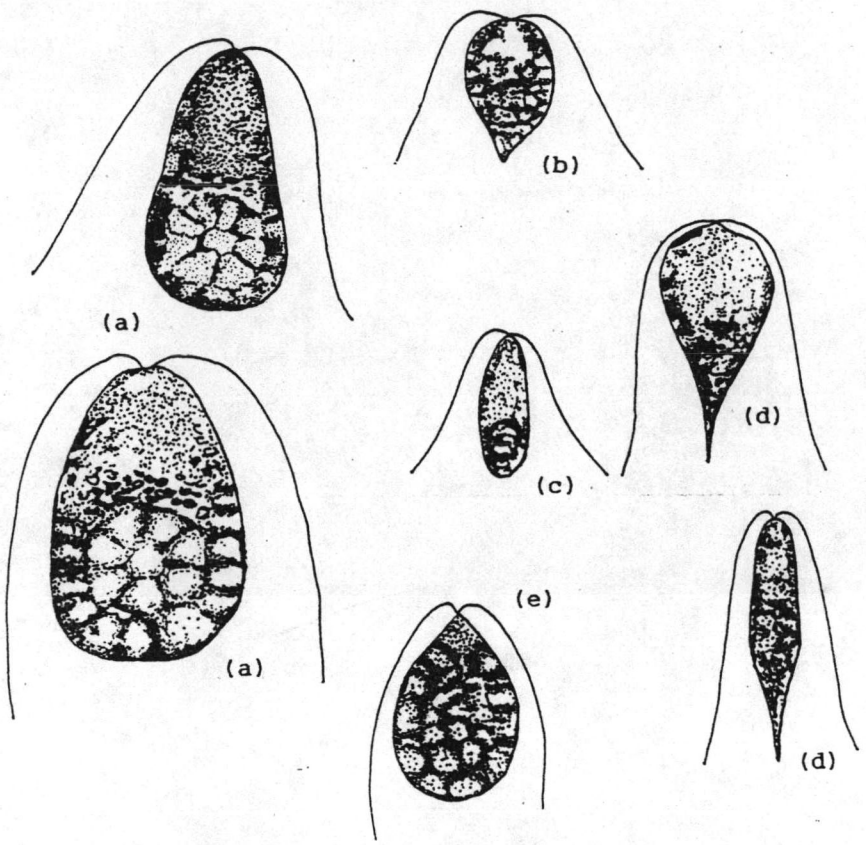

Figure 1. Picture of Dunaliella spp. (rebuild from Lerche, 1937)

(a) D. salina

(b) D. euchlora

(c) D. minuta

(d) D. piercii

(e) D. parva

However, it was later found that 29 species and several varieties and forms of Dunaliella were found and revision (Massyuk, 1973 cited by Borowitzka and Borowitzka, 1988a). Because of many species name and some unnecessary species names have occured, the confusing species description and misnamed were appeared in the culture collection. For example, Borowitzka and Borowitzka (1988a) suggested that all Dunaliella strain that appear red due to accumulation of large amounts of β-carotene under extreme environmental conditions are D. salina and they explained that D. bardawil (Ben-Amotz and Avron, 1983) is actually strain of D. salina while D. salina of Ben-Amotz and Avron (1982) is probably D. parva, to which all strains are assigned that accumulate lesser amounts of carotenoids and that appear yellowish under extreme conditions. Levin (1990) also proposed that D. bardawil was technically invalid of D. salina.

On the other hand, Ben-Amotz (personal communication) quoted that the major difference between D. bardawil and D. salina is the presence of distinguishing extra chloroplastic eye spots, and the related active phototaxis of D. bardawil in comparison to D. salina. Both species can accumulate high amounts of chloroplastic β-carotene as related to light intensity and retarded growth conditions.

However, there were many reports similarly suggested that the capability to accumulated β-carotene and turn to red color in extreme condition are the most important characters of D. salina (Ben-Amotz and Avron, 1989a; Loeblich, 1982; Borowitzka and Borowitzka, 1988a; Moulton et al, 1987a and Al-hasan et al., 1987). Loeblich (1982) proposed that D. salina is an organism

that has the capability of turning red with a carotenoid to chlorophyll ratio grater than 6:1. Some strains of Dunaliella currently called D. salina were not accepted if cells do not turn red at salinity up to 25% NaCl or low amount of carotenoids per cell. Finally, he recommended the only way to be sure that an isolate of Dunaliella are D. salina is to grow it at a salinity greater than 15% NaCl under high light intensity and observe whether it is capable of turning red, or alternatively, to isolate single red cells from high salinity natural water samples.

β-carotene Synthesis and Factors Effect the β-carotene Accumulation of D. salina

Carotenoids are yellow-orange pigments found in plants and animals. Carotenoids are classified into two groups: [1] carotene, pure hydrocarbon polyenes such as α, β and γ carotene, and [2] xanthophylls, oxygenated hydrocarbons such as lutein, zeaxanthin and astaxanthin (Simpson et al., 1985; Latscha, 1990). Carotenoids serve several functions in nature. They protect photosynthesis organism against photodynamic destruction, act as auxiliary light adsorbers for photosynthesis and direct phototaxis, and serve as provitamin A for animals (Liaaen-Jensen, 1990).

β-carotene is a C-40 carotenoid oxygen-free unsaturated hydrocarbon found in several plant and algae. The biosynthesis

of β-carotene can be divided into four stages (Goodwin, 1980; Shaish et al., 1990):

1. formation of geranylgeranyl pyrophosphate form mevalonic acid
2. condensation to form phytoene
3. desaturation of phytoene to lycopene
4. cyclization of lycopene to form β-carotene

The β-catotene accumulated in D. salina has been showed to be composed of mostly two isomers, all-trans and 9-cis, in approximately equal amounts. The ratio of the 9-cis to the all-trans isomer increases with increasing in the light intensity . The isomerization reaction which leads to the production of the 9-cis isomer occurs early in the path of carotene biosynthesis, at or before formation of all-trans phytoene (Ben-Amotz et al., 1988).

A detailed study of β-carotene biosynthesis of Dunaliella was concluded by Ben-Amotz et al. (1987) and Shaish et al. (1990). The biochemical pathway is shown in Figure 2.

Massive amounts of β-carotene are located in a large number of chloroplastic, lipoidal globules located in the interthylakoid space (Figure 3). This is in contrast to the low β-carotene content of plants and algae which is located within the chloroplast membranes (Ben-Amotz and Avron, 1989a). When the light intensity is increased more than normal required for growth, growth rate is limited, and β-catotene accumulates to the highest levels (Ben-Amotz and Avron, 1983).

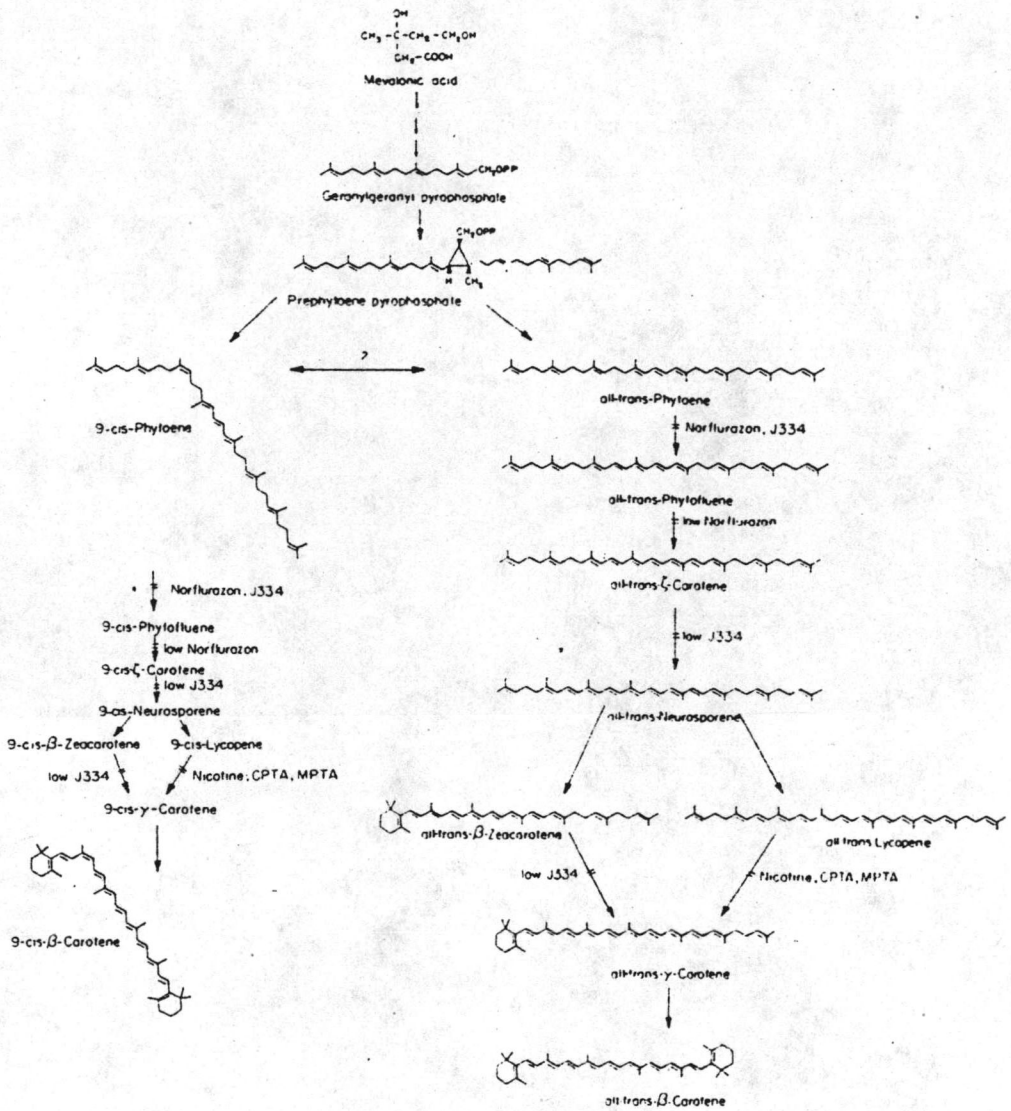

Figure 2. Postulated pathway of all-trans and 9-cis β-carotene biosynthesis in Dunaliella bardawil. (Shaish et al., 1990)

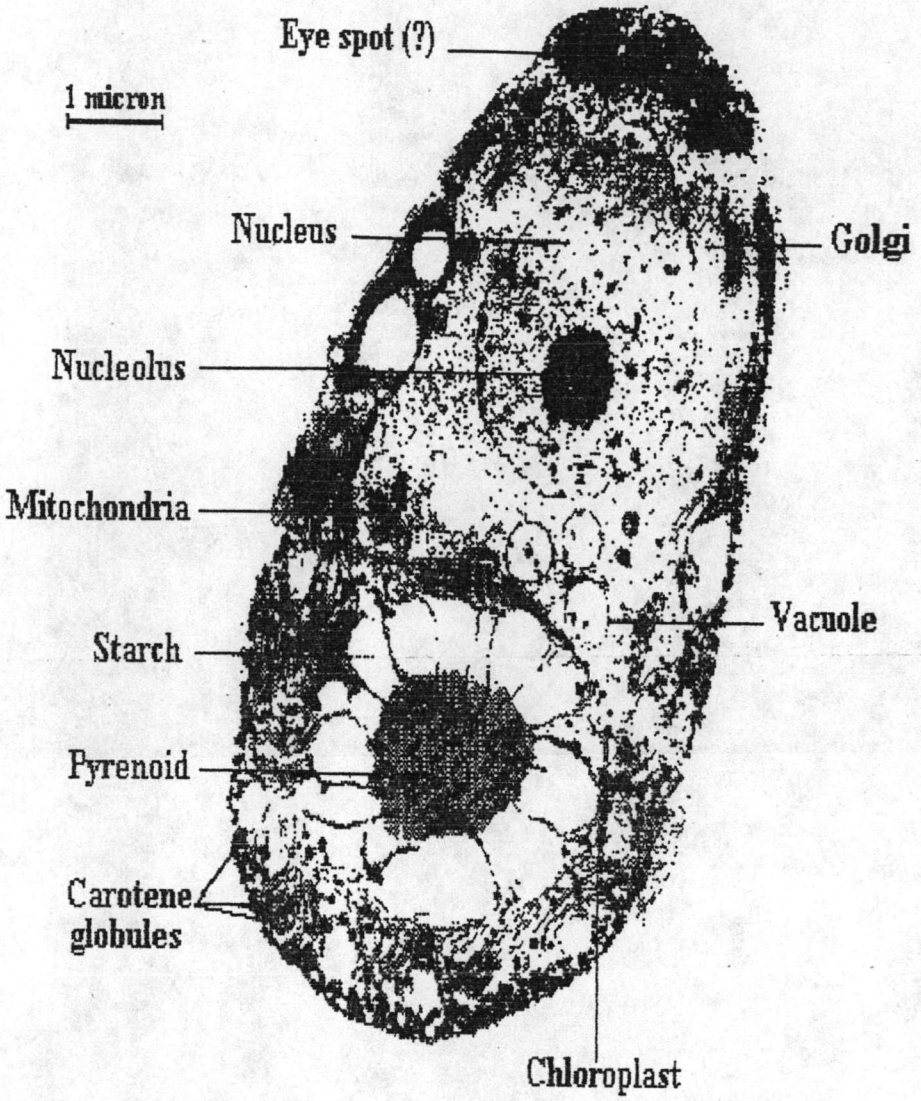

Figure 3. Electron micrograph of a section throuh β-carotene rich Dunaliella bardawil (Ben-Amotz and Avron, 1990).

Photosynthesis activity of Dunaliella is greater in blue light (440 nm) than in red light (675 nm) (Loeblich, 1982; Ben-Amotz and Avron, 1989b). Ben-Amotz et al. (1989a) found that the β-carotene accumulation of Dunaliella is machanism to protecting the alga from excess irradiation. The high β-carotene strains of D. bardawil could resist to photoinhibition of photosynthesis oxygen evolution when compared with low β-carotene strains. Low β-carotene strain of D. bardawil exposed to long periods (8 h) of high blue light shows photobleaching of both β-carotene and chlorophyll while high β-carotene strain was fully protected from photobleaching. No photoprotection was observed with red light which is not absorbed by β-carotene. They suggested that the protection could be due to a simple screening effect by orange-red color of β-carotene.

The environmental stress such as high light intensity, high NaCl concentration, extreme temperatures, extreme pH value or nutrient deficiency were important factors for β-carotene content in the cell (Loeblich, 1982; Ben-Amotz and Avron, 1983; Al-Hasan et al, 1987; Borowitzka and Borowitzka, 1988a; Junmin, 1990). Under the optimum environmental conditions, D. salina can accumulated up to 20% (w/w) of β-carotene in the cells (Yamaoka et al, 1992).

Dunaliella grows well in an inorganic medium with no requirement of any organic factors (Ben-Amotz and Avron, 1989a). The major nutrient for mass cultivation are nitrigen, phosphorus, sulphate, magnesium, potassium, calcium, Fe-chelated complex and micronutrient. In most media containing salt or seawater

addition of micronutrient is not needed because they are present in excess for the algal requirement.

Nitrate is the best nitrogen source for Dunaliella cultivation (Ben-Amotz and Avron, 1983; Yamaoka et al., 1992). One of the advantageous way to limit the growth rate for maximizing β-carotene production is by limiting the nitrogen supply (Ben-Amotz and Avron, 1989a; Shaish et al., 1991). For example, when reducing nitrate concentration from 5 mM to 0.5 mM, D. bardawil could increased β-carotene content from 0.5% to 5% (Ben-Amotz and Avron, 1989a).

When salinity increase, not only β-carotene but also lipid composition will changed. The total lipid content decrease with increasing salinity and proportions of constituent linolenic acid (18:3) both in total lipids and in the galactolipids and of palmitoleic acid (16:1) in phosphatidylglycerols are increased (Al-hasan el al., 1987). For photosynthetic characteristics, carbonic anhydrase (CA) activity was found higher in β-carotene rich D. salina light dependent rate of exygen evolution and photoinhibition depends on the concentration of β-carotene in D. salina cells (Gómez-Pinchetti et al., 1992)

Biotechnology of Cultivating D. salina

Ben-Amotz and Avron (1989a) proposed that the green alga Dunaliella is probably the most successful microalga for outdoor cultivation. The following characteristic of Dunaliella make it

an attractive candidate for commercial mass cultivation (Vonshak, 1990; Ben-Amotz and Avron, 1989a):

(a) Its ability to accumulate relatively high concentrations of β-carotene, known to be in high demand and have a high commercial value.

(b) Its ability to thrive under extreme conditions such as 6-12% NaCl provides a selective advantage, wherein development of other algae or predators in open raceway ponds is prevented.

(c) Under suitable growth conditions Dunaliella accumulates massive amounts of one highly priced product, β-carotene (over 10% of the algal organic weight), in addition to glycerol (around 20-40%) and the remaining algal meal.

(d) Lacking a cell wall, dried Dunaliella is easily and fully digestible by animals and humans.

An important technical problem for mass cultivation of Dunaliella is the harvesting (Vonshak, 1990; Ben-Amotz and Avron, 1989; Borowitzka and Borowitzka, 1988a). Because of tiny cell size (less than 20μ), Filtration method is not practical for Dunaliella harvesting. Filter screens with pores around 1 μm are required to obtain efficient harvesting (Kormanik and Cravens, 1979). The algae clogged the filter rapidly by forming a layer of mucous material which prevents further filtration unless backwashing is performed frequently. Repeated backwashing breaks the algae and releases glycerol and organic matter to the medium (Naghavi and Malone, 1986 cited by Ben-Amotz and Avron, 1989a). Several harvesting methods have been tried such as high pressure

filtration through sand filter, cellulose fibre, diatomaceous earth, the use of salinity-dependent buoyancy properties in stationary or moving salt gradients, alkaline flocculation, exploitation of the phototactic ang gyrotactic responses of the algae (Borowitzka and Borowitzka, 1988a).

Centrifugation and flocculation are frequently methods for Dunaliella harvesting. Flocculation by adding chemicals such as aluminium sulphate (alum) (Cordero et al., 1990), Chitosan (Promjaroen et al, 1992), ferrous and ferric sulphate, ferric chloride, lime, etc. have been examined. As with most other microalgae, alum at concentration about 150 mg/l was found to be the most efficient chemical agent for flocculation of Dunaliella (Ben-Amotz and Avron, 1989a). However, the flocced algae cannot be used for food grade unless the flocculant is safe or is completely released from the algae prior to utilazation. Until now, harvesting by centrifugation has been used for commercial production of Dunaliella in Israel and China. This method is safe for food but require high energy inputs and high production cost.

To increase of β-carotene yield, production and selection of high β-carotene mutants of D. bardawil were induced by UV irradiation (Shaish et al., 1991). The selection of high β-carotene mutant were terminated by strong blue irradiation. Cells that could resistant to high blue light were refer to the high β-carotene mutant. β-carotene production of mutant was very high when compared with wild type strain.

Products of Dunaliella cultivation

Unlike the cultivation of others green unicellular algae and blue green alga such as Spirulina, Dunaliella culture was aimed for the production of β-carotene and glycerol in addition to proteinous meal (about 33% protein (Liangchen, 1990)) which may be used as feed (Ben-Amotz and Avron, 1980) or added in food (Finney et al., 1984).

β-carotene is the most important product of Dunaliella. It is currently used for widely propose such as food coloring agent (yellow-orange color), pro-vitamin A or ratinol in animal feed (Ben-Amotz et al, 1986), as an additive to cosmetics sun-screen products, multivitamin preparations and health food products (Ben-Amotz and Avron, 1990). For example, adding dry Dunaliella or an extract of this alga (high β-carotene) to a vitamin-A deficient chick diet showed that it is an excellent source of the vitamin and in addition a yolk color-enhancing agent (Ben-Amotz et al, 1986).

There are several research on prevention and regression cancer by algal β-carotene (Shklar and Schwartz, 1988; Schwartz et al, 1988). The safety of Dunaliella for food use has been examined by Mokady et al (1989). They suggested that multigeneration feeding study in rat may be indicative of the safety of D. bardawil for human consumption.

Natural β-carotene from Dunaliella or vegetables compose of two dominant sterioisomers, 9-cis β-carotene and all-trans β-carotene (Shaish et al., 1990). The proportion of 9-cis β-

carotene of Dunaliella can reach 50% of total β-carotene (synthetic β-carotene is compose of >99% all-tran β-carotene). The sterioisomeric mixture of β-carotene, as found in D. bardawil, has been shown to be preferentially accumulated, over that of synthetic all-trans β-carotene, in chick and rat tissue (Ben-Amotz et al., 1989b cited by Ben-Amotz et al., 1991). The physicochemical properties of 9-cis β-carotene differs from those of all-trans β-carotene. All-trans β-carotene is practically insoluble in oil and is easily crystallized, while 9-cis β-carotene is much more soluble in hydrophobic solvents, very difficult to crystallize, and generally an oil in its concentrated form (Ben-Amotz and Avron, 1990).

Most of the commercially available β-carotene is synthetic. The synthetic product is sold for approximately US$600 per kg to an estimated market volume of around US$200 million per year; the algae β-carotene, being a natural product, is sold for more than twice this value (Ben-Amotz and Avron, 1990). In recent years, Dunaliella products has developed an increasing market demand of natural β-carotene (Borowitzka and Borowitzka, 1990).

The market size of natural β-carotene from Dunaliella is still difficult to estimate but all of production facilities around the world are reporting a continuous increase in production and demand (Vonshak, 1990).