# CULTIVATION OF *DUNALIELLA BARDAWIL* RICH IN CAROTENOIDS AND STUDIES ON NUTRITIONAL AND BIOLOGICAL ACTIVITIES

**A THESIS**
***submitted to the***
**UNIVERSITY OF MYSORE**

***In fulfillment of the requirements for the degree of***
**DOCTOR OF PHILOSOPHY**
**in Biotechnology**

***By***

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*Dedicated to,*

*My dear parents*
*&*
*Beloved husband*

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# DECLARATION

I hereby declare that this thesis entitled **"CULTIVATION OF *DUNALIELLA BARDAWIL* RICH IN CAROTENOIDS AND STUDIES ON NUTRITIONAL AND BIOLOGICAL ACTIVITIES"** submitted to the **UNIVERSITY OF MYSORE**, for the award of the degree of **DOCTOR OF PHILOSOPHY** in **BIOTECHNOLOGY**, is the result of research work carried out by me in the **Plant Cell Biotechnology Department**, **Central Food Technological Research Institute, Mysore** under the guidance of **Dr. G. A. Ravishankar** during the period April 2005 to till date.

I further declare that the work embodied in this thesis is original and has not been submitted previously for the award of any degree, diploma or any other similar title.

Place: Mysore **Vanitha. A**

Date:

**Dr. G. A. RAVISHANKAR,**
**Scientist & Head,**
**Plant Cell Biotechnology Department**

## CERTIFICATE

This is to certify that the thesis entitled **"CULTIVATION OF *DUNALIELLA BARDAWIL* RICH IN CAROTENOIDS AND STUDIES ON NUTRITIONAL AND BIOLOGICAL ACTIVITIES"** submitted by **Ms. Vanitha. A,** to the **University of Mysore** for the award of the degree of **Doctor of Philosophy** in **Biotechnology**, is the result of work carried out by her in **Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore** under my guidance during the period April 2005- till date.

Place: Mysore
Date:

**G. A. RAVISHANKAR**
Research Supervisor

# Abstract

Carotenoids are being intensively investigated regarding their potential to prevent chronic diseases and vitamin A deficiency. *Dunaliella* is a eukaryotic photosynthetic micro-algae belonging to Chlorophyceae, which can produce β-carotene in large quantities under stress conditions. The study presented in this thesis consists of development of a cultivation methodology for the production of *D. bardawil* in outdoor conditions, determination of its efficiency to accumulate carotenoids under different experimental conditions, evaluation of stability of carotenoids in biomass, safety analysis of biomass in rat models, evaluation of its biological activity, bioavailability and vitamin A conversion efficiency in rat experimental models. An efficient outdoor cultivation and harvesting methodology was developed for both in indoor and outdoor condition. *D. bardawil* produced upto 4% β-carotene in outdoor culture condition. Analysis of the biomass revealed 22% protein, 27% carbohydrate, and 8 % fat content on dry weight basis.

*D. bardawil* cultures when exposed to high light intensity of 30-35 Klux showed accumulation of carotenoids. Among the carotenoids, β-carotene was predominant along with other carotenoids such as lutein, lycopene and α-carotene. The enhancement of β-carotene accumulation positively correlated with the up regulation of phytoene synthase, phytoene desaturase and lycopene cyclase mRNA transcripts when the cells were subjected to high light intensity.

Feeding trials, for experimental rats, were carried out to assess the safety and efficacy of *D. bardawil* biomass as a source of β-carotene. The study revealed that *D. bardawil* biomass was safe in single (5g $kg^{-1}$ body weight) as well as multiple doses (100mg & 1000mg $kg^{-1}$ body weight for 90 days) in experimental rats. *In vivo* experiments demonstrated that *D. bardawil* β-carotene is as efficient as synthetic β-carotene in terms of retinol formation and its accumulation in serum and liver. *D. bardawil* β-carotene showed higher level of β-carotene accumulation in liver compared to synthetic β-carotene in case of repeated doses. The carotenoids of *D. bardawil* biomass were as efficient as synthetic β-carotene, against $CCl_4$ induced hepatic and renal damage.

This study demonstrated that *D. bardawil* could be mass cultivated to produce β-carotene rich biomass that could be safely used as a nutritional supplement.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| α | alfa |
| β | beta |
| μ | micro |
| $^{0}$C | Degree centigrade |
| *D. bardawil* | *Dunaliella bardawil* |
| *D. salina* | *Dunaliella salina* |
| DW | Dry weight |
| FW | Fresh weight |
| GC | Gas Chromatography |
| HPLC | High Performance Liquid Chromatography |
| TLC | Thin Layer Chromatography |
| hrs | Hours |
| Klux | Kilolux |
| L | Litre |
| dL | Deci liter |
| M | Molar |
| g | Gram |
| kg | Kilogram |
| μg | Microgram |
| μM | Micromolar |
| mg | Milligram |
| min | Minute(s) |
| mL | Milliliter |
| mM | Millimolar |
| OD | Optical Density |
| rpm | Revolution per minutes |
| SD | Standard deviation |
| SE | Standard error |
| ROS | Reactive Oxygen Species |
| b.w. | Body weight |

# *INTRODUCTION & REVIEW OF LITERATURE*

# Contents

## 1.0. General introduction to algae

Algae are relatively simple aquatic organisms that capture light energy through photosynthesis, use it to convert inorganic substances into organic matter. Algae have been regarded as simple plants, but they actually span more than one domain, including both Eukaryota, belonging to Chlorophyceae, Rhodophyceae etc., (*Chlorella*, *Dunaliella* etc) and Prokaryota, belonging to cyanophyceae group (Blue-green algae eg*., Spirulina*) (Gupta, 1981). In general, algae are classified into seven groups or divisions (Table 1). Algae range from single-celled organisms (micro algae) to multicellular organisms, some with fairly complex differentiated form. The complex forms are known as macro algae which includes the marine forms such as seaweeds. They are devoid of well differentiated structures such as leaves, roots, flowers, and other organ structures that characterize higher plants (Dawson, 1966; Fritsch, 1977). All algae have photosynthetic machinery basically derived from the cyanobacteria (Dawson, 1966; Fritsch, 1977), and so produce oxygen as a byproduct of photosynthesis.

**Table 1. Classification of algae with important example**

| Division | Brief description of different classes | Example |
|---|---|---|
| Chlorophyta | Chlorophyceae, green algae, Large group of very differentiated forms, pigments resemble that of higher plants | *Chlorella, Dunaliella, Haematococcus sp* |
| Euglenophyta | Euglenophyceae, green flagellates, mainly fresh water forms | *Astasia longa* |
| Chrysophyta | Xanthophyceae or golden brown algae, mainly fresh water.<br>Chrysophyceae or yellow brown algae, mainly fresh water forms.<br>Bacillariophyceae or diatoms, characterized by strong silicified cell membranes, fresh water and marine | *Fragillaria pinnata*<br>*Prymensium parvam* |

| | | |
|---|---|---|
| Pyrrophyta | Desmophyceae, mainly marine forms<br>Dinophyceae, free living marine unicellular organisms<br>Cryptophyceae, small poorly known group, marine or fresh water forms | *Prorocentrum micans*<br>*Dinophysis* |
| Phaeophyta | Phaeophyceae or brown algae, thallus with high differentiation, microscopic to complicated filamentous bodies, majority found in littoral zones | *Ectocarpus,*<br>*Fucus,*<br>*laminaria* |
| Cyanophyta | Cyanophyceae or blue green algae, fresh or marine forms | *Spirulina* |
| Rhodophyta | Rhodophyceae or Red micro algae, Seaweeds, thallus is highly differentiated, found in littoral zones; commercially important agar and carrageenan are the important polysaccharides from this group. | *Porphyridium sp,*<br>*Euchema,*<br>*Gelidium,*<br>*Gracilaria,* |

(Adopted from Levring , 1979)

**1.1. History of algae as food**

Historical records suggest that people collected macro algae and seaweeds for food around 2,500 years ago in China (Tseng, 1981). Europeans have collected seaweeds for food for 500 years. Of the macro algae, the most widely consumed throughout the world has been the membranaceous red alga *Porphyra*. This alga commonly known as "Nori", "amanori" or "hoshinori" in Japan and "purple laver" in the West. This one genus of red algae represents the largest tonnage of aquacultural product in the world (McCoy, 1987) and was the first marine macro algae to be cultivated by man. Nori has been grown in Tokyo Bay for nearly 300 years (Lobban et al, 1985). It is directly eaten in soups or as a vegetable or used as a condiment. Presently China and Japan are the two major growers (Mumford, 1990). The Japanese grow over 500,000 tons of Nori per year and consume over 100,000 tons directly per year. The Nori industry in

Japan employs over 60,000 people and is estimated to support over 300,000 people (McCoy, 1987). The Chinese also have a very large Nori industry. Major commercial centers for Nori include Marinan Islands, Saipan, and Guam. However, the world's largest and most technically advanced Nori farm facilities are present in the Philippines (McCoy, 1987). Nori is also consumed eaten in Europe, mainly in salads. The alga has also been fried in fat, boiled and even baked into bread. The British used to seal the fresh algae in barrels for use as food by whaling crews (Lerman, 1986). At present in Asia the turnover of Nori industry is about US$ one billion (Pulz and Gross, 2004).

The blue green alga *Spirulina* is another important algae, was eaten by the Aztecs in Mexico, who called it "Tecuitlat" (Farrar, 1966). The same algae forms the part of food of the Kanembou tribe north of Lake Chad in Central Africa, who make it into sauce called "Dihe". Another blue-green alga, *Phylloderma sacrum* is eaten in several region of Java. In India, Burma, Thailand and Vietnam various species of *Oedogonium* and *Spirogyra* are eaten (Becker and Venkataraman, 1982). Most people in the United States ingest red or brown algae products everyday in chocolate milk, toothpaste, candy, cosmetics, ice creams, salad dressing, and many other household and industrial products (McCoy, 1987). *Chlorella* is mainly sold in health food stores and as a fish feed (Hills and Nakamura, 1978).

### 1.2. Micro algae as a source of high value metabolites

Some of the commercially important algal species and their importance as a valuable product have been summarized in Table 2. The major biologically active constituents present in algae belong to the following groups.

#### 1.2.1. Pigments

Chlorophyll, carotenoids, algal tannins, fucoxanthins, phycocyanin and phycoerythrin are some of the important pigments that can be extracted from algae for its use.

#### i. Carotenoids

Carotenoids are organic pigments that are naturally occurring in plants and some other photosynthetic organisms like algae, some types of fungi and bacteria. Carotenoids are a group of fat soluble pigments (Ikan, 1991; Mastuno and Hirao, 1989) which are

isoprenoid polyenes. There are over 600 known carotenoids, which are split into two classes, xanthophylls and carotenes (Cunningham and Gantt, 1998). Carotenes are made up of carbon and hydrogen, without the oxygen group. Carotenoids with molecules containing oxygen, such as lutein and zeaxanthin, are known as xanthophylls (Cunningham, 2002; Hirschberg, 2001). Carotenoids form an important group of colorant too. Algae belonging to chlororphyceae contain α-carotene, β-carotene, lutein, violaxanthin and neoxanthin with some species also accumulating astaxanthin (Johnson and Schroeder 1995; Grung et al, 1992). In Rhododphyta, the predominant carotenes are lutein, zeaxanthin, α and β-carotenes, while in Pheophyta, the main pigments are β-carotene, violaxanthin and fucoxanthin (Shahidi et al, 1998). The pigments are usually associated in lipid globules located in the inter thylakoid space of the chloroplasts within plastids (Ben-Amotz and Avron, 1983) but occur as extra plastidic carotenoids in green algae *Haematococcus* (Lang, 1968) *Dunaliella* produces carotenoid during all stages of growth, while *Haematococcus* synthesizes carotenoids during the formation of aplanospores after cessation of growth.

**ii. Chlorophylls**

Chlorophylls are widely distributed in microalgae. Chlorophyll and phycocyanobilins are tetrapyrrol group of compounds. Chlorophyll a and chlorophyll b are the most abundant pigments in plants and in green algae. Besides chlorophyll a, brown algae and diatoms contain pigments similar to chlorophyll: the chlorophyll c and chlorophyll c esters (Garrido et al, 2000). Chlorophyll is used as a stable natural color additive in food products (Hutchings, 1994).

### 1.2.2. Protein

The different algal forms are known as rich sources of protein. Nutritional quality of algal protein is very high compared to conventional plants that we use in our regular diet (Becker, 2007). Protein content in the algal forms range from 15-65 % w/w. For example, *Spirulina* contains 60-70% (Becker and Venkatraman, 1982, Becker 2007), *Chlorella* sp containing 50-58%, *Dunaliella* containing up to 57%, *Porphyra* 28-39% by dry weight (Becker, 2007), and *Ulva* can also yield 26% protein (Burtin, 2003). Algal proteins are considered as single cell proteins. The advantages of these proteins

compared to plant and animal proteins are that these are simple and easily digestible (Becker, 2007).

### 1.2.3. Lipids

Algae contain a high proportion of omega-3 fatty acids concentrated in the galactolipid fractions (Khotimchenko, 2003; Guschina and Harwood, 2006). These fatty acids are very well known for their anticancer properties and play an integral role in cell membrane function and development of the brain and eyes. They are also associated with reduced risk of heart diseases and possibly in a reduced likelihood of behavioral problems, depression and inflammatory conditions, such as rheumatoid arthritis (Ruxton, 2004). The unicellular *Porphyridium cruentum* contains high concentration of arachidonic acid and eicosapentanoic acid (Cohen et al, 1988; Cohen and Cohen, 1991), whereas in the diatom *Phaeodactylum tricornutum* eicosapentanoic acid is more than 35% (Velso et al, 1991, Youngmanitchai and Ward, 1991). *Spirulina* is a richest source of gamma linolenic acid (GLA, 11% dry weight) that has great significance in pharmaceutics (Borowitzka 1988, 1994).

### 1.2.4. Polysaccharides

The commercial phycocolloids (all are polysaccharides) are extracted from brown and red marine macro algae or seaweeds. Alginate is extracted from the kelp (brown sea weeds), whereas agar and carrageenans are extracted from red algal species. Other polysaccharides found in marine algae include fucoidans (group of sulfated, fucose-rich polysaccharides) and laminarins (β-glucans present in brown algae), which are biologically active (Berteau and Mulloy, 2003; Fitton, 2003).

### 1.2.5. Amino acids

The single alga can serve as a source of several essential amino acids (Becker, 2007). This is mainly because they produce all the amino acids required for biological system. The pleasant savory flavor of some algae is due to sodium glutamate. Japanese chemist Ikeda (1909) discovered this and is a common flavoring agent found in the macro algae, *Laminaria* sp. Glutathione is a commonly found constituent of all macro algae. *Sargassum thumbergeii (*1.482g $100g^{-1}$) and *Ishige okamurai* (3.082 g $100g^{-1}$) were found to be exceptionally high in glutathione content (Kakinuma, 2001).

### 1.2.6. Antioxidants

Carotenoid pigments have long been considered to be antioxidants, although it is only in the last 10 years or so that investigators have begun to study their ability to interact with and quench free radical reactions either *in vitro* or *in vivo* (Burton and Ingold, 1984, Krinsky 1989, 1994a & b). There is increasing interest in the use and measurement of antioxidant capacity in food and pharmaceutical preparations and in clinical studies. The interest is mainly due to the role of reactive oxygen species (ROS), because these are involved in ageing process and pathogenesis of several diseases (Cao and Prior, 1998; Ames et al, 1993; Poli, 1993). ROS have also been known to damage proteins, carbohydrates and DNA in both *in vitro* and *in viv*o models (Halliwell and Gulteridge, 1990). These free radicals attack unsaturated fatty acids of biomembrane which results in lipid peroxidation, leading to desaturation of proteins and DNA, which causes series of deteriorative changes in the biological systems leading to cell inactivation (Reviewed by Krinsky, 1994a & b; Stahl and Sies, 2005). The antioxidants may act by raising the levels of endogenous defense by up regulating the expression of genes encoding the enzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase etc., (Serafin, 2006).

Burton and Ingold (1984) reported the antioxidant activity of β-carotene at different concentration (0.05-5 mM) with varying partial pressure of oxygen ($O_2$: 2-100%i.e., 15 to 750 torr). At low oxygen pressure (15 torr), β-carotene is an effective antioxidant by trapping free radicals. At higher oxygen pressure (> 150 torr) β-carotene losses its antioxidant activity and shows an autocatalytic, prooxidant effect particularly at higher concentrations. The same observation was confirmed by different workers (Stocker et al, 1987; Vile and Winterbourn, 1988).

Most of the edible algal forms are rich sources of one or the other antioxidant form. They accumulate high amount of antioxidant principles (e.g., β- carotene in *Dunaliella*, astaxanthin from *Haematococcus* etc) because, they have to survive in high stress conditions compared to higher plants. *Enteromorpha* and *Kappaphycus* are good sources of ascorbic acid. *Caulerpa* sp are good sources of antioxidant enzymes such as catalase, peroxidase and superoxide dismutase. Antioxidant activity of *Spirulina* and *Dunaliella* are well documented both *in vitro* and *in vivo* (Miranda et al, 1998; Chidambara Murthy et al, 2005b).

### 1.2.7. Minerals and vitamins

Potassium, sodium, calcium, magnesium, iodine and other essential elements are found in high concentrations in algae (Kikunaga et al, 1999). The content varies with species, but algae are rich in tocopherol, B vitamins and vitamin A. *Porphyra* species are especially rich in vitamin D (Aaronson, 2000). Algae are also known to contain both water soluble and fat-soluble vitamins. *Spirulina* and Nori are the two important sources of cyanocobalamine (vitamin $B_{12}$) (Berg et al, 1991). *Dunaliella*, *Spirulina* contains β-carotene, which is important precursor of vitamin A (Borowitzka, 1988; Borowitzka and Borowitzka, 1989). Excretion of vitamins $B_{12}$, $B_1$ and biotin (Nakamura and Gowans, 1964), folic acid ($B_9$) and pantathonic Acid ($B_5$) (Aaronson et al, 1980) by fresh water *Chlamydomonas* cells has been reported.

### 1.2.8. Algae as a source of pharmaceuticals

Heparins like sulphated polysaccharides from red algae, was used to inhibit herpes and immuno-deficient virus (Gonzalez et al, 1987). Carrageenan and other sulphated polysaccharides inhibit both DNA and RNA virus *in vivo* and *in vitro (*Gonzalez et al, 1987). Antibiotics and antiviral compounds are the important class of valuable products obtained from algae (Borowitzka, 1994, 1995).

Cyanobacterial extracts have shown activity against *Herpes simplex* virus type-II and also against respiratory syncytial virus at higher concentrations (Lau et al, 1993). Cyanobacteria also produces a number of cytotoxic compounds namely, tubericidin and toyocamycin from *Streptomyces* (Patterson et al, 1991). Scytophycin B from *Scytonema pseudohoffmanni* has shown cytotoxic effect against KB (a human nasopharyngeal carcinoma) cell line at the concentration of 1 ng $mL^{-1}$. Indocarbazoles isolated from *Nostoc* has also shown the activity in the human carcinoma cell lines (Knubel et al, 1990). The acutiphycins from *Oscillatoria acutissima* and other macrolides have shown activity against KB and lung carcinoma cell lines (Brachi et al, 1984). *Spirulina* and *Dunaliella* extracts have shown anticancer activity against oral cancer cell line as well as in case of tobacco induced buccal cancer in human volunteers (Shklar and Schwartz, 1988). This may be due to the presence of both β□-carotene and phycocyanin in algal extract. The algal β□- carotene (Sude et al, 1986) and phycocyanin (Gerwick et al, 1994) has shown the anti cancer property in oral carcinogeneis.

Caulerpenyne is a sesquiterpene isolated form marine alga *Caulerpa taxifolia* has shown antiproliferative and apoptotic activity in human neuroblastoma cell lines (Caves et al, 2006). Red alga *Amphiroa zonata* has shown the presence of palmitic acid, which has shown antitumor activity in both *in vivo* and *ex vivo* (Laycock et al, 1989).

**Table 2. Reported literature on production of high value metabolites of biological significance from algae**

| Algal sp | Compounds of Interest | Reference |
|---|---|---|
| *Dunaliella sp* | β-carotene, Glycerol, Protein | Del campo et al, 2007; Ben-Amotz and Avron, 1990 |
| *Spirulina* | High protein, Essential amino acids, vitamin B complex and E, Gamma linolenic acid, β-carotene, Phycocyanin, Chlorophyll | Becker 2007; Becker and Venkataraman, 1982 |
| *Haematococcus* | Astaxanthin | Del campo et al, 2007; Higuera-Ciapara et al, 2006 |
| *Botryococcus* | Hydrocarbon | Banerjee, 2002; Dayanand et al, 2005 |
| *Porphyridium* | Phycoerythrin | Dufosse et al, 2005; Kathiresan, 2007 |
| *Chlorella* | Lutein, Protein, minerals | Becker and Venkataraman, 1982 |
| *Scenedesmus* | Protein, Essential amino acids | Becker and Venkataraman, 1982 |

| | | |
|---|---|---|
| *Kappaphycus* | Protein, iron, α-tocopherol, ascorbic acid, β - carotene | Moore et al, 1988<br>Fayaz et al, 2005 |
| *Enteromorpha* | Protein, ascorbic acid, Iron | Moore et al, 1988 |
| *Porphyra* | Protein, Essential amino acids, vitamins | Moore et al, 1988 |

**1.3. Culture conditions and commercial production of microalgae**

A micro alga is generally referred to as organism that is less than 2mm in diameter (Gupta, 1981). Today, micro algae are cultivated for food or for products synthesized by the algal cells. The history of cultivation of these small plants date back to the Aztecs (Sommer, 1988). The micro algae are rich in protein, carbohydrates, amino acids, trace elements and vitamins (Waaland, 1981).

In the modern days unialgal cultures are becoming very important industrially. The first unialgal cultures were achieved by Beijerinck (1890) with *Chlorella* v*ulgaris*. In the 1950's the Carnegie Institute made a major research program to study the potential for mass culturing of *Chlorella* to fight against the world hunger. In general, algae exhibit high growth rates and yields i.e., approximately 30 tonnes of dried cell powder is produced in one year from one-acre pond (Hills and Nakamura, 1978). Commercial large-scale culture of micro algae commenced in the early 1960's in Japan with the culture of *Chlorella* (Tsukada et al, 1977) followed by the establishment of *Spirulina* in Lake Texcoco, Mexico by Sosa Texcoco S.A. (Durand-Chastel, 1980). The blue-green algae (=cyanobacteria) *Spirulina*, was traditionally harvested from natural lakes as a protein source by the Aztec Indians and some North American tribes. *Spirulina* was initially regarded as a nuisance in the ponds of Mexico. But in late 1960's this species was rediscovered as a valuable food source. First experimental harvesting of *Spirulina* was carried out in Mexico to feed the poor people of the country. However, a much more lucrative market existed in the United States and Japan as a health food. Today the major use of *Spirulina* is for the extraction of phycocyanin, a blue photosynthetic pigment. The pigment has commercial uses as a natural food color and cosmetic ingredient. In 1977 Dai Nippon Ink and Chemicals Inc. established a commercial *Spirulina* plant in Thailand, and by 1980 there were 46 large-scale

factories in Asia producing more than 1000 kg of micro algae (mainly *Chlorella*) per month (Kawaguchi, 1980) and in 1996 about 2000 tonnes of *Chlorella* were traded in Japan (Lee, 1997).

The large-scale production of cyanobacteria commenced in India at about the same time (Becker and Venkataraman, 1982; Venkataraman and Becker, 1985). In the area of algal biotechnology at Central Food Technological Research Institute, India, studies have been carried out on mass cultivation of *Spirulina* and *Scenedesmus* with the focus on processing of algal biomass for use as a source of protein, vitamins, minerals and nutraceuticals. *Spirulina* technology has been transferred to several industries in India, which produce algae of international quality. Several formulations of *Spirulina* are already available in the Indian market. Algae are also a good source of colorants such as phycocyanin, which is a blue pigment of importance obtained from *Spirulina* (Becker and Venkataraman, 1982).

Commercial production of *Dunaliella* sp as a source of β-carotene became the third major micro algal industry when production facilities were established by Western Biotechnology Ltd and Betatene Ltd in Australia in 1986. Other commercial plants soon followed in Israel and in USA. *Dunaliella* species are grown for the photosynthetic pigment, β-carotene, a vitamin A supplement. It is grown in high salinity ponds in California, Hawaii, Israel and Australia (Oren, 2005).

Thus in a short period of about 30 years the industry of micro algal biotechnology has grown and diversified significantly (Borowitzka, 1999). A common feature of most of the algal species produced commercially (i.e. *Chlorella*, *Spirulina a*nd *Dunaliella*) is that they grow in highly selective environments which mean that they can be grown in open-air cultures and still remain relatively free of contamination by other algae and protozoa. Thus, *Chlorella* grows well in nutrient-rich media (Soong, 1980), *Spirulina* requires a high pH, > 9.0 and bicarbonate concentration (Belay, 1997) and *Dunaliella* grows at very high salinity (Borowitzka and Borowitzka, 1988).

### 1.4. Algae cultivation methods

Most of the literatures available till date are concerned with the physiology and growth conditions for cultivation of algae under indoor conditions to obtain compounds of interest and market demand (Boussiba and Vonshak, 1991; Lu et al,

1995; Dufosse et al, 2005; Spolaore et al, 2006; Del Campo et al, 2007, Raja et al, 2007).

Micro algae can efficiently utilize the energy from the sun, water and $CO_2$ from the air to convert into biomass. They exhibit properties that make them well suited for use in a commercial scale production. Many species exhibit rapid growth and high productivity and many micro algal species can be induced to accumulate substantial quantities of high value metabolites.

The advantages of utilization of algae for its commercial use have been widely accepted due to following reasons.

**i.** They are photosynthetic forms and hence can trap solar energy.

**ii.** Algae have short life cycle and can be multiplied fast.

**iii.** Algae can be easily manipulated for desired components compared to plants.

**iv**. They are rich sources proteins, vitamins, minerals and other bioactive molecules.

**v.** Their simple physiology makes them to easily acclimatize to different environmental condition.

**vi**. They can be scaled up to commercially viable levels.

All these aspects are very well observed in case of commercialized algal forms like *Chlorella*, *Spirulina* and *Dunaliella.*

Most of the culture systems in use today are either closed or open system. The cultivation systems are selected based on the nature of the organism and depending on its requirements such as media composition, pH and salt content. In open cultivation methods, tanks or ponds are built using cement or plastic with polymer lining inside depending on the algal form. In case of *Dunaliella,* high salt content and raise in pH upon growth will make it less susceptible for contamination and hence it is possible to cultivate in open-air conditions.

Alternate method is closed culture systems. Though it is too expensive, contamination by other species and protozoa can be virtually eliminated in closed systems such as the tubular photo bioreactors (Chaumount et al, 1988). The growth conditions can also be optimized and closely controlled, resulting in higher cell densities and better carotenoids yield per unit volume compared to open-air cultures. On the other hand, closed systems require pumping of the culture for circulation. However, the limiting factors are high capital cost and a higher operating cost. Cultivation of

*Haematococcus* sp in closed systems is a good example because of its neutral pH requirement and highly prone to contamination. Some of the commonly used cultivation systems employed for *Dunaliella* are presented below in Table 3.

**Table 3. Cultivation techniques employed for different algal systems worldwide**

| Type of culture system | Type of tank | Major Location |
|---|---|---|
| Open culture systems | Extensive open ponds | Australia, Myanmar and Mexico |
| | Raceway ponds | China, Chile, India, Israel, USA, Vietnam |
| | Continuous culture tanks | USA and China |
| | Very large, shallow ponds | Australia |
| | Natural ponds | Australia, Ukraine |
| | Earth well lined unstirred ponds | Australia, Chile |
| | Paddle wheel driven raceway ponds | Israel, Japan, USA |
| Closed culture systems | Tubular photobioreactors | Germany, Israel |
| | Large bags | China, Japan |

(Adopted from Raja et al, 2007)

### *1.5. DUNALIELLA*

The scientific records of the brine algae *Dunaliella* date from one hundred and fifty years ago when the French engineer, Dunal, showed interest on the reddish coloration of the salt crystallizers in the Mediterranean salt fields (Oren, 2005). Dunal examined the samples of red brine under microscope; he saw that they were red-colored, biflagellate cells, which he identified as *Haematococcus*, because they resembled similar algal cells. Later scientist, Teodoresco closely examined similar cells from salt ponds in Rumania and noted that unlike *Haematococcus*, they lacked cell walls. He gave them a new generic name, *Dunaliella*, in honor of the French scientist Dunal (Borowitzka and Borowitzka, 1988).

Massyuk (1973) systematically examined the taxonomic details, revised the genus and recognized a total of 29 species as well as a number of forms and varieties (Borowitzka and Siva, 2007). Some of the important and extensively found species

are *Dunaliella bardawil, D. salina, D. bioculata, D. teritolecta, D. parva, D. granulata* and *D. primolecta*. Commercially cultivated strains for the production of natural carotenoids are *D. bardawil* and *D. salina.*

The classification of *Dunaliella* is given below.

**Table 4. Taxonomical classification of *Dunaliella*.**

| | |
|---|---|
| **Super kingdom** | Eukaryota |
| **Kingdom** | Viridiplantse |
| **Subkingdom** | Phycobionta |
| **Division** | Chlorophyta |
| **Class** | Chlorophyceae |
| **Order** | Dunaliellales |
| **Family** | Dunaliellaceae |
| **Genus** | *Dunaliella* |

(Adopted from Borowitzka and Siva, 2007)

### 1.5.1. Morphology of *Dunaliella*

*Dunaliella* is a unicellular biflagellate alga with two equal and long flagella, contains one large cup shaped chloroplast, which occupies half of the cell volume (Fig. 1). *Dunaliella* are generally ovoid in shape, 4-10 μm wide and 6-15 μm long (Ben-Amotz and Avron, 1983; 1990). The cells are motile and chloroplast contains a large pyrenoid surrounded by polysaccharide granules (a storage product). The chief morphological character of *Dunaliella*, in contrast to other members of chlorophyta is that it lacks a rigid polysaccharide cell wall. Cell is a natural protoplast, enclosed by thin elastic membrane, which is responsible for its rapid change in shape and response to osmotic changes (Ben-Amotz and Avron 1983; Ben-Amotz et al, 1982; Borowitzka and Siva. 2007).

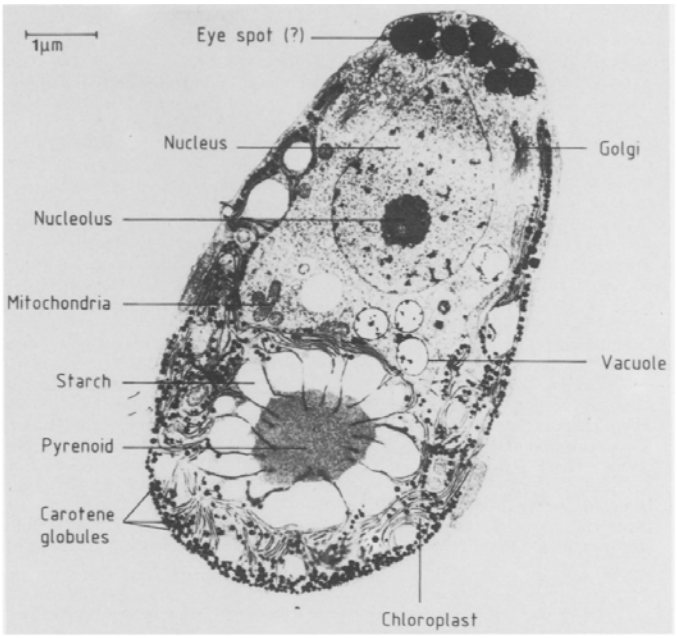



**Fig. 1. Electron micrograph section of *Dunaliella bardawil*** showing the lack of a cell wall with the presence of only a thin cellular membrane, the single, large cup-shaped chloroplast with its photosynthetic thylakoid membranes, pyrenoid and starch, and the numerous β-carotene globules within the chloroplast (Adopted from Ben Amotz and Avron, 1990)

### 1.5.2. Reproduction in *Dunaliella*

*Dunaliella* multiplies by longitudinal division of the motile cell or by fusion of two motile cells to form a zygote. Fusion of two equally sized gametes to form a zygote was reported by many of the researchers (Hamburger 1905; Teodoresco 1906). Detailed study on sexual reproduction of *Dunaliella* was reported by Lerche (1937), who witnessed the zygote formation in five of six species studied (*D. salina, D. parva, D. peircei, D. euchlora* and *D. minuta*). The formation of zygote in *Dunaliella* can be induced by reducing the salt concentration from 10 to 3.0 % in culture medium (Lerche, 1937).

In the process of sexual reproduction, the flagella of the two cells come closer, with the gametes forming a cytoplasmic bridge and ultimately fuse to form the zygote. Thus formed zygote has a thick outer layer and can withstand exposure to freshwater and also survive prolonged period of dryness. These zygotes germinate with the release of up to 32 haploid daughter cells through a tear in the cell envelope. Lerche (1937) performed a series of experiments in which carotenoid rich red cells were crossed with green cells, enableling them to form zygote. Possibility of formation of asexual resting cysts by *D. salina* was explained by Hamburger (1905). Later Loeblich (1969) reported the formation of such cysts in media with low salinity.

When exposed to extreme conditions, such as low or high salinity, some strains of *Dunaliella* form a palmella stage (Lerche (1937). A comprehensive account of *Dunalliella* taxonomy, morphology, reproductions are recently dealt in a review by Oren (2005) and Borowitzka and Siva (2007).

### 1.5.3. *Dunaliella* cultivation

*Dunaliella* was reported to be growing near Montpellier, Mediterranean coasts of France. Later in 19$^{th}$ century, other biologist observed algae similar to the one reported by Dunal in hyper salinity lakes near Crimea (Butschinsky 1897), Algeria (Blanchard 1891), Lorrine, France (Florentin 1899) and Romania (Bujor 1900). First quantitative occurrence of the algae was reported in lakes of Dead Sea, cell density of $4\times 10^4$ cells ml$^{-1}$ was observed on surface water (Kaplan and Friedmann, 1970).

There are two commercially important species of *Dunaliella* i.e*., D. salina* and *D. bardawil.* In early years they were used for the production of glycerol and protein but now the focus is on carotenoids especially the β-carotene and xanthophyll, lutein. The halotolerant alga *D. bardawil* possesses the unique ability to accumulate a very high content of β-carotene. The amount of β-carotene in *D. bardawil* under controlled conditions can be manipulated by different growth conditions, to vary from about 3 to 8% (Ben-Amotz and Avron, 1983). The β-carotene found in *D. bardawil* contains almost equal amount of cis and trans β-carotene owing to 90% of total carotenoids, with the rest composed mostly of lutein and other carotenes (Johnson et al, 1996; Ben-Amotz et al, 1982).

*Dunaliella* demonstrates a remarkable degree of environmental adaptation to salt and is widely distributed in natural habitat. It is generally found in oceans, salt marshes, and salt water ditches near sea. It can survive in a range of salt (sodium chloride) concentration ranging from 0.1M (less than sea water) to saturation level (5.0 M). Under optimum growth conditions doubling time of *Dunaliella* is 5 h, which can go up to 3 days under salinity limitations (Ginzburg and Ginzburg, 1981). Initially cells can grow in low salt concentration and upon growth they can adapt to 2-3 fold hypertonic and hypotonic change in salt concentrations (Ben-Amotz and Avron 1983; 1990).

### 1.5.4. Carotenoids and other chemicals from *Dunaliella*

Carotenoids are the major nutraceutical constituent in *Dunaliella*. *Dunaliella* sp are the richest known source of dietary β-carotene (Ben-Amotz and Avron, 1983). In addition to this, alga contains a mixture of natural carotenoids. Some of the major carotenoids include: β-carotene, α-carotene, lutein, zeaxanthin and cryptoxanthin. β-carotene is accumulated as lipid globules in the interthylakoid spaces of the chloroplasts in *Dunaliella* (Vorst et al, 1994). They protect the algae from damage caused during excessive irradiances by acting as a light filter. Carotenoids also prevent the formation of reactive oxygen species, quenches the triplet-state chlorophyll or the formation of singlet oxygen ($^1O_2$) (Telfer, 2002). The isomeric composition of β-carotene from *Dunaliella* contains more than 50% of 9-cis-β-carotene (Johnson et al, 1996). β-carotene obtained from *Dunaliella* has many advantages like increased absorption and high efficiency (Dufosse et al, 2005). The chemical composition of this alga is listed in the following table.

**Table 5. General composition of *Dunaliella* sp**

| Compounds of Interest | Composition (dry weight) | Reference |
|---|---|---|
| β-carotene | 3 – 8% | Ben-Amotz and Avron, 1983 |
| Protein | 50-60% (green cells)<br>30% (red cells) | Borowitzka and Borowitzka, 1988 |
| Carbohydrate | 40% (green cells)<br>11% (red cells) | |
| Lipid | 6% (low salinity)<br>18% (high salinity) | |
| Fatty acids | Palmitic acid (31%),<br>Oleic acid (13%),<br>Linoleic acid (20%),<br>γ- Linolenic acid (17%) | |
| Thiamine, pyridoxine, riboflavin, nicotinic acid, biotin and α-tocopherol | Detectable levels | |

### 1.5.5. Biosynthesis and accumulation of carotenoids in *Dunaliella*

Carotenoids are isoprenoids synthesized by the isoprenoid pathway. Isopentanyl pyrophosphate (IPP) is the common precursor of many of the isoprenoid compounds. Several reviews stated that the initial steps of carotenoid synthesis are common to all carotenogenic organisms (Fraser and Bramley, 2004; Sandmann, 2002; Hirschberg, 2001). $C_{20}$ - geranylgeranyl pyrophosphate (GGPP) is synthesized through a series of condensation steps. The condensation of two GGPP molecules forms the precursor of most carotenoids, 15-cis-phytoene ($C_{40}$). This reaction is carried out by phytoene synthase, the first enzyme in the pathway specifically found in carotenogenic organisms (Armstrong et al, 1990).

Conversion of phytoene to lycopene occurs through two desaturation steps, carried out by two desaturases, phytoene desaturase (PDS) and z-carotene desaturase (ZDS). Lycopene can be cyclized by several different lycopene cyclases to generate carotenes with either β- or ε-ionone end groups (Fig. 2). Through two rounds of hydroxylation, β-carotene and α–carotene are converted to the xanthophylls, zeaxanthin or lutein respectively (Moise et al, 2005). Reports on the enzymes and proteins involved in β-carotene regulation in *Dunaliella* are very scanty. β-carotene is accumulated in intraplastid lipid globules (Gonzalez et al, 2005), which are stabilized and maintained by a peripherally associated 38 KD protein called the carotene globule protein (CgP). Probably, CgP is involved in stabilizing the globules within the chloroplast (Katz et al, 1995). Rabbani et al, (1998) suggested that induction of CgP and deposition of triacylglycerol are in parallel with β-carotene accumulation. In addition, inhibitors of lipid biosynthetic pathway affected the β-carotene formation (Katz et al, 1995). All these reports imply that the formation and stabilization of globules are the main factors associated with β-carotene accumulation.

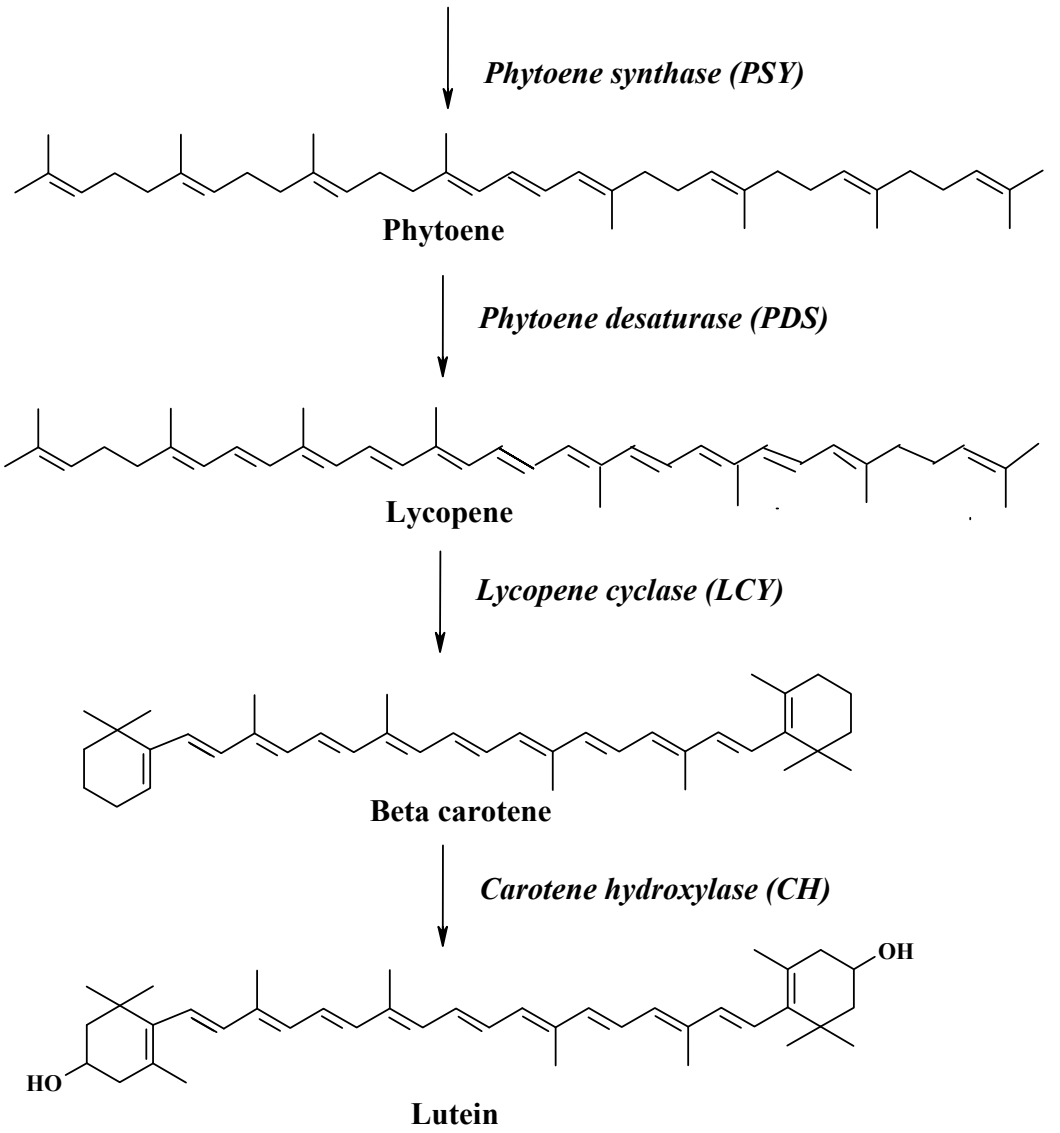



**Fig. 2. Brief description of carotenoid biosynthetic pathway in *Dunaliella bardawil*.** (Re-drawn based on literatures, using ISIS draw) The carotene biosynthesis enzymes phytoene synthase (*PSY*), phytoene desaturase (*PDS*), lycopene cyclase (*LCY*), and carotenoid hydroxylase (*CH*) are known to be the key steps in the carotenogenesis.

## 1.6. CAROTENOIDS

### 1.6.1. Distribution of carotenoids

Carotenoids are colored lipid-soluble compounds, which are found in higher plants and algae. In nature about 600 carotenoids have been isolated and characterized (Yeum and Russel, 2002; Stahl and Sies, 2005). The total carotenoid production in nature has been estimated to be approximately 100 million tonnes per annum by all the living organisms (Krinsky and Johnson, 2005). They are distributed widely in fruits, flowers, birds, insects and marine animals (Krinsky and Johnson, 2005). Carotenoids are synthesized *de novo* by higher plants, mosses, liverworts, algae, photosynthetic and non-photosynthetic bacteria and fungi (Del campo et al, 2007). All the carotenoids in photosynthetic tissues are located in the grana of the chloroplast and consist of the same major group of pigments. Major ones are β -carotene, lutein, violaxanthin and neoxanthin and smaller amount of α-carotene, β-cryptoxanthin, zeaxanthin and antheraxanthin (Ladygin 2000). In photosynthetic organisms the carotenoids are most often masked by chlorophyll present in chloroplast (Goodwin, 1979).

### 1.6.2. Advantages of chemical Vs biological carotenoids

Synthetic β-carotene mainly consists of all-*trans* β-carotene, whereas naturally occurring β-carotene is made up of all-*trans* β-carotene and 9 *cis* β-carotene and to a lesser extent as 13 *cis* β-carotene. There are several advantages of chemical synthesis for carotenoid production. Chemical synthetic technology can produce carotenoids of exceptional purity and consistency and the overall costs of production of these carotenoids are quite low. Chemical synthesis produces mixtures of stereo isomer, some of which may not be found in nature, may not be as active as the naturally occurring carotenoid isomer, may not be desired by the consuming public, or may have undesired side effects (Ausich, 1997). *Dunaliella* containing equal mixture of cis and trans isomers had a greater ability to prevent methyl-linoleate peroxidation than synthetic β-carotene in *in vitro* model study (Levin and Mokady, 1994).

### 1.6.3. Carotenoids and Health

Carotenoids are responsible for the red color of tomatoes and the orange color of carrots. The greater the intensity of the color of the fruit or vegetable, the more

carotene it contains. Apart from their aesthetic role, dietary carotenoids, or foods rich in these colorful pigments, are considered to be beneficial in the prevention of a variety of major diseases, including certain cancers and eye diseases. (Ames et al, 1993; Krinsky and Johnson, 2005). Carotenoids are currently being manufactured for animal and human consumption. Carotenoids are used as pigments to color the skin or egg yolks in poultry, to color the flesh of fish grown under aquaculture conditions and to color the shells of crustaceans. Carotenoids are important to humans and other animals as precursors of vitamin A and retinoids. In addition, they act as antioxidants, immunoenhancers, inhibitors of mutagenesis and transformation, inhibitors of premalignant lesions, screening pigments in eye and nonphotochemical fluorescence quenchers. Increased dietary intake of carotenoids is associated with decreased risk of macular degeneration and cataracts, some cardiovascular diseases and cancers (Krinsky, 1993). In general the beneficial effects of carotenoids can be attributed to its antioxidant activity, which includes preventing lipid peroxidation, anticancer, antiaging, cardiovascular diseases, eye health etc., The other benefits of the carotenoids includes growth inhibition of tumor cell lines, antimutagenic activity, protective effects on genotoxicity like, DNA damage, formation of micro nucleated cells, sister chromatid exchanges, and prevention of chromosomal aberrations, translocations, mutagenesis or even death of the cell. The various aspects of genotoxicity have been modified by the addition of carotenoids. The carotenoids that have been most studied in this regard are β-carotene, lycopene, lutein and zeaxanthin. Lutein and zeaxanthin are thought to have an additional role of absorbing the damaging blue light that enters the eye, thus preventing light-associated damage, such as the development of age-related macular degeneration and cataracts (Reviewed by Rodrigues and Shao, 2004).

### 1.6.4. Biological action of carotenoids as a vitamin A precursor

Carotenoids are being intensively investigated regarding their potential to prevent chronic disease and vitamin A deficiency (VAD) (von Lintig et al, 2005). In humans, VAD leads to night blindness in milder forms, while more severe progression results in corneal malformations, e.g., xerophthalmia. Besides visual defects, this deficiency affects the immune system, leads to infertility or causes malformations during embryogenesis. Being essential for vision, in vertebrates the vitamin A derivative

retinoic acid (RA) is a major signal-controlling molecule in a wide range of biological processes (Underwood, 2004). VAD is still a major problem particularly in developing countries. Vitamin A demand can be met either by supplementing vitamin A or carotenoids with provitamin A activity. All naturally occurring vitamin A in the food chain derives from provitamin A conversion and the world's population mainly relies on carotenoids from staple food sources to meet vitamin A requirements. Retinoids, vitamin A (retinol) metabolites and analogs, are physiological regulators of a large number of essential biological processes including embryonic development, vision, reproduction, bone formation, metabolism, hematopoiesis, differentiation, proliferation and apoptosis (Sun and Lotan, 2002).

#### 1.6.4.1. Pathways of carotene cleavage

There are two major pathways for the conversion of vitamin A from β-carotene the central cleavage and the eccentric cleavage pathway (Glover, 1960). The central cleavage mechanism splits β-carotene at the central double bond (Castenmiller and West, 1998) by a specific enzyme, β-carotene 15,15'-oxygenase (E.C. 1.13.11.21), to yield two molecule of retinal in intestinal cells and liver cytosol (Goodman and Olsen, 1969). The cleavage product, retinaldehyde, can be reversibly reduced to retinol (vitamin A) or irreversibly oxidized to retinoic acid (Fig. 3) (Olson and Lakshman, 1990). In eccentric cleavage pathway, β-carotene is cleaved to a molecule of retinal and β-apocarotenals with different chain lengths by cleavage at random position of its conjugated double bonds. The aldehydes were further cleaved to the short-chain carbonyl compounds or oxidized to retinoic acid by the β-oxidation pathway (Wang et al, 1991). However several studies have shown exclusive central cleavage of β-carotene in the intestines of guinea pig (During et al, 1998), pig (Nagao et al, 1996, Prince and Frisoli, 1993), rat, hamster (Devery and Milborrow, 1994) and human beings (van Vliet et al, 1995, 1996).

The extent to which central and eccentric cleavage pathways contribute to vitamin A formation was studied using pig intestinal homogenate (Nagao et al, 1996) and they have found that 94% of the β-carotene consumed was converted to retinal, and no formation of β-apocarotenals was observed. These results clearly indicated that the enzyme preparation of pig intestinal mucosa converted β-carotene to retinal exclusively by central cleavage. The same was confirmed by an *in vivo* study using

rats (Barua and Olson, 2000). Therefore, central enzymatic cleavage of β-carotene has an essential role to provide vertebrates with vitamin A.

Recently, German scientists successfully cloned and sequenced cDNAs encoding enzymes having β-carotene 15,15'-oxygenase activity from *Drosophila* (von Lintig and Vogt, 2000) and chicken duodenal tissue (Wyss et al, 2000 and 2001).

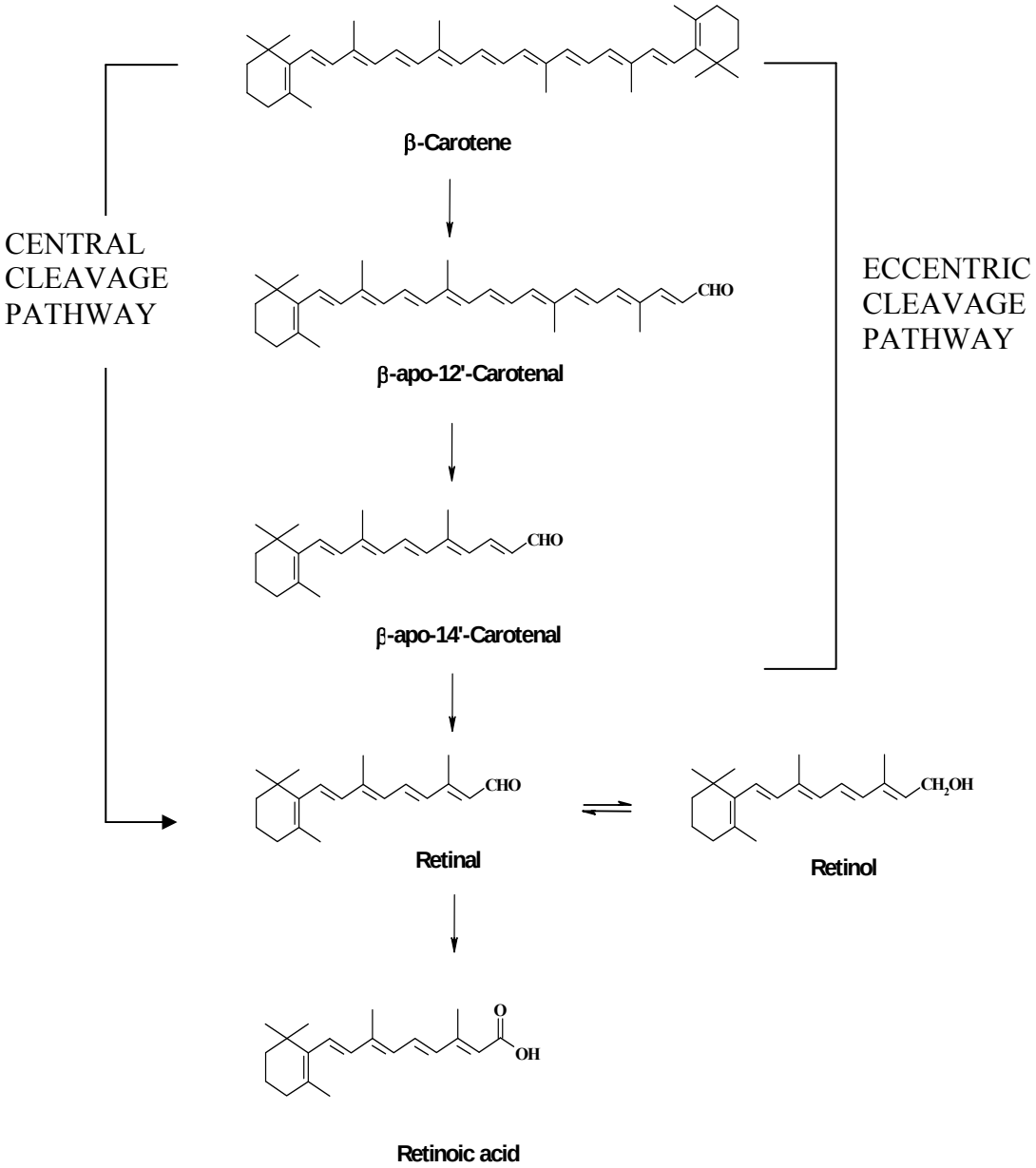



**Fig 3. Schematic representation of cleavage pathway of β-carotene to retinol in biological system** (re-drawn based on literatures, using ISIS draw). In central cleavage mechanism β-carotene is converted to two molecules of retinal by a single step. In eccentric cleavage β-carotene is converted to one molecule of retinal along with apocarotenals through a sequential steps.

### 1.6.5. Carotenoid bioavailability

Bioavailability is defined as the fraction of an ingested nutrient that is available for utilization in normal physiological functions or for storage (Jackson, 1997). Published information on carotenoid bioavailability is based mainly on measurement of carotenoids in serum or plasma after ingestion. It was noted that at steady state, plasma carotenoids amount to approximately 1% of the total body content of carotenoids, whereas the highest concentration of β-carotene was found in the liver (Schmitz et al, 1991).

### 1.6.6. Methods to determine bioavailability of carotenoids

The methods for determining bioavailability of carotenoids are reviewed by Yeum and Russell (2002). Some of the methods include Serum/Plasma response after carotenoid ingestion (Rock and Swendseid, 1992; Yeum and Russell, 2002), Chylomicron response after carotenoid ingestion (Johnson and Russell, 1992; van Vliet et al, 1995; van den Berg and van Vliet, 1998)), Oral-Fecal Balance Technique (Yeum and Russell, 2002), Stable Isotope Application (Blomstrand and Werner, 1967; Goodman et al, 1966) and Macular Pigment Density Measurement (Yeum and Russell, 2002).

#### 1.6.6.1. Serum/Plasma response after carotenoid ingestion

Most research has concentrated on determining serum or plasma concentrations of provitamin A carotenoids, especially β-carotene. Comparatively little is known about the occurrence, function, and bioavailability of non-provitamin A carotenoids. Plasma or serum carotenoid responses (concentration vs. time curves) have been widely used to measure carotenoid bioavailability, because this method provides an estimate of relative bioavailabilities using simple procedures. In this method quantitated amounts of carotenoids are ingested and changes in serum concentration of carotenoids are measured at various time intervals following ingestion (Parvin et al, 2000). Serum response curves are drawn using either single or multiple doses. A rise in serum concentration followed by a fall is generally measured. However, in chronic dose trials, serum carotenoid concentrations reach a constant elevated level of various magnitudes (Yeum and Russell, 2002).

### 1.6.6.2. Chylomicron response after carotenoid ingestion

Carotenoid concentrations in triglyceride-rich lipoprotein (TRL) fractions (mixtures of chylomicrons and very low density lipoproteins) in the intestine have also been used to estimate the β-carotene absorption and conversion to retinyl esters (van Vliet et al, 1995). Advantages of this method over the serum response curve method are that (*a*) the method accounts for intestinal conversion to retinyl esters; (*b*) it improves the distinguishability of newly absorbed carotenoids from endogenous pools; and (*c*) it allows for the use of smaller doses (Yeum and Russell, 2002). Potential limitations of this approach is that food matrices that are slowly digested result in slow rates of carotenoid absorption and thus yield little or no rise of carotenoids in the TRL fraction. As observed with serum response curves, TRL response curves are highly variable (van Vliet et al, 1995; van den Berg and van Vliet, 1998), especially among subjects, even when treatment conditions are highly standardized. This may be due to variability in carotenoid absorption as well as in the kinetics of chylomicron secretion and clearance depending on the individual.

### 1.6.6.3. Oral-Fecal balance technique

Comparison of carotenoid consumption with its fecal excretion (i.e., balance) has been used for the estimation of absorption of carotenoids, particularly from foods. Balance studies involve the estimation of carotenoid intake and the collection and analysis of all feces for carotenoids over a period of time, because there is no urinary excretion of either free or conjugated carotenoids and there is negligible loss with exfoliation from skin. The balance method has major limitations: It does not account for (*a*) carotenoid degradation in the upper (chemical oxidation) or lower (microbial degradation or alteration) regions of the gastrointestinal tract (*b*) the excretion of endogenously secreted carotenoids. Therefore, it is not surprising that oral-fecal balance studies have yielded considerable variation in estimates of carotenoid absorption, even with the similar carotenoid sources or preparations. In an attempt to overcome this limitation, Bowen et al, (1993) modified the method by using gastrointestinal lavage (washout) after allowing a defined period for digestion and absorption. The advantage of this approach is that it controls the residence time of nonabsorbed carotenoids in the lower gut, thus limiting micro floral degradation.

However, the duration of the allowed absorption period in this approach is arbitrary and it may alter gastrointestinal physiology (Yeum and Russell, 2002).

#### 1.6.6.4. Stable isotope application

The development of stable isotope labeled carotenoids has made it possible to (*a*) distinguish between dosed and endogenous carotenoids, (*b*) assess the extent of intestinal conversion of vitamin A, (*c*) estimate absolute absorption and post absorptive metabolism, and (*d*) use doses that are low enough to avoid influencing endogenous pools (Novotny et al, 1995). In this method single doses of deuterated ($^{2}H$) or $^{13}C$-enriched β-carotene were administered to subjects under standardized conditions. Serial blood samples were drawn at baseline frequently over the first 16 hr, then less frequently, if post absorption data are required. Because the absorption peak typically occurs at 4–5 hr after dosing, frequent sampling (at least hourly) is needed during this period to obtain accurate area under curve kinetic parameters. Hence, stable isotope labeling approaches appear to be the best approach for studying carotenoid bioavailability (Yeum and Russell, 2002).

#### 1.6.6.5. Macular pigment density measurement

The oxygenated carotenoids, lutein and zeaxanthin, are a major macular pigment of the human retina. Macular pigment density, which can be assessed to know its relationship with carotenoid intake, may be a functional indicator of the bioavailability of lutein or zeaxanthin (Bone et al, 1988, 2000, 2001; Snodderly and Hammond, 1999).

### 1.6.7. Carotenoid absorption and metabolism

After consumption of carotenoid-containing foods, carotenoids are released from their food matrix, absorbed and incorporated into mixed micelles, which consist of bile acids, free fatty acids, monoglycerides, and phospholipids. Absorbed β-carotene, and presumably the other provitamin A carotenoids, can undergo oxidative cleavage in intestine as well as in other organs such as liver. Carotenoids appear to be absorbed by the mucosa of the small intestine (mainly in the duodenum) via passive diffusion (Hollander and Ruble, 1978; Parker, 1996) and gets packaged with triacylglycerol-rich chylomicrons. Provitamin A carotenoids, such as β-carotene, α-carotene and β-

cryptoxanthin, are partly converted to vitamin A, primarily retinyl esters, in the intestinal mucosa, and both carotenoids and retinyl esters are incorporated into chylomicrons and secreted into lymph for delivery to the blood stream, where the chylomicrons are rapidly degraded by lipoprotein lipase. The resulting chylomicron remnants containing carotenoids are rapidly taken up by the liver (Parker, 1996).

Under normal nutritional conditions, liver acts as the storage organ of vitamin A. Up to 80% of the total retinol plus retinyl esters are stored in the liver (Blomhoff et al, 1991). In the hepatocytes, retinyl esters undergo hydrolysis to release free retinol, which then binds with retinol-binding protein (RBP) and gets stored in the hepatic stellate cells (also called Ito cells), from where vitamin A will be mobilized in case of necessity (Wake, 1994). Stellate cells account for >90% of the hepatic retinol and retinyl esters stores. Retinol reesterification occurs in stellate cells, with deposition of the retinyl esters in cytoplasmic lipid droplets, along with other lipids. In the target cells of the whole body, the cells perform retinol uptake, and then retinol undergoes metabolic activation, ultimately supporting the biosynthesis of all trans retinoic acid, 9 cis retinoic acid and 14-hydroxy-retro-retinol. Although the liver is the major site of retinyl esters storage, it is not the sole site (Shiota et al, 2006).

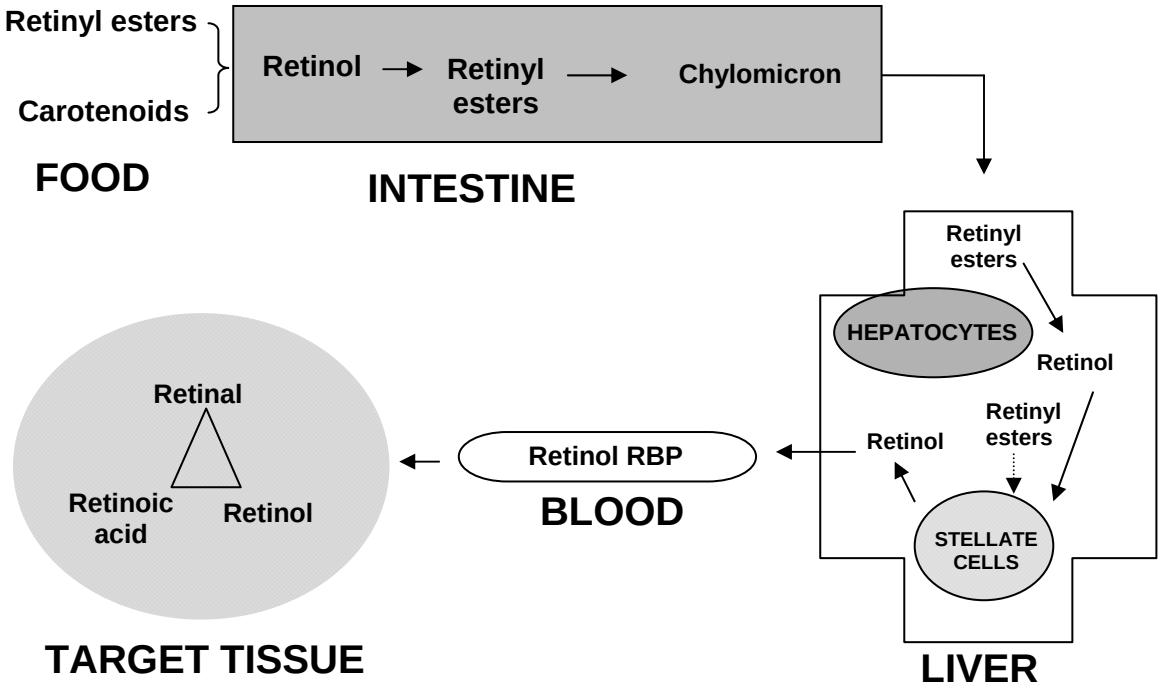



**Fig. 4. Schematic representation of vitamin A absorption, digestion, transport to the liver and delivery to target tissues.** Carotenoids or the retinyl esters taken up along with the food are cleaved to retinol in the intestine and transported to various organs through the circulation and finally stored in the liver in different inter convertible forms of retinol.

### 1.6.8. Bioconversion

Carotene bioconversion is defined as the proportion of bioavailable carotene converted to retinol. Often, the term covers both the bioavailability and the bioconversion process. Provitamin A carotenoids are converted to retinol by the action of 15-15'-carotenoid dioxygenase mainly in the intestine and liver. van Vliet et al, (1995) suggested that the ratio of the response of retinyl esters to β-carotene might be a good indicator of intestinal β-carotene conversion. Of absorbed β-carotene, 60–70% was mainly converted to retinyl esters, but several details with respect to the cleavage reaction remains to be elucidated (van Vliet et al, 1995). There is no information about the carotenoids that escape bioconversion in intestinal enterocytes. Preformed vitamin A and carotenoids are released from the food matrix by the action of enzymes present in the stomach and intestine. The bioavailability of the two forms of vitamin is related to the food matrix in which they are contained and to the overall status of the organism. Moreover, the absorption efficiency is higher for preformed vitamin A (80–90%) than for carotenoids (50–60%) (Yeum and Russell, 2002).

#### 1.6.8.1. Factors affecting carotenoid bioavailability and bioconversion

There are a number of factors that influence the bioavailability of carotenoids (Reviewed by De Pee and West, 1996; Castenmiller and West, 1998). Briefly they include species of carotenoids, molecular linkage, amount of carotenoids consumed in a meal, matrix in which the carotenoid is incorporated, effectors of absorption and bioconversion, nutrient status of the host, genetic factors, host-related factors, and mathematical interactions.

The bioavailability and provitamin A activity of the various carotenoids and geometrical isomers of carotenoids differ. The absorption and bioconversion of all-*trans*-β-carotene is higher than that of 9-*cis*-β-carotene (De Pee and West, 1996). The vitamin A activity of other provitamin A carotenoids is lower than that of β-carotene (Castenmiller and West, 1998). *In vitro* studies suggest that lutein interferes with the conversion of β-carotene to retinol (van Vliet et al, 1996) and this may explain, in part, the low conversion of β-carotene to retinol from dark green leafy vegetables (De Pee et al, 1995).

Due to the low bioavailability of carotenoids, it has been calculated that 1 μg of retinol is provided by 26 μg of β-carotene from dark-green leafy vegetables and

carrots and by 12 μg from yellow and orange fruits (De Pee et al, 1998a). Intake of dietary fat has a positive effect on β-carotene bioavailability and dietary fiber has a negative effect, alcohol intake seems to interfere with the bioconversion of β-carotene to retinol (De Pee and West, 1996). Nutrient status and genetic factors related to the host may explain some of the differences observed during bioavailability. Effects of season, sex, age and smoking largely explains the differences in long and short term intakes of carotenoids (Castenmiller and West, 1998).

### 1.7. Health benefits from the carotenoids of *Dunaliella*

At the thirty-fifth meeting of the WHO Committee, it was concluded that carotene isolated from *Dunaliella* would be acceptable for food additive use if it were of sufficient purity to meet the specifications for synthetic β-carotene (available online at http://www.inchem.org/documents/jecfa/jecmono/v32je07.htm). Acceptance of algal biomass or crude extracts of carotene from algal sources for use as food additives would be contingent on the provision of evidence of the safety of such materials.

*Dunaliella bardawil* biomass was used as vitamin A supplement in retinol deficient rats (Ben Amotz et al, 1986). Ben Amotz et al (1996) studied the beneficial effect of *D. bardawil* supplementation in the diet to reduce the oxidizing effect of the whole body irradiation. In another study, children's who were near the Chernobyl accident were supplemented daily with 40mg β-carotene mixture from *D. bardawil* for a period of three months. The increased blood oxidation levels were decreased upon supplementation with *Dunaliella* (Ben-Amotz et al, 1998).

The mammary tumor strain of mice when fed with β-carotene rich algae *D. bardawil* markedly inhibited spontaneous mammary tumourigenesis and also the progression of mammary tumors (Nagasawa et al, 1989 a and b; 1991). *Dunaliella* also showed growth promoting activity on normal mammary glands (Fujii et al, 1993). An extract of *Spirulina-Dunaliella* algae was shown (Schwartz et al, 1988) to prevent tumor development in hamster buccal pouch when applied topically thrice a week for 28 weeks. After 28 weeks, the animals given vehicle and untreated controls showed gross tumors of the right buccal pouch. Animals fed with β-carotene demonstrated significant reduction in tumor number and size, whereas algae treated animals showed complete absence of gross tumors.

Fabregas (1999) studied the *in vitro* inhibition of viral replication with extracts from the *D. tertiolecta* and *D. bardawil.* The viral hemorrhagic septicemia virus (VHSV) of salmonid fish and the African swine fever viral (ASFV) replication were inhibited by the extracts of *Dunaliella*. This inhibition was suggested to be due to sulfated polysaccharides present in the exocellular extracts from micro algae. However, the inhibition of viral replication did not correlate with the percentage of sulfatation of the exocellular polysaccharides. Hence studies are required in the future to utilize algae as a potential supplement in the prevention of different viral diseases.

Supamattaya (2005) studied the effects of commercially available *Dunaliella* extract on growth, immune functions and disease resistance in black tiger shrimp (*Penaeus monodon*). Small shrimp (1–2 g body weight) or juvenile shrimp (12–15 g body weight) were utilized in the study. Shrimps when fed with 125–300 mg of the *Dunaliella* extract/kg diet for 8 weeks showed higher weight gain and survival compared to the control. There was no significant difference in total hemocyte count and phenoloxidase activity among treatment. Shrimp fed with 300 mg of the extract/kg diet exhibited higher resistance to viral infections than other groups and also became more tolerable to the stress (low dissolved oxygen condition). Color intensity of boiled shrimp and total carotenoid and astaxanthin levels were highest in groups fed with 200–300 mg of the *Dunaliella* extract/kg diet. In conclusion, the *Dunaliella* extract showed beneficial effects as a shrimp feed supplement.

### 1.8. Bioavailability of carotenoids from *Dunaliella* on human subjects

#### 1.8.1. Short-term studies

Ben-Amotz and Levy (1996) studied the effect of *D. bardawil* cells on humans for 14 days. After 14 days, serum analysis showed mainly oxycarotenoids and to a lesser extent all-trans β-carotene and α-carotene, but no 9-cis β-carotene was detected.

In a study conducted by Jensen et al, (1987), on the bioavailability of cis- and trans-β-carotenes, 16 healthy adults, who had been on a low-carotene diet for ten days, were fed with either β-carotene extracted from *D. salina* alga containing approximately equal amounts of all trans β-carotene and 9 cis-β-carotene, or β-carotene in the form of fresh carrots containing predominantly trans-β-carotene, or avocado oil-placebo capsules. Subjects were randomly divided into three groups: they consumed daily in a single dose either β-carotene capsules or 207.3 g carrots containing 24 mg β-carotene

in each; or β-carotene free placebo capsules for seven days. The serum analyses showed trans β-carotene to be the predominant isomer in serum before and during all the treatments. Serum trans-β-carotene concentrations were significantly increased in the β-carotene capsules and carrot fed groups. Cis-β-carotene concentrations were increased in the carrot and placebo groups. These data demonstrate a predominant absorption of intact trans-β-carotene over intact cis-β-carotene into human serum even when approximately equivalent amounts of these isomers were ingested (Jensen et al, 1987).

### 1.8.2. Long-term studies

In a case report by Tamai et al, (1995) thirty male volunteers were given daily either 60 mg of synthetic all trans β-carotene or β-carotene derived from *D. bardawil* or a placebo for 44 weeks. The plasma levels of β-carotene reached a maximum after two weeks of administration and stabilized thereafter in the subjects who took the β-carotene preparations. The all trans β-carotene level in the synthetic carotene treated group was almost twice that of the *Dunaliella* group. The plasma 9-cis carotene levels were found to be higher in the all-trans β-carotene group than in the *Dunaliella* group, despite no intake of the 9-cis forms in the all-trans group and the higher intake of the 9-cis forms in the *Dunaliella* group. This finding suggests that isomerization of the all-trans form to the 9-cis form may occur in the body either during or after absorption.

The daily administration of 60 mg of β-carotene preparation to healthy young male volunteers (30 mg of all-trans β-carotene and 30 mg of 9-cis β-carotene) was performed and β-carotene concentrations were determined in the plasma. In conclusion, the bioavailability of β-carotene derived from *D. bardawil* was preferential to all-trans β-carotene, although a small amount of the 9-cis form was detected in the plasma and blood cells (Morinobu et al, 1994)

Neuman and associates (1999) have used dry powder of the β-carotene enriched alga *D. bardawil* (64 mg/day β-carotene for one week) to the patients with exercise-induced asthma (EIA) to evaluate the effect of supplemental carotenoids on the extent of this effect. Among this, 53% patients of *Dunaliella* treatment showed protection against EIA.

## 1.9. Commercial scale production of carotenoids from *Dunaliella* and global market

The estimated market size for β-carotene is up to 100 tonnes per year and the estimated price is >750 euros/ kg (Pulz et al, 2001). The commercial production of β-carotene from *Dunaliella* is a well-exploited industry. These production units are located in areas where solar irradiance is maximum, cloudiness is minimal, climate is warm and near the hyper saline areas (Ben-Amotz, 1999). Today there are number of companies that produces β-carotene from *Dunaliella*. Australia is the major country producing over 80% of the world's natural β-carotene (Curtain, 2000). The major contributors are Betatene Ltd, Western Biotechnology and Aqua carotene Ltd. Betatene Ltd has an extensive system for cultivation of *D. salina* for β-carotene production at Whyalla in South Australia covering an area of 300 acres (Schlipalius, 1991). They grow the algae in very large and shallow (approx. 20 cm deep) ponds constructed on the bed of hypersaline coastal lagoon or formed by artificially expanding a lagoon (Curtain et al, 1987; Borowitzka and Borowitzka, 1988). Production rate and nutrient requirement depends upon the environmental condition and varies throughout the year. Inner Mongolia Biological Eng and Tianjin Lantai Biotechnology in China, Cyanotech Ltd in Hawaii and Nature Beta technologies in Israel are the other leading companies involved in the commercial production of carotenoids from *Dunaliella*. In India ABL biotechnologies and Parry Nutraceuticals are in this line (Del Campo et al, 2007; Dufosse et al, 2005).

## 1.10. IPR issues

*Dunaliella* being a commercially important organism for the production of valuable products like protein, glycerol and carotenoids, protection of intellectual property rights has gained importance. Most of the patents obtained recently are on the novel formulations, like water-based formulations, carotenoids rich extracts for antioxidant activity and extraction of carotenoids from *Dunaliella*. Selected patents in the area of *Dunaliella* cultivation and carotenoid production (http://ep.espacenet.com/) has been compiled and presented in Table 6. These studies have bearing in the future applications of the technology for both basic and applied research. The search for value addition and improvement is bound to continue in view of the fact that *Dunaliella* could continue to be a commercially important alga.

**Table. 6. Selected patents in the area of *Dunaliella* cultivation and production of valuable products**

| Title | Inventors | Patent Number |
|---|---|---|
| A modified medium for producing the *Dunaliella* species for production of β-carotene | Chidambara Murthy KN; Mahadeva Swamy M; Ravishankar GA. | 587/DEL/2004 (Indian patent) |
| Therapeutic uses of *Dunaliella* powder | Shaish A; Harats D | EP1522310 |
| Synthesis of glutathione from *D. salina* | Ding Q. | CN1611595 |
| Method for cultivating green alga *D. salina* of liking for salt extremely | Zhang F; Ma Ruoxin. | CN1446904 |
| Method for culturing and producing *Dunaliella* and agent which used for thalasso therapy containing *Dunaliella* obtained by the culturing and producing method as main ingredient | Takenaka Hiroyuki; Shimokawa A | JP2003325165 |
| Medium for the production of β-carotene and other carotenoids from *D. salina* (ARL 5) and a strain of *D. salina* for production of carotenes using the novel media | Srinivasa VN; Rajagopal B; Parthasarathy S | US6936459 |
| Transgenic *D. salina* as a bioreactor | Lexun X; Weidong JP; Zhong G; Jianmin W. | US2003066107 |
| Method for producing natural β-carotene using *D. salina* | Cho Man Gi; Kim Won Suk. | KR2002012351 |
| *Dunaliella* alga-containing food | Hayashi Katsuhiko | JP2001149040 |
| Production of miso containing *Dunaliella* phycobiont | Tanaka Yoshio | JP2000217535 |
| Extraction of *Dunaliella* alga body | Tanaka Yoshio | JP2000175696 |
| Rigid capsule food containing *Dunaliella* algal material | Tanaka Yoshio | JP9000203 |
| Isolation of carotenoid(s) from sea water algae, e.g. *Dunaliella* | Dragoljub B; Helmuth MF. | DE4342798 |
| Method for desalting dry powder of algal | Tanaka Yoshio. | JP7000147 |

| | | |
|---|---|---|
| body belonging to genus *Dunaliella* | | |
| Sugar coated tablets containing *Dunaliella* algae and process for the production thereof | Tanaka Yoshio. | HK90292 |
| Encapsulated food containing *Dunaliella* algae and process for the production thereof | Tanaka Yoshio. | HK72192 |
| Production of β-carotene by high density culture of chlorophyceae *Dunaliella* | Yamaoka Y. | JP6046884 |
| Production of vacuum-packed food containing *Dunaliella* algae | Tanaka Yoshio. | HK53492 |
| Process for making soft capsule from algae *Dunaliella* | Danaka Yoshio. | KR9109680B |
| Process for making solid capsule containing algae *Dunaliella* | Danaka Yoshio. | KR9109679B |
| Process for making foods containing of algae *Dunaliella* | Danaka Yoshio. | KR9109678B |
| *Dunaliella* algae-containing flexible encapsulated food and production thereof | Tanaka Yoshio. | JP1215264 |
| *Dunaliella* algae-containing rigid encapsulated food and production thereof | Tanaka Yoshio. | JP1215263 |
| Method of determining absolutely dry biomass of *Dunaliella* | Rudik Valerij F; Gudumak Valentin S | SU1507261 |
| *Dunaliella* algae feed supplement | Avron M; Ben-Amotz A**;** Samuel E. | AU596839B |
| Strain of algae *D. salina* teod calv-834 - producer of protein-carotene biomass | Rudik Valerij F; Obukh Petr A; Vasilij SM. | SU1324627 |
| Extraction of carotenoids from algae of the genus *Dunaliella* | Thomas PW. | AU6926087 |
| Cultivation of *D. salina* for β-carotene production | James KP; Dominic MT. | AU6533186 |
| Extraction of carotenoids from algae of the genus *Dunaliella* | Thomas PW. | AU6926087 |
| Method of obtaining β-carotene from | Kurtis K S; Kharvi S | SU1531851 |

| | | |
|---|---|---|
| suspension of algae *Dunaliella* in solution on sodium chloride with concentration not below 3 M | | |
| Cultivating method of *Dunaliella* (green alga) | Nishitoi Mutsumi | JP57159484 |
| Cultivation of *Dunaliella* | Okuda Masao; Nakai Takeshi; Tsuyoshi W; Shiyuuzou T. | JP56051980 |
| Composition containing 9-cis-.β-carotene in high-purity and method of obtaining the same | Takehiko S; Kunio Y; Nobuko O. | CA2234332 |
| High purity β-carotene and process for obtaining same | David BT; Randall J; Robert A; Leonard AK; Steven RL; Zhengjie ZL; James MP; Chris JK | US2002082459 |
| High purity β-carotene | Geoffrey HW. | WO9410140 |
| Building model of producing β-carotene by cultivating marine algae | Jianguo Liu; Chaoyuan Wu. | CN1095102 |
| Method of *Dunaliella* collecting and β-carotene extracting | Sheng Yuan; Huilan Qin; Guanghua Yu. | CN1084848 |
| Tocopherol cyclase isolated from *Chlorella protothecoides*, *D. salina* and wheat leaves | Grueninger F; Hochuli Erich; Matzinger PK. | US5432069 |
| Method for leaching carotene from *Dunaliella* | Jingqi Teng; Jichun Meng; Shuhai Xia. | CN1059716 |
| Method for extracting carotene from *Dunaliella* and its extract | Xihai Wu; Dexin Shu; Liancheng Guo. | CN1044277 |
| Production of glutathione by *Dunaliella*, a green algae | Yamaoka Y; Takimura Osamu. | JP2234691 |
| Process for making solid state foods for algae *Dunaliella* | Danaka Yoshio. | KR9109681 |
| Production of glycerol β-carotene and proteinous nutrients from algae of the | Avron M; Ben-Amotz A. | NZ184303 |

| | | |
|---|---|---|
| *Dunaliella* species | | |
| A method for preparing β-carotene | Ivanovych CS; Rudas OM. | UA74111 |
| Fish feed additive containing *Dunaliella* powder and cyclodextrin and process for producing the same | Tanaka yoshio | AU652396B |

**Patents on genes involved in the carotenogenesis pathway in *Dunaliella***

| | | |
|---|---|---|
| Gene for coding phytoene dehydrogenase of *D. salina* | Jiang Jianguo; Zhu Yuehui | CN1670212 |
| Gene for coding lycopene β-cyclase (Lyc-B) of *D. salina* | Jiang Jianguo ; Zhu Yuehui | CN1670211 |
| Encoding gene of synthetase for phytoene of Dushi salt alga | Jiang Jianguo Yan Yuan | CN1563068 |

### 1.11. Future prospects for β-carotene production

There is a growing global demand for natural carotenoids due to its proven health benefits. With this increased awareness by the vitamin manufacturers as well as the consuming public, there is increasing interest in the biological production of carotenoids by many companies. *Dunaliella* is the only organism which produces massive amount of carotenoids and feasible for commercial production. This alga may be a boon for children with malnutrition and vitamin A defeciency. There are increasing awareness and opportunities for the expanded use of carotenoids for vitamin A and dietary supplement formulations.

Research has been performed to develop specialized strains amenable to large-scale production. In addition processes have been developed for the efficient extraction of β-carotene from the algal cells. There are several examples of the use of recombinant DNA technology to increase carotenoid productivity. Genes involved in the entire carotenoid biosynthetic pathway from geranylgeranyl diphosphate to zeaxanthin from the core isoprenoid enzymes were introduced into baker's yeast, *Saccharomyces cerevisiae* (Verwaal et al, 2007). Additional genes from the core isoprenoid enzymes were added to the same strain. The new recombinant strains developed produced more than 57% carotenoids than the control. The overproduction of carotenoids has also

been studied in bacteria *Bacillus subtilis* (Nishizaki et al, 2007) and *E. coli* (Kim et al, 2006)

Other researches targeted towards the production of carotenoids in higher plants. Over expression of the gene for phytoene synthase were found to increase the carotenoid levels in higher plants (Ausich et al, 1997). The gene for phytoene synthase from tomatoes were isolated, characterized and when reintroduced into tomatoes, resulted in increased carotenoid synthesis in tomato fruits (Fraser et al, 2002). Several researches in this area lead to the findings such as increased β-carotene, decreased lycopene (Romer et al, 2002), a three fold increase in phytoene, lycopene and β-carotene content (Fraser et al, 2002; Dharmapuri et al, 2002). The expression of undesired characters like ketocarotenoids in leaf, but not in fruit (Ralley et al, 2004), sense suppression, dwarf plants and premature lycopene accumulation (Fray et al, 1995) were also evident in addition to the success.

The transgenic potato tubers (*Solanum tuberosum* L) also known as 'golden tubers', developed by expressing an *Erwinia uredovora crtB* gene encoding phytoene synthase, produced a six fold increased β-carotene and 19-fold high lutein content than the control tubers (Ducreux et al, 2005).

Another important technology is of the 'Golden Rice', the genes used for the creation of golden rice were, *psy* (photoene synthase), *lcy* (lycopene cyclase) both from *Daffodil* (*Narcissus pseudonarcissus*) and *ctr1* from the soil bacterium *Erwinia uredovora.* (Datta et al, 2003; Hoa et al, 2003; Paine et al, 2005). Even though it is undergoing a number of intellectual property right issues around the world, with an expectation to help vitamin A deficiency, agricultural and nutritional (safety) aspects of golden rice are under consideration for its effective utility.

The production of β-carotene by the alga *Dunaliella* sp. is a well-developed technology. There are few companies producing *Dunaliella* on large scale and marketing the products. Since it can be grown in a controlled environment there is a possibility of using genetically modified *Dunaliella* for various purposes. The major focus for future research would be to engineer the algae to produce still higher amount of carotenoids and also to regulate the production of different carotenoids by manipulating the carotenoid pathway using recombinant DNA technology. The progress made in the area of genetic transformation demonstrates the possibility to genetically modify this organism for desired traits. The first step in this direction was

done by Degui and co workers (2003) who showed the possible expression of hepatitis B surface antigen gene in *Dunaliella salina.* The hepatitis B surface antigen (HBsAg) gene was introduced into the cells by electroporation and in future this will be useful against hepatitis B viral infections. This is an important step towards the production of useful foreign proteins in the alga. Further improvements in the area of algal transformations, cloning of new genes and promoters from algae including *Dunaliella*, would certainly lead the algal biotechnology area to a new horizon.

The cultivation of this alga for the carotenoid rich biomass, its biological significance and safety aspects are the main focus of the present work. In view of β- carotene demand and possibility of utilization of algal source for large-scale carotenoid production, the present study was undertaken with the following objectives.

### 1.12. Objectives of the study

- To optimize a cultivation process of *D. bardawil*.
- Analysis of genes involved in light regulated synthesis of carotenoids in *D. bardawil.*
- Studies on the involvement of selected metal ions in the regulation of carotenoid biosynthesis in *D. bardawil.*
- Biological activity of *Dunaliella* carotenoids in *in vitro, in vivo* and cell culture models.

# *MATERIALS & METHODS*

# Contents

## SECTION I

## CULTIVATION, GROWTH, CAROTENOGENESIS AND NUTRITIONAL COMPOSITION OF *D. BARDAWIL* BIOMASS

### 2.0. Material used for experiments

#### 2.0.1. Algal genotype used in the present study

The different genotypes used in the study include,

i. *Dunaliella bardawil* was isolated from the Sambar salt lake of Rajasthan, India.

ii. *Dunaliella bardawil* strains V-101, V-102, AP-504, Ne-17 and T-34 were gift from Dr. Rengaswamy, DOS in Botany, Annamalai University, Chennai.

#### 2.0.2. Glasswares

All glasswares used for the experiments viz., conical flasks, culture tubes, culture bottles, measuring cylinders, volumetric flasks etc, were from Borosil or Vensil Ltd, Mumbai, India.

#### 2.0.3. Chemicals used for experiments

All the chemicals used were of analytical grade obtained from Hi-media Laboratories, Qualigens Fine Chemicals and SISCO Research Laboratory Chemicals, Mumbai, India. Chemicals used for large-scale cultivation were of commercial grade purchased from M/s Mysore pure chemicals, Mysore, India. Standard β-carotene, lycopene, lutein, retinol, primers and cell culture media chemicals were procured from Sigma Chemicals Ltd, St Louis, Missouri, USA. Solvents used were of analytical and HPLC grade, obtained from Qualigens Fine Chemicals, Mumbai; Rankem Pvt Ltd, Mumbai and E. Merck Ltd, Mumbai, India. Different diagnostic kits used for biochemical analysis were obtained from Span Diagnostics Ltd, Bangalore, India. Diet used for experimental animals were from Amrut Laboratory Feed Product, Bangalore, India.

## 2.1. Maintenance of *Dunaliella bardawil* cultures and *in vivo* culture conditions

### 2.1.1. Maintenance of the germplasm

The axenic cultures of *D. bardawil* were maintained in AS-100 media (Vonshak, 1986) with modification. Chemical composition of the medium (AS-100) is presented in Table-7. The Tris buffer ($1gL^{-1}$) used in AS-100 medium was replaced with $NaHCO_3$ ($4.0gL^{-1}$). The pH was adjusted to 7.5 using a pH meter (Cyber Scan 510, Oakton, USA) prior to autoclaving at 121ºC, for 20min. The solid medium was prepared by gelling with 1.5% (w/v) tissue culture grade agar (Hi-media, Mumbai, India), in test tubes and in Petriplates, each containing 15mL of the medium. The tubes and flasks were closed with cotton plugs. The slant, Petriplates and flasks were inoculated with *D. bardawil* cultures, under aseptic conditions in laminar airflow cabinet (Airflow control systems, Bangalore, India). Algal pure cultures were maintained in solid medium as well as in liquid medium in Erlenmeyer flasks.

**Table 7. Chemical composition of AS-100 media**

| Chemicals | ($g\ L^{-1}$) |
|---|---|
| $MgSO_4$ | 2.44 |
| $CaCl_2$ | 0.3 |
| $KH_2PO_4$ | 0.05 |
| KCl | 0.6 |
| $NaNO_3$ | 1.0 |
| NaCl | 50.0 |
| Tris buffer | 1.0 |
| Trace metal solution | |
| $H_3BO_3$ 3.426 $gL^{-1}$ | |
| $CoCl_2.6H_2O$ 1.215 $mgL^{-1}$ | |
| $MnCl_2.4H_2O$ 0.432 $mgL^{-1}$ | |
| $ZnCl_2$ 31.5 $mgL^{-1}$ | 10 mL of stock |
| Conc.$H_2SO_4$ 1.0 mL | |
| $(NH_4)_6Mo_7O_2.4H_2O$ 31.19 $mgL^{-1}$ | |
| Chelated Iron solution (10g of $Na_2EDTA$ dissolved in 500 mL of hot water + 0.81g of $FeCl_3.6H_2O$ in 500mL of 0.1N HCl, mix and make up to 1L) | 3 mL of stock |

### 2.1.2. Growth of *Dunaliella* in AS 100 medium

The cultures were maintained at 20-22$^0$C in Erlenmeyer flasks under 12:12 hrs light/dark cycle of light (1.5-2 Klux), provided by universal-white lamps. The cultures were shaken manually once a day. Light intensities were measured using a lux meter

(TES 1332, Digital lux meter, Thaiwan). The growth of *D. bardawil* was monitored by the following parameters.

### 2.1. 3. Parameters for measurement of growth

***i. Cell count***

Growth of *D. bardawil* was measured in terms of cell number and counted using haemocytometer after fixing the cells by adding a drop of dilute hydrochloric acid (0.1N HCl). Cell count was expressed as number of cells/mL. In order to know the growth, optical density (OD) was also measured at 590 nm.

***ii. Fresh weight (FW) and Dry weight (DW)***

The fresh weight of algae was determined after centrifugation of the culture at 5000 rpm for 10 min and removing the excess of moisture using blotting paper. In order to determine the dry weight, algal cells after centrifugation were washed with distilled water three to four times to remove salt and other media chemicals and dried in a hot air oven (Sanyo, Electric Biomedical Co., Ltd, Japan) at 60 ± $2^0$C till a constant weight was attained on a glass Petridish. Biomass was expressed as gram per liter of culture.

Specific growth rate was calculated using the formula,

$$= \frac{\text{Final biomass} - \text{Initial biomass}}{\text{Initial biomass}} \text{ X } \text{Number of days}$$

***iii. Estimation of pigments***

***a. Chlorophyll content***

*D. bardawil* cells were taken into a graduated tube and centrifuged at 5000 rpm for 15 minutes. A known amount of pellet was homogenized with acetone using pestle and mortar. The supernatant was centrifuged at 3000 rpm for 10 minutes. The supernatant was collected and absorbance was measured spectrophotometrically (Shimadzu-160A, Japan) at 645 nm and 661.5 nm against acetone blank. Concentration of chlorophyll a and b were calculated by the equation of Lichtenthaler (1987) and expressed as mg $g^{-1}$ biomass.

$$\text{Chl a} = 11.24 \times OD_{661.5} - 2.04 \times OD_{645.0}$$

$$\text{Chl b} = 20.13 \times OD_{645.0} - 4.19 \times OD_{661.5}$$

***b. Carotenoid content***

Carotenoids were extracted with acetone as mentioned above and analyzed using High Performance Liquid Chromatography (HPLC).

### 2.1.4. Identification and estimation of carotenoids

#### 2.1.4.1. Identification of carotenoids by Thin Layer Chromatography

*D. bardawil* biomass was taken in mortar along with glass powder and acetone, subjected for mechanical grinding for 2-3 minutes, until the biomass becomes colorless. Whole process was carried out under yellow light in order to minimize the loss due to photo degradation. The whole solution was centrifuged at 5000 rpm for 5 minutes and the supernatant was collected, concentrated and redissolved in acetone. This extract was spotted on silica gel TLC plates (Merck Ltd, 20 × 20 cm, 0.5mm thickness) and the carotenoids were fractionated using acetone: hexane: acetic acid (7:3:0.1). The individual carotenoids and the total extract were identified using HPLC as explained below.

#### 2.1.4.2. Identification of carotenoids by High Performance Liquid Chromatography (HPLC)

The total extract and individual carotene resolved through TLC was identified using high-performance liquid chromatography on a Bondapak C18 column (5$\mu$ × 250 mm) with methanol: acetonitrile: chloroform (47:47:6) as mobile phase at a flow rate of 1mL $min^{-1}$. Parameters were controlled by a Shimadzu LC10 AS liquid chromatograph equipped with a dual pump and a photodiode array detector (Model SPD-10A) set at 450 nm. The recorder Shimadzu C-R7A chromatopac was set at a chart speed of 2.5 cm $min^{-1}$. Samples were (10µl) injected with Rheodyne 7125 injector. Peak identification was achieved by comparing with their respective standards (Sigma, USA) and confirmed by spiking the standards with individual samples.

### 2.1.5. Growth and carotenoids production in *D. bardawil* under different culture conditions

In order to achieve high growth and induce accumulation of carotenoids, algal cells were cultured under different culture conditions like light, salt and different metal ions, after a growth period of 7±2 days.

#### 2.1.5.1. Influence of light on the growth and carotenoid production

To study the effect of light intensity on growth and carotenogenesis efficiency, cultures were grown under different light regimes as given below.

(a) Culturing algae in controlled conditions with a light intensity of 1.5-2Klux provided by cool white fluorescent lamps and a temperature of 20-22° C with a 12:12 hrs light/dark cycle (Indoor laboratory culture condition).

(b) Culturing algae in outdoor ponds equipped with a shade net for achieving a light intensity of 15-20 Klux at a temperature of 22-28° C.

(c) Culturing algae under direct sunlight in outdoor ponds subjected to a light intensity of 30-35 Klux at a temperature of 30-35° C.

The cultures were daily monitored for cell count, chlorophyll and carotenoid contents as explained earlier (Section 2.1.3 and 2.1.4).

#### 2.1.5.2. Influence of sodium chloride on the growth and carotenoid production

To study the effect of sodium chloride content on growth and carotenogenesis in *D. bardawil* cells, the cultures were grown in different concentration of NaCl ranging from 0.5 to 4.0 M. The cultures were monitored for cell count, chlorophyll and β□-carotene content.

#### 2.1.5.3. Influence of carbon dioxide on growth of *D. bardawil*

To study influence of carbon dioxide on the growth of *D. bardawil*, the cultures were grown in two-tier flask as explained by Tripati et al (2001). A two-tier culture vessel consisting of two 250 mL small neck Erlenmeyer flask was employed. The lower compartment of the flask contained 100mL of 3M buffer mixture ($KHCO_3$/ $K_2CO_3$ at 73; 27), to generate 2% $CO_2$ partial pressure was used. The upper chamber was inoculated with media containing known inoculum of *D. bardawil* cells. The cultures were monitored for cell count, chlorophyll and β□- carotene content.

**2.1.5.4. Influence of metal ions on the growth and carotenogenesis in *D. bardawil***

Micronutrients/metal ions play a crucial rule in growth of any organism. In order to know the effect of various divalent metal ions on the growth of *D. bardawil,* growth was monitored with different metal ions like Fe (0.2-2.0 mM), Zn (0.2-1.0 μM) and Mn (2.0-10 μM). Effect of these micronutrients on growth and carotenogenesis was studied.

**2.1.6. Scale up studies in outdoor conditions**

Once the culture was established in the laboratory conditions of 1.5-2Klux light intensity and a temperature of 20-22$^0$C, scale up studies was carried out. Gradually the indoor grown cultures of *D. bardawil* was scaled up to 500mL and 1L flasks, 5L and 20L carboys and finally transferred to rectangular open glass tanks (40cm height X 40cm long X 20cm wide) with 20-25L culture capacity. During transferring of cultures from indoor to outdoor, initially carboys were closed with cotton plugs and the closures were removed gradually during 2$^{nd}$ and 3$^{rd}$ cycle to expose the cultures completely to an open system.

In continuation of scale up studies, *D. bardawil* culture was grown in circular cement tank of 500L capacity with culture volume of 200 ± 50L. Culture tank was maintained in natural environmental conditions of temperature and light. The high light caused bleaching and death of the algal cells, hence direct sunlight on the culture was avoided during vegetative growth phase by covering with green house shade net. The cultures were daily mixed in order to prevent settling of cells at the bottom of the tank.

The cultures from the circular tanks were transferred to raceway tanks of 5.0m$^2$ of 500L culture capacity for vegetative growth. Initially 250L media was inoculated with 40 ± 5L of green culture of14-15 days old, having cell count of ~ 40.0 × 10$^4$ cells mL$^{-1}$ under 50 % cut-off light (15-20 Klux) provided by green house shade net. The cultures were mixed occasionally using paddle wheel at 10-12 rpm, for ten minutes, twice or thrice a day depending on the light intensity. After a growth period of 20 days the cultures were kept for carotenogenesis by direct exposure to sunlight under outdoor condition of 30-35 Klux light intensity. The contaminations observed under outdoor conditions such as *Chlorella*, Protozoa and Diatoms were controlled by the addition of sodium chloride (2.0% w/v) to the medium.

### 2.1.7. Harvesting of algal cells

*D. bardawil* cells are 4-10 μm wide, 6-15 μm long and flagellate organisms with high motility and hence it is difficult to harvest this algae unlike other algal forms. The harvesting was achieved either by batch or continuous centrifugation.

#### *2.1.7.1. Batch Centrifugation*

To harvest the algal biomass in a batch mode, bowl centrifuge was used at a speed of 5000rpm (M/s West Folia, Germany). Thirty liter of culture was manually fed at a time to the centrifuge. The rotor speed was 5000 rpm with flow of the culture adjusted to 5L $hr^{-1}$. Biomass was collected in a cone shaped rotor and the media collected after the centrifuge was recycled for further cultivation.

#### *2.1.7.2. Online centrifugation*

Online centrifugation was used to harvest large amount of culture. A batch of 500 L culture was fed to online centrifuge (M/s Sharples, UK) at the rate of 8-10 L $hr^{-1}$, at an average speed of 7500 ± 1500 rpm. Biomass settled on the inner side of cylindrical tube was collected and the media was recycled.

### 2.1.8. Drying of *D. bardawil* biomass

Different drying methods were employed to study the effectiveness of drying processes, so that the method should not affect the concentration of pigment, in *Dunaliella,* the carotene content. The different drying methods employed were oven drying, sun, shade, freeze and spray drying.

***i. Sun drying*:** The wet biomass of *Dunaliella* was spread as a thin layer of ~0.5 cm thickness on an aluminum tray. Samples were kept under direct sunlight (~27 ± 3 $^0$C) for 5-6 hr. Sample was analyzed for moisture content and also for the carotene content. After complete drying total loss of carotenoids were recorded and percentage loss of pigments were calculated.

***ii. Shade drying****:* This was carried out in a similar manner as described above except the trays were kept in shade avoiding direct sunlight (20-25$^0$C).

***iii. Oven drying:*** The wet biomass of *Dunaliella* was spread as a thin layer of ~0.5 cm thickness on an aluminum tray inside hot air oven (Sanyo, Electric Biomedical Co., Ltd, Japan). Algal biomass was dried at 45 ± $2^0$C till a constant weight was achieved. The dried biomass was analyzed for moisture and carotene content.

***iv. Freeze-drying*:** This was carried out in a freeze drier (Model-10XB, Lyophylization Systems Inc. USA) for 7 hours by spreading the sample in a tray. The samples were analyzed for moisture and pigments content after freeze drying.

***v. Spray drying:*** Algal biomass was subjected for spray drying using spray drier (Bowen Eng Inc., New Jersey USA). Feed rate of sample containing 15-20% solids was at 100 mL $min^{-1}$ (6 L $Hr^{-1}$). Inside temperature was maintained at 160± $5^0$C and outlet was maintained at 80±$5^0$C. Sample was fed using the blower at an air pressure of 2.2-2.5 kg $in^{-2}$.

**2.1.9. Storage of dry biomass**

Equal weight (5g) aliquots of dried biomass were placed in brown glass vials, filled with nitrogen gas and stored at –80, 0, 25 and $37^0$C for 3 months under complete dark conditions. Aliquots (50 mg) of powder were drawn every two weeks for analysis.

**2.1. 9.1. Color changes during storage**

The color change of carotenoid powder during storage was studied by using a Hunterlab color measurement system (Labscan XE, USA), which was used to determine the Hunter L, a and b values. 'L' was used to denote the brightness of the powder, while 'a' and 'b' denotes the intensity of red and yellow color respectively.

**2.1.9.2. Analysis of carotenoids in stored samples**

Aliquots of samples were analyzed for the carotenoid content by HPLC as explained earlier (section 2.1.4).

### 2.1.10. Nutritional composition of *Dunaliella bardawil* biomass

#### *2.1.10.1 Moisture content*

Moisture content was estimated by the method described by Raghuramulu et al (1983). A known amount of algal biomass was dried in hot air oven at 70 ± $2^0$C to get a constant weight. The dried biomass was cooled in a desiccator and weighed. The loss in weight was recorded and expressed as percentage of moisture content.

#### *2.1.10.2. Ash content*

Ash content was estimated according to the method described by Raghuramulu et al (1983). A known weight of the algal material was initially charred on a silica crucible and placed in a muffle furnace at 400-450 $^0$C for 6 hours till the charred material becomes white. The dish was allowed to cool to room temperature in a desiccator till a constant weight is obtained. The difference in initial and final weight was taken as total ash content.

#### *2.1.10.3. Estimation of mineral content*

The mineral content was estimated by the method described by Raghuramulu et al (1983). The ash obtained as above was dissolved with a small amount of water (0.5 to 1.0mL) and mixed with 5mL concentrated Hydrochloric acid to prepare the solution. The mixture was evaporated to dryness, redissolved in 5mL HCl, dried and repeated again. Finally dissolved in 4mL of HCl and filtered through whatmann filter (#40) paper and made to known volume. The resulting ash solution was aspirated into the flame generated atomic absorption spectroscopy (AAS; Perkin-Elmer, USA, Model-3100) and the determination of metals and minerals were executed with the following operation conditions (Table 8) with acetylene air flame (acetylene pressure-75psi and air pressure 8psi) on the flame generating system.

**Table. 8. The conditions of atomic absorption spectroscopy**

| Metal analyzed | Hollow cathode lamp | Wavelength | Lamp Current (mA) | Slit width (nm) |
|---|---|---|---|---|
| Ca | Ca | 422.7 | 10 | 0.7 |
| Fe | Fe | 248.3 | 20 | 0.2 |
| Cu | Cu | 324.8 | 15 | 0.7 |
| Mg | Mg | 285.2 | 06 | 0.7 |

| | | | | |
|---|---|---|---|---|
| K | K | 404.4 | 12 | 0.7 |
| Na | Na | 589.0 | 08 | 0.2 |
| Zn | Zn | 213.9 | 10 | 0.7 |

***2.1.10.4. Crude fiber***

Crude fiber was determined by the method described by Mahadevaswamy (1996). A known quantity of algal biomass was taken in a conical flask to which, 50 mL of 1.25 % sulphuric acid was added and boiled for 30 minutes. The mixture was filtered and the residue was washed with distilled water till free from acid. The residue was quantitatively transferred into the original flask and digested for 30 minutes with 50 mL of 1.25 % sodium hydroxide solution. The digested residue was filtered and washed with distilled water and quantitatively transferred to a previously weighed silica crucible. The crucible was dried at $110^0$C for 3 hours and weighed again. The difference in weight before and after drying was expressed as crude fiber content.

***2.1.10.5. Estimation of protein***

Protein content of the biomass was calculated using total nitrogen content, which was estimated by micro-Kjeldhal method (Raghuramulu et al, 1983). A known amount of algal sample was digested in concentrated sulphuric acid with a catalyst (copper sulphate, potassium sulphate and selenium dioxide) for 6-8 hours, until the solution becomes clear. The digested solution was cooled and made up to a known volume. An aliquot was distilled by adding excess of 40% (w/v) sodium hydroxide. The liberated ammonia was absorbed in 2 % (w/v) boric acid and titrated against 0.014N hydrochloric acid. The nitrogen content was expressed as crude protein by multiplying with factor 6.25.

***2.1.10.6. Estimation of lipids***

***2.1.10.6.1. Extraction and purification of lipid***

Lipids were extracted, purified and quantified according to the method of Mahadevappa and Raina (1978). Fifty grams of cells was sonicated with 10 mL water, and lipids were extracted with chloroform:methanol (2:1) solvent mixture at room temperature for 3-4 h. The water phase was removed by allowing the two phases to separate using a separating funnel and the chloroform: methanol layer was collected.

The organic layer was flash evaporated to dryness and the difference in weight before and after drying was expressed as total lipid. The lipid fraction was dissolved in 1 ml chloroform and stored at -20$^0$C for further analysis.

***2.1.10.6.2. Separation of lipids***

Neutral and polar lipids were resolved from the total extract by thin-layer chromatography (TLC) as described by Mahadevappa and Raina (1978). Initially neutral lipids were separated using petroleum ether: diethyl ether: acetic acid (80:20:1) in which polar lipids would remain at the origin. The resolved neutral lipids (excluding the origin) were scrapped off the plate, re-extracted in chloroform: methanol (2:1) and re-chromatogramed individually in the same solvent system. The polar lipid present at the origin was likewise scrapped off, extracted in chloroform: methanol (2:1) and the lipids thus resolved were chromatographed in chloroform: methanol (4:1) to resolve polar lipids. Individual fractions were scrapped off the plate and quantified using gas chromatography.

***2.1.10.6.3. Quantification of lipid fractions***

The TLC plates were lightly sprayed with 50% $H_2SO_4$ and charred to locate the various lipid components. The relative concentrations of these components were determined by an automatic TLC scanner (Model 2, mounted on a Flourimeter, Model III; Turner Associator, CA, USA). Identification of the lipids on the plates was carried out by comparing with authentic standards and with the use of specific spray reagents (Siakator and Rouser, 1965). Sugar (Dubious et al. 1956), protein (Lowry et al. 1951), phosphorous (Lowry et al. 1954) and sterol (Lowry 1968) in the lipid were estimated by standard procedures.

***2.1.10.6.4. Identification of lipids by Gas Chromatography (GC)***

Fatty acid methyl esters were prepared using the method of methanolic boron triflouride and were analyzed by gas chromatography. A GC model Shimadzu-15A, (Shimadzu Corporation, Kyoto, Japan) equipped with a 15% capillary diethylene glycol succinate (DEGS) (Shimadzu Corporation Kyoto, Japan) column (30 m X 0.25 mm i.d.) and a flame ionization detector, was used. Nitrogen flow was 1 mL min$^{-1}$, and the temperature programming was 120$^0$C (1 min) with a 10$^0$C min$^{-1}$ increase to

$180^0$C (4 min), and then $4^0$C $min^{-1}$ to reach $280^0$C (2 min). The injector and detector temperatures were set at $250^0$C. Peaks were identified by comparison with authentic fatty acids methyl ester standards (Sigma).

### *2.1.10.7. Estimation of carbohydrate*

Carbohydrate was estimated as described by Raghuramulu et al (1983). Known amount of algal biomass (1g) was hydrolyzed by keeping it in waterbath for 3 hrs with 5 mL of 2.5N HCl. The mixture was cooled and neutralized with $Na_2CO_3$ until effervescence ceases. The mixture was made to 100mL and centrifuged. Aliquots of the supernatant (100, 200, 300µL….) were taken in different tubes, and made to 1mL. To this 1mL phenol, followed by 5mL $H_2SO_4$ was added to each tube. The blank was run with 1 mL of distilled water. After cooling (10 minute at room temperature), the brown color developed was measured at 490 nm against reagent blank. Glucose (5-25 µg) was used as standard. The amount of carbohydrate was calculated using standard graph.

## SECTION II

## ANALYSIS OF GENES INVOLVED IN CAROTENOID BIOSYNTHESIS PATHWAY DURING LIGHT INDUCED CAROTENOGENESIS

### 2.2.1. Growth and cultivation of *D. bardawil*

In this section the effect of light intensity on growth and carotenogenesis, the regulation of genes involved during carotenogenesis were studied. *D. bardawil* cultures were grown under the different light regimes as given below.

(a) Culturing algae in controlled conditions with a light intensity of 1.5-2Klux provided by cool white fluorescent lamps and a temperature of 20-22° C with a 12:12 hrs light/dark cycle (Indoor laboratory culture condition).

(b) Culturing algae under sunlight in outdoor ponds equipped with a screen for achieving a light intensity of 15-20 Klux at a temperature of 22-28° C.

(c) Culturing algae under direct sunlight in outdoor ponds subjected to a light intensity of 30-35 Klux at a temperature of 25-30° C.

The cultures were daily monitored for cell count, chlorophyll and carotenoid contents as explained earlier (Section 2.1.3 and 2.1.4).

### 2.2.2. Isolation of genomic DNA from *D. bardawil*

Genomic DNA was isolated from algal cells using the Gen Elute Plant Genomic DNA isolation kit (Sigma, USA).

### 2.2.3. RNA Extraction and transcript analysis

Total RNA was isolated using an RNA isolation kit (Ambion, USA) from aliquots of frozen cells harvested at different stages of carotenoid accumulation subjected to different light intensities. To avoid possible RNase contamination, all plastic wares were treated with 0.1% diethyl pyrocarbonate and the working area, electrophoresis tank, and other required materials were treated with RNase Zap (Sigma, USA). The quality and concentration of RNA were checked on denaturing agarose gel and quantified by measuring absorbance at 260 nm in a UV spectrophotometer (Sambrook et al, 1989). All RNA samples were subjected to DNase (DNAfree, Ambion, USA) treatment to avoid possible amplifications from contaminant genomic DNA.

The primers for all the candidate genes were designed using Primer3 software (Rozen and Skaletsky, 2000) are listed in Table 9. A control PCR was run on extracted RNA samples to check for the absence of genomic DNA. First strand cDNAs were synthesized using first strand synthesis kit (Ambion USA) from 400ηg of total RNA in 10 µL of final volume, using M-MuLV reverse transcriptase and oligo-dT (18-mer) primer following the instructions of the manufacturer. One µL of cDNA was used in semi quantitative RT-PCR reaction using specific primers and specific annealing temperatures (Table 9).

### 2.2.4. Polymerase chain reaction (PCR)

PCR was performed using primers designed for particular genes (Table 9). The PCR mixture (25 µL) contained 50 ng of cDNA prepared from different treated *D. bardawil* as the template, 1X PCR buffer, 2.5 mM of dNTPs and 1 unit of *Taq* DNA polymerase (MBI Fermentas), 25 pmoles of each primer (Genosys, Sigma USA).

PCR for phytoene synthase (*PSY),* phytoene desaturase (*PDS)* and lycopene cyclase (*LCY)* gene was performed at initial denaturation at 94$^{o}$ C for 5 min followed by 35 cycles of 1 min denaturation at 94$^{o}$ C, 1 min annealing at different annealing temperatures for candidate genes and 1 min extension at 72$^{o}$ C with a final extension of 72$^{o}$ C for 10 min. PCR for β-carotene hydroxylase (*CH*) was carried out as described above with annealing at 55$^{o}$ C for 1 min. The thermal cycler used was Primus 25 PCR system (MWG, AG Biotech, Germany). An aliquot of 12.5 µL from each PCR reaction was fractionated on a 1.5% (w/v) agarose gel in Tris acetate EDTA (TAE) buffer.

**Table 9. Primers and the PCR conditions used for different genes**

| Target gene amplified | Primer Name | Primer sequence 5' – 3' | RT PCR Product size (bp) | Source | Ann.Temp |
|---|---|---|---|---|---|
| *Dunaliella* sp. phytoene synthase mRNA | PSYF | ATGTCTATGATGGATGCCAGGAG | 442 | DQ463305 | 50$^{0}$C |
| | PSYR | CTTCCAGCGCAGTACATTGC | | | |
| *Dunaliella bardawil* mRNA for phytoene desaturase. | PDSF | TCTTTGGTGCTTACCCCAAC | 664 | Y14807 | 48$^{0}$C |
| | PDSR | TTGCTACCATGTCGCTCTTG | | | |

| | | | | | |
|---|---|---|---|---|---|
| *Bixa orellana* lycopene cyclase mRNA | LCYF | CTTGATGCTACGGGCTTCTC | 420 | AJ549288 | $50^0$C |
| | LCYR | AGCTAGAGTCCTTGCCACCA | | | |
| *Haematococcus pluvialis* carotenoid hydroxylase mRNA, | CHF | CTACACCACAGCGGCAAGTA | 521 | AF162276 | $55^0$C |
| | CHR | GCCTCACCTGATCCTACCAA | | | |
| *Dunaliella salina* actin mRNA | ACTF | GTGCCCATCTACGAGGGTTA | 357 | AF163669 | $50^0$C |
| | ACTR | GGAGTTGAAGGTGGTGTCGT | | | |

### 2.2.5. Agarose gel electrophoresis

**i. Preparation of 50X TAE buffer (1000mL)**

242.0 $mgL^{-1}$ Tris base, 57.1 $mgL^{-1}$ glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0) was dissolved and made to 1L.

**ii. Preparation of DNA loading dye (6X)**

0.25% Xylene cyanol, 0.25% Bromophenol blue and 30% Glycerol was mixed well and stored at $4^O$C

The gel casting boat was sealed with adhesive tape and the comb was placed on it for making the wells. Agarose (Electrophoretic grade SRL, India) was used at 1.5% (w/v) level and melted in 1X TAE. Agarose was allowed to cool to about $50^O$C and poured into the sealed gel casting boat. The gel was allowed to set for 30 min. The comb and adhesive tapes were removed and the gel was placed in the electrophoresis tank (Bangalore genei, India). Electrophoresis was carried out at 80V using a power pack (Consort Power Pack- E861, Belgium). The tank was filled with 1X TAE buffer to the electrode chamber to cover the gel to a depth of about 1 mm. The samples were loaded by mixing 10 µL aliquot with 2 µL of loading dye. The samples were run at 50 volts till the loading dye reaches ¾$^{th}$ of the gel. The gel was removed from the tank and placed in ethidium bromide (5µg $mL^{-1}$) solution for 15 min, subsequently destained with distilled water to remove unbound dye.

Ethidium bromide stained gels were observed under UV light and photographed with a Digital Imaging System (HeroLab, Germany). The transcript abundance of *PSY*, *PDS*, *LCY* and *CH* were quantified using the intensity histogram.

## SECTION III

## SAFETY AND TOXICITY EVALUATION OF *D. BARDAWIL* BIOMASS IN ALBINO RATS

### 2.3.1. Acute oral toxicity study in rats

The study was designed to investigate the toxicological effects of oral administration of *D. bardawil* biomass after a single oral dose. The toxicological symptoms were monitored for 15 days. This will provide information on the health hazards likely to arise from a short-term exposure by the oral route.

#### 2.3.1.1. Maintenance of rats for experimentation

Five male and five female rats of 6-8 weeks old, weighing 80-100g rats were used in the study. The rats were acclimatized for 7 days and the test substance; *D. bardawil* biomass (5000mg $Kg^{-1}$ b.w) was freshly prepared in distilled water (vehicle) and administered orally. The control group animals were treated with only distilled water.

The animals were caged in a group of 5 according to sex; in polypropylene cages fitted with wire mesh tops and paddy husk bedding. The room was well ventilated with a temperature of 24± $2^0$C, with 60% relative humidity and 12hr light/dark cycle. The standard rat pellet feed and water was given *ad libitum*. All experimental animals were observed for 15 days after *D. bardawil* oral administration. Observation was made 3 times on the day of dosing and twice daily thereafter for the remaining 14 days. Body weights were recorded initially and weekly.

#### 2.3.1.2. Biochemical observations

The following clinical laboratory determinations were made in all the animals at the end of the experiment. The organs were weighed and collected at the termination of the experiment. Haematological observations like Red blood cell (RBC) count, White blood cell (WBC) count, Hemoglobin (Hb) count, Platelet count, Neutrophils (N), Lymphocytes (L), Monocyte (M), Eosinophils (E) and Basophils (B) count were determined using baker hematology system.

Serum biochemistry parameters like total protein, serum aspartate aminotransferase (AST), serum alanine amino transferase (ALT) and serum alkaline phosphatase (ALP) were measured using Span diagnostic kits, India.

### 2.3. 2. Sub chronic (90 days repeated) oral toxicity study in rats

The toxicological effects of 90 days repeated oral administration of *D. bardawil* biomass in albino rats was assessed. This will provide information on the possible health hazards likely to arise from repeated exposure over a limited period of time.

### 2.3. 2.1. Maintenance of rats for experimentation

Thirty healthy rats (15male and 15 female, in a group of five each) were acclimatized for 7 days. The first group was kept as control given only the vehicle i.e. distilled water. Second group was given 100mg $kg^{-1}$ b.w. *D. bardawil* biomass and third group was administered with 1000mg $kg^{-1}$ b.w. *D. bardawil* biomass for 90 days. The rats were observed daily for behavior, appearance, toxicological signs and symptoms, if any. Body weight and feed consumption was recorded at weekly intervals.

### 2.3.2.2. Biochemical observations

All the biochemical estimations were carried out as explained earlier (section 2.3.1.2)

## SECTION IV

## BIOAVAILABILITY AND BIOCONVERSION OF CAROTENOIDS FROM *D. BARDAWIL - IN VITRO, INVIVO* AND CELL LINE STUDIES

### 2.4.1. *In vitro* bioavailability of carotenoids from *D. bardawil* biomass by simulated (*in vitro*) digestion method

The standard procedure given by Garrett et al, (1999b) was used for the *in vitro* digestion. Briefly the homogenized meal was subjected to acidification (pH 2) with 1M HCl and 2 mL of porcine pepsin (40 mg $mL^{-1}$ in 0.1M HCl). The homogenate was transferrrred to a clean amber bottle and incubated at $37^0$C in a shaking water bath (95 rpm) for 1hour. Next, the pH of the partially digested sample was raised to 5.3 by the addition of sodium bicarbonate (0.9M), followed by the addition of a mixture of bile extract and pancreatin (9mL containing 2mg $mL^{-1}$ pancreatin and 12mg $mL^{-1}$ bile extract in 100mM sodium bicarbonate solution). The pH of the sample was increased to 7.5 by the addition of 1N sodium hydroxide and 10mL aliquots of the incompletely digested meal were transferred to three amber glass bottles sealed and incubated at $37^0$C for 2 hours to complete the intestinal phase of the *in vitro* digestion. The digesta was centrifuged and the aqueous fraction was extracted three times with hexane. All the fractions were pooled and evaporated to dryness in a rotary evaporator (Buchi Rotavapour/R-205, Flawil, Switzerland). The residue was dissolved in 500µL of mobile phase solvent, filtered through a 0.25 $\mu$m membrane filter and analyzed by HPLC for carotenoids with the conditions as explained earlier (section 2.1.4).

### 2.4.2. *In vivo* bioconversion of carotenoids from *D. bardawil*

#### 2.4.2.1. Bioconversion of *D. bardawil* carotenoids to vitamin A by intestinal perfusion method

The study technique of Wang et al, (1993) with a modification was employed to study the *in vivo* bioconversion of carotenoids from *D. bardawil*. The experiment was carried out by perfusing β-carotene extract from *D. bardawil* biomass into the intestine of rat and monitoring the level of retinol conversion for a varied interval of 15, 30, 60 and 120 min.

### 2.4.2.2. Maintenance of rats for experimentation

The male Wister strain rats (weighing about 100-120g each) were used for this study. These animals were housed under the following conditions: temperature- 25±2$^0$C, relative humidity 55±5%, and a 12 hr light/dark cycle. The animals were housed in polypropylene cages (3 rats/cage) on soft sawdust bedding, fed with commercial basal diet (Amrut feeds, Bangalore, India) and water *ad libitum*. After 7 days of acclimatization, rats were deprived of diet for 12 hours before using for perfusion study.

### 2.4.2.3. Experimental design

The animals were anaesthetized using diethyl ether anaesthesia. Through the midline abdominal incision, the proximal end of intestine was flushed with a normal saline and then with the known volume of carotenoid extract from *D. bardawil* biomass. To prevent the perfusate from washing back into the stomach or continuing to large intestine, both distal and proximal ends were tied immediately. The water soluble carotenoid extract was prepared as below. A known concentration of carotenoid extract in acetone was dried under nitrogen flush. To the dried extract 20 µL of Tween-20 was added and vortexed vigorously. This is made to known volume by addition of phosphate buffer saline to get a final concentration. This carotene preparation was perfused and the whole intestine was removed from the group of rats in a definite interval of 15, 30, 60 and 120 mins.

### 2.4.2.4. Analysis of carotene and vitamin A

The perfusate from the intestine was decanted to a graduated tube, and intestine was excised from the rat. The extraction of carotenoids and vitamin A was carried out according to Schmitz et al, (1991). Briefly, intestine was homogenized with ethanol: water (1:1) with BHT, then saponified using 5mL of 10% NaOH in ethanol for 30 min at 60$^0$C. To the saponified mixture 10 mL of water was added and extracted with hexane. The hexane fraction was dried and redissolved in acetone. HPLC analysis was carried out at 450 nm for carotenoids and at 320 nm for vitamin A with the conditions as explained earlier (section 2.1.4). The percentage increase in retinol contet was monitored during different time interval.

### 2.4.3. Studies on bioavailability of carotenoids from *D. bardawil* biomass by feeding trials

#### 2.4. 3.1. Maintenance of rats for experimentation

The male Wistar strain rats (weighing about 100-120g each) were used for this study. The animals were housed under the following conditions: temperature 25 ± $2^0$C, relative humidity 55 ± 5%, and a 12 hr light/dark cycle. The animals were housed in polypropylene cages (4 rats/cage) on soft sawdust bedding. Throughout the experiment, rats were given commercial basal diet (Amrut feeds, Bangalore, India) and water *ad libitum*. After 7 days of acclimatization, rats were deprived of diet for 12 hours before administering β-carotene (single dose study). Otherwise rats were received same diet throughout the experimental period (multiple dose study). All the experiments were carried out under the regulation of Institute Animal Ethical Committee.

#### 2.4. 3.2. Test compound and levels of dose administration

*D. bardawil* biomass {100µg β-carotene equivalent (~3.5mg D. bardawil biomass) per kg body weight} was ground with minimal amount of water to form uniform slurry. Standard β-carotene (100µg $kg^{-1}$ body weight) was prepared in a water-soluble form and used for the experiment**.**

#### 2.4. 3.3. Single dose studies

The groups of rats (n=36) were administered orally with single dose of *D. bardawil* biomass and with synthetic β-carotene after overnight fasting. Each group was further divided in to 8 subgroups (n= 4) and a control group to measure the retinol and β-carotene concentration of serum and liver at different intervals. Control group was fed with the same amount of water. Rats from control and in each treatment group at 0, 2, 4, 6 and 8 hr after gavage were sacrificed with diethyl ether anesthesia. Blood was collected directly from the heart and kept at $4^0$C. Serum was separated by centrifugation at 2500 rpm for 20 min, and stored at -$70^0$C for further analysis. The livers were removed and washed with ice-cold saline and stored at -$70^0$C until it is processed for carotene and vitamin A estimation. All the animals were fasted till the last blood drawing.

The quantity of retinol converted was measured in terms of area under curve (Parvin et al, 2000). The area under curve is the serum concentration Vs time of a known drug/product. It is also known as the total amount of drug/product absorbed by the body at definite time period. It is very useful to study the relative efficacy of different drug/product at a given time. The area under curve was calculated using the software 'origin'.

**2.4. 3.4. Multiple dose study**

For multiple dose study, three groups of rats (n=4) were individually caged in metabolic cages. Two groups were administered daily with doses of either synthetic β-carotene or *D.bardawil* biomass for a period of 7 days. Clinical signs and general appearances were checked daily and body weights were measured once in a week. Urine and faecal matter from each rat was collected into amber bottles everyday and preserved at -70$^0$C until analysis. Urine was collected daily in amber colored bottles containing 2mL of toluene (to prevent bacterial growth) from day 2 onwards. Urine volume was determined by measuring cylinder and corrected for the added toluene. Faecal matter was collected separately from the individual rats for 3 days and weight was noted down. Rats were weighed initially and at the end of the experiment. Before the day of necropsy, the animals were deprived of food overnight and sacrificed by anaesthetizing with diethyl ether. Blood was collected directly from the heart and kept at 4$^0$C. Serum was separated by centrifugation at 2500 rpm for 20 min, and stored at -70$^0$C for further analysis. The livers were removed and washed with ice-cold saline and stored at -70$^0$C until the analysis.

**2.4. 3.5. Estimation of carotenoids and vitamin A in intestine and liver**

The extraction of carotenoids and vitamin A was carried out according to Schmitz et al, (1991) as explained earlier (section 2.4.2.4). The HPLC estimation was carried out at 450 nm for carotenoids and at 320 nm for vitamin A.

**2.4.3.6. Analysis of carotenoids and vitamin A in urine and faecal matter**

Aliquots of urine and faecal samples from each rat were taken for analysis either fresh or frozen at –70$^0$C. Urine samples were filtered through Whatmann No.1filter paper and faecal matter (1g) was homogenized with 2 mL water and extraction was carried out as explained earlier (2.4.2.4).

### 2.4.3.7. Estimation of serum triglycerides

Triglyceride in the serum was estimated using kits from Span diagnostics, India.

### 2.4.4. Biological activity of *D. bardawil* carotenoids on primary cell lines

#### 2.4.4.1. Maintenance of experimental rats

The maintenance of rats was given in earlier section 2.4.2.2.

#### 2.4.4.2. Isolation of rat intestinal epithelial cells

Overnight, fasted rats were sacrificed by ether anesthesia. The small intestine was removed and flushed gently with normal saline containing 1.0mM dithiothretol. Intestinal epithelial cells were prepared according to Upreti et al (2005). In brief, the caecal end of the intestine was ligated and solution A containing 1.5mM KCl, 96mM NaCl, 27mM sodium citrate, 8mM $KH_2PO_4$ and 5.6 mM $Na_2HPO_4$ (pH 7.3) was filled after clamping the other end with artery forceps. The intestine was then immersed in Solution A and incubated at $37^0$C for 15 min in a constant temperature shaker bath. After incubation the intestine was emptied and fluid discarded. The intestine was then filled with solution B containing 1.5mM EDTA and 0.5mM dithiothretol in Phosphate buffer saline (pH 7.2) and immersed in solution A for incubation. After 4 min incubation, the contents were emptied into a plastic centrifuge tube to recover the first epithelial cell population. The process of filling the intestine with solution B and collecting the washings were repeated twice. All the cell population were pooled and centrifuged at 900rpm for 5 mins and washed twice with 4mM EDTA solution containing 15mM β-mercaptoethanol to remove phosphate buffer. The resulting cell mass is known as primary intestinal epithelial cells. These cells were suspended in Dulbecco's Modification of Eagle's (DME) medium (pH 7.2 containing100 IU $mL^{-1}$ penicillin and streptomycin and 10% fetal bovine serum). These cells were grown in a tissue culture plates on an incubator under 5% $CO_2$ atmosphere at $37^0$C with 95% relative humidity.

#### 2.4.4.3. Trypan blue dye exclusion assay to study the cell viability of intestinal epithelial cells

An aliquot of cells (100μL) were dispersed in phosphate buffer saline (pH 7.0) and mixed with trypan blue (100μL containing 0.2mg $mL^{-1}$) for 1 min (Altman et al,

1993). The stained (non-viable cells) and non-stained cells (viable cells) were observed under the microscope and counted separately using haemocytometer. The percentage of viable cells was calculated using the formula,

% of viable cells = (Total no. of cells –Total no. of dead cells) / total no. of cells X 100

**2.4.4.4. Treatment of cells to study the uptake of carotene and conversion to vitamin A in cell lines**

*D. bardawil* carotenoid extract and synthetic β-carotene was prepared in phosphate buffer saline containing 5 and 10 μM of carotenoid content as explained in section 2.4.2.3. Approximately equal numbers of (5 X $10^7$ cells/mL) intestinal epithelial cells were suspended in media with and without carotene extract and incubated at $37^0$C with 5 % $CO_2$ and 95% relative humidity. Aliquots of sample s were withdrawn at different time periods (30, 60, 120 and 180 min) and the cells were pelleted by centrifugation at 8000rpm for 10 mins. The carotenoids and vitamin A in the cell pellet were extracted with acetone and analyzed by HPLC conditions as explained in section (2.1.4) at 450 nm for carotenoids and at 320 nm for vitamin A.

## SECTION V

## BIOLOGICAL ACTIVITY OF *D. BARDAWIL* BIOMASS ON $CCl_4$ INDUCED TOXICITY: BENEFICIAL ATTRIBUTES OF *D. BARDAWIL* AND ITS POTENTIAL TO MODULATE EXPERIMENTALLY INDUCED DISEASE CONDITIONS IN DIFFERENT ORGANS

### 2.5. Modulatory effect of *D. bardawil* on $CCl_4$ induced toxicity

The possible beneficial attributes of *D. bardawil* biomass and its potential to modulate experimentally induced disease conditions on blood parameters, liver and kidney were studied in this section.

#### 2.5.1. Experimental design

Albino rats of Wister strain (120–150g body weight) bred in the Animal House of Central Food Technological Research Institute were used for the study. Animals were grouped into six groups each consisting of 6 rats (3 males and 3 females, maintained separately). The carotenoid rich biomass of *D. bardawil* was given at two different doses, i.e., 2.5 and 5.0 g $kg^{-1}$ body weight (approximately equivalent to 50 and 100 mg of β-carotene $kg^{-1}$body weight) and synthetic β-carotene (50 mg $kg^{-1}$ body weight) as a single dose for a period of 14 days.

The six groups are as follows,

Group-1 Normal (receiving normal basal diet without toxin treatment),

Group-2 Control (receiving normal basal diet with toxin treatment),

Group-3 *D. bardawil* biomass was fed at 5g $kg^{-1}$ body weight,

Group-4 *D. bardawil* biomass was fed at 2.5g $kg^{-1}$ body weight along with $CCl_4$,

Group-5 *D. bardawil* biomass fed at 5g $kg^{-1}$ body weight with $CCl_4$ and

Group-6 was treated with synthetic β-carotene at 50mg $kg^{-1}$ body weight along with $CCl_4$.

The dosage of $CCl_4$ was decided based on earlier reports (Chidambara Murthy et al, 2002). The animals of all the groups except group1 and 3 were given single dose of $CCl_4$ (2mL $kg^{-1}$ b. w.) on $15^{th}$ day dissolved in olive oil (1:1). Animals of group1 and 3 were given same dose of olive oil as a vehicle.

### 2.5.2. Maintenance of rats for experimentation

The animals were housed in a room with a barrier system, and maintained under the following conditions: temperature 24±1$^0$C, relative humidity 55±5%, and a 12 hr light/dark cycle. The animals were housed in polypropylene cages (3 rats/cage) on soft sawdust bedding. Throughout the experiment, rats were given commercial basal diet and water *ad libitum*. All the experiments were carried out under the regulation of Institute Animal Ethical Committee.

### 2.5.3. Test compound and administration dose levels

*D. bardawil* biomass was ground with minimal amount of water to form uniform slurry. Experimental groups were fed with either *D. bardawil* biomass or synthetic β-carotene once a day for 14 days by forced feeding using an oral gavage method. On 15$^{th}$ day the animals were administered with single dose of $CCl_4$ (2mL kg$^{-1}$ b.w) in olive oil, and sacrificed after 6 hrs of the dosage.

### 2.5.4. Biochemical screening

Clinical signs and general appearances were checked daily and body weights were measured once in a week. Before the day of necropsy, the animals were deprived of food overnight and sacrificed by anaesthetizing the animals with ether. Blood was collected from the animals and the serum obtained was analyzed. The organs for the analysis were transferred to ice-cold containers for various biochemical estimations. A piece of liver was stored in 10% formalin solution for histopathological examination.

### 2.5.5. Serum biochemistry

Serum aspartate aminotransferase (AST) and serum alanine aminotransferase (ALT) were measured by the DNPH method (King 1965). Serum alkaline phosphatase (ALP) activity was assayed by the method of King and Armstrong (1988) using the commercially available kits (M/s SPAN diagnostic reagent kits, Mumbai, India). Serum Creatinine and Bilirubin content was estimated using diagnostic kits from SPAN diagnostics Ltd, India.

### 2.5.6. Liver parameter evaluation

The liver parameters were analyzed by estimating the lipid peroxidation and histopathological examination.

#### 2.5.6.1. Lipid peroxidation

Liver tissues were homogenized in 0.1 mol $L^{-1}$ Tris-buffer (pH 7.4) and centrifuged. The particle-free homogenate was used for various biochemical analyses. Extent of lipid peroxidation was measured by quantifying the malondialdehyde formed in terms of thiobarbituric acid reactive substances (TBARS) and expressed in terms of nmol $mg^{-1}$ protein (Buege and Aust, 1978).

#### 2.5.6.2. Histopathological studies

A portion of tissue was fixed in freshly prepared Bovin's fluid (Saturated solution of picric acid 80%, formaldehyde (commercial) 15 % and glacial acetic acid 5 %). The tissues were processed according to the method of Lillie (1965). Six μm thick paraffin sections were prepared and stained with hematoxylin and eosin for histopathological examination.

### 2.5.7. Kidney parameter evaluation

Kidney parameters studied include the lipid peroxidation in renal tissues and creatinine estimation in serum.

#### 2.5.7.1. Lipid peroxidation

Kidney tissues were homogenized in 0.1 mol $L^{-1}$ Tris-buffer (pH 7.4) and centrifuged. The particle-free homogenate was used for the biochemical analysis. Extent of lipid peroxidation was measured by quantifying the malondialdehyde formed in terms of thiobarbituric acid reactive substances (TBARS) and expressed in terms of nmol $mg^{-1}$ protein (Buege and Aust, 1978).

#### 2.5.7.2. Estimation of serum creatinine

Serum Creatinine, indicator of kidney damage was estimated using diagnostic kit from SPAN diagnostics Ltd, India

### 2.5.8. Estimation of protein

Protein content was determined using the method of Lowry et al, (1951).

## 2.6. Statistical analysis

All the *in vitro* experiments were carried out three times in triplicates. Results were expressed as mean ± SD in case of *in vitro* experiments, and mean ± SE in case of *in vivo* analysis. Analysis of variance (ANOVA) was used for comparison of mean values and the values are considered as statistically significant at $p < 0.05$ or 0.01.

# *RESULTS*

# *&*

# *DISCUSSION*

## Contents

## SECTION I

## CULTIVATION, GROWTH, CAROTENOGENESIS AND NUTRITIONAL COMPOSITION OF *D. BARDAWIL* BIOMASS

### Background

Algae are very diverse group of organisms inhabiting many ecosystems ranging from marine and fresh water environments to desert sands, hot springs to snow and ice (Guschina et al, 2006). Some algal species are important source of food and pharmaceutical products. *Dunaliella* sp are halotolerant, unicellular marine algae, which can be cultivated in a media with high NaCl concentrations, ranging from 0.2% to saturation level (Ben-Amotz and Avron, 1981). The remarkable feature of this alga is its ability to accumulate large amounts of commercially important compounds such as β-carotene and glycerol (Ben Amotz and Avron 1981, 1983, Borowitzka and Borowitzka 1988), which has been detailed in chapter I. In the present study an indigenous strain of *D. bardawil* was used. The success of algal cultivation mainly depends on the establishment, maintenance and growth of the culture in different climatic and environmental conditions. Different parameters such as nutrients, light, temperature, carbon dioxide and essential macro and micronutrients play a pivotal role in the growth of any algae. Several researches (Ben Amotz and Avron, 1981, 1983, Ben Amotz 1995) have been carried out for the production of carotenoids from *Dunaliella* species. The studies point out that nutritional imbalance, particularly lack of nitrogen, high salinity and light intensity play a major role in the carotenogenesis of *Dunaliella*. Hence in this section, studies were conducted to evaluate the influence of above mentioned parameters on growth and carotenogenesis of *D. bardawil*. A successful scale up study of algae is a prerequisite to achieve its commercial feasibility. Hence an outdoor culture methodology was developed to achieve efficient growth and carotenogenesis in large scale. Apart from that, this section also deals with harvesting and drying methods for algal biomass, stability analysis of carotenoids during different storage conditions and nutritional evaluation of the algal biomass.

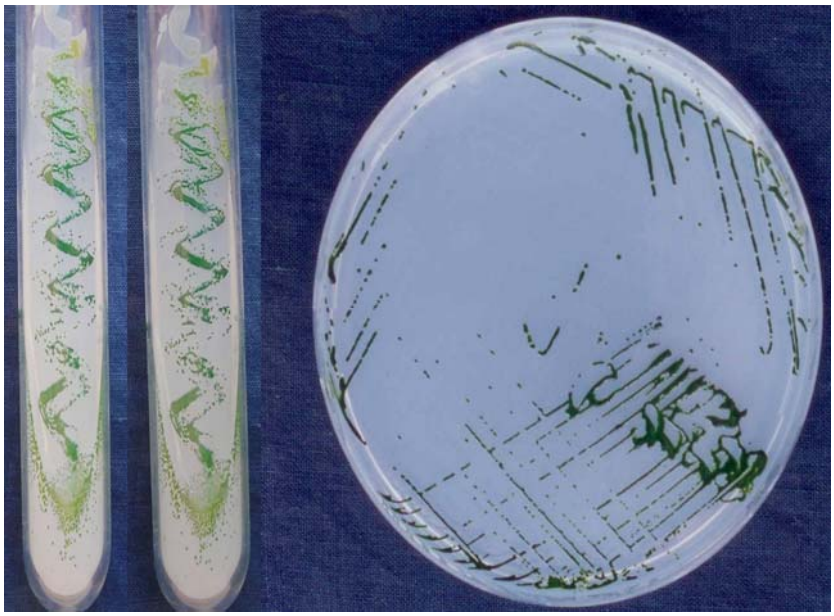
### 3.1.Maintenance of the germplasm

#### 3.1.1. *D. bardawil* growth under laboratory condition

The cultures of *D. bardawil* were maintained in agar slants and Petriplates (Fig 5). The suspension cultures were prepared from these axenic cultures. Erlenmeyer flasks (150mL) containing 30 mL AS100 media (Table 7, material and methods, Sec 2.1.1) were inoculated with the algae from the fully grown slants. These cultures were maintained at 20-22$^0$C under light intensity of 1.5-2 Klux provided by universal white lamps (Philips, India). During the vegetative phase a maximum of 20 ± 5 g $L^{-1}$ biomass (wet weight) was obtained on 30$^{th}$ day with a cell count of 10X10$^6$ cells $mL^{-1}$ (Fig 6).

The cultures were sub cultured once in every 30 days with AS 100 media (Table 7, material and methods 2.1.1) with *D. bardawil* cultures of ~ $10^6$ cells $mL^{-1}$ as inoculum. This provided a culture density of ~40 X $10^4$ cells initially. Further, these cultures were maintained in Erlenmeyer flasks of 150 and 250mL culture capacity (Fig. 7).

Initial experiments were carried out to study the effect of photoperiod on growth during vegetative stage. The results revealed that the growth was higher in case of continuous light (1.5-2 Klux) than 16 hr photoperiod of same light intensity. Hence in all further studies the cultures were maintained in continuous light intensity.



**A** **B**

**Fig 5. *D. bardawil* growing in (A) slant and (B) in Petriplate after 10 days of inoculation.** *D. bardawil* inoculated into solid media and grown at 20-22$^0$C under light intensity of 1.5-2 Klux.

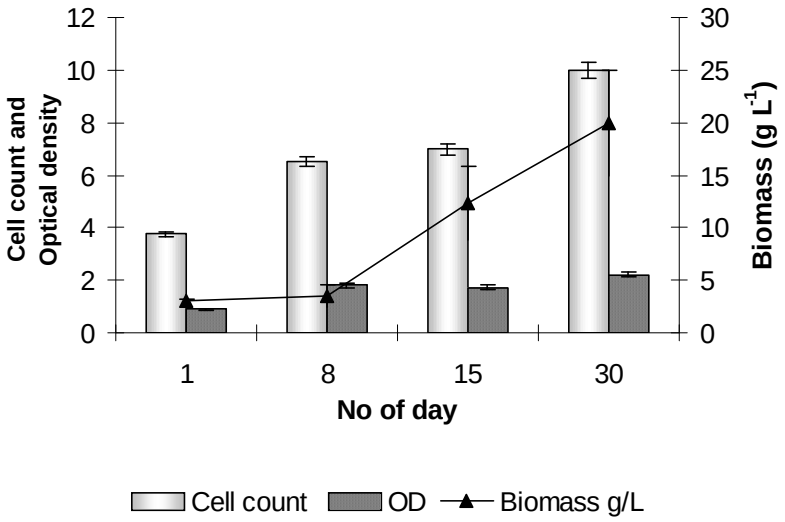



**Fig 6. Growth of *Dunaliella bardawil* in 150mL flasks under indoor condition at 20-22$^0$C temperature and 1.5-2 Klux light intensity.** The parameters studied for the growth include Cell count ($10^6$ cells $mL^{-1}$), Optical density (OD) and the total biomass weight (wet weight).

**Fig 7. *D. bardawil* vegetative stage culture growing in 150mL flask under indoor condition of 20-22$^0$C and a light intensity of 1.5-2 Klux.**

### 3.1.2. Chlorophyll and carotene content in the vegetative stage of *Dunaliella bardawil* under laboratory condition

Chlorophyll content was estimated from cultures grown in 150mL flasks for a period of 30 days under laboratory culture conditions. The time course data on accumulation of chlorophyll is presented in figure 8. Chlorophyll a and b content was found to be 200 ± 12 mg $100mg^{-1}$ wet biomass and 75 ± 9 mg $100mg^{-1}$ wet biomass respectively on $30^{th}$ day. In indoor culture the initial carotene content was 50± 8μg $100mg^{-1}$ wet biomass (0.05%) and after 30 days this was 136 ± 15 μg $100mg^{-1}$ wet biomass (0.13%, Fig 8).

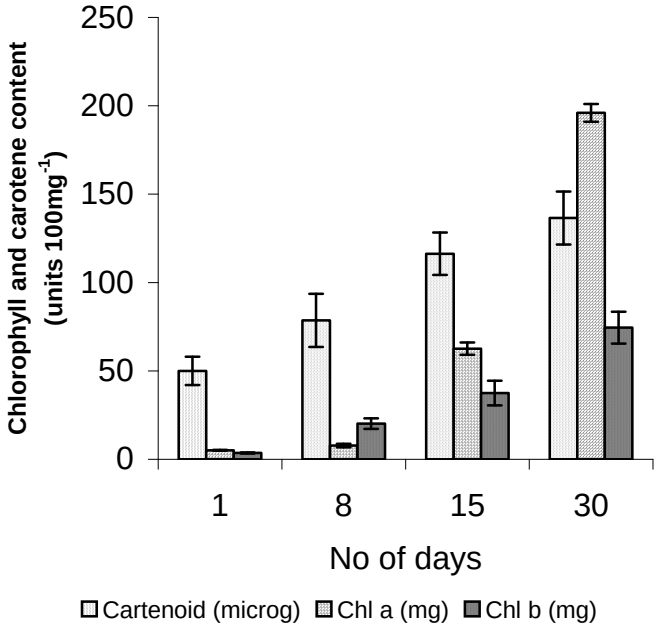



**Fig 8. Chlorophyll a, b and carotenoid content in *Dunaliella bardawil* grown under 20-22$^0$C and light intensity of 1.5- 2.0 Klux.**

### 3.1.3. Growth and carotene content in different *Dunaliella* species under indoor laboratory culture condition

The growth (Fig 9) and carotenoid profile (Fig. 10) of different *Dunaliella* sp are compared with *D. bardawil* cultured in modified AS100 medium. Among the studied strains Ne-17, T-34 and AP-504 strain showed a comparable amount of carotene yield, however the growth was maximum in *D. bardawil* strain.

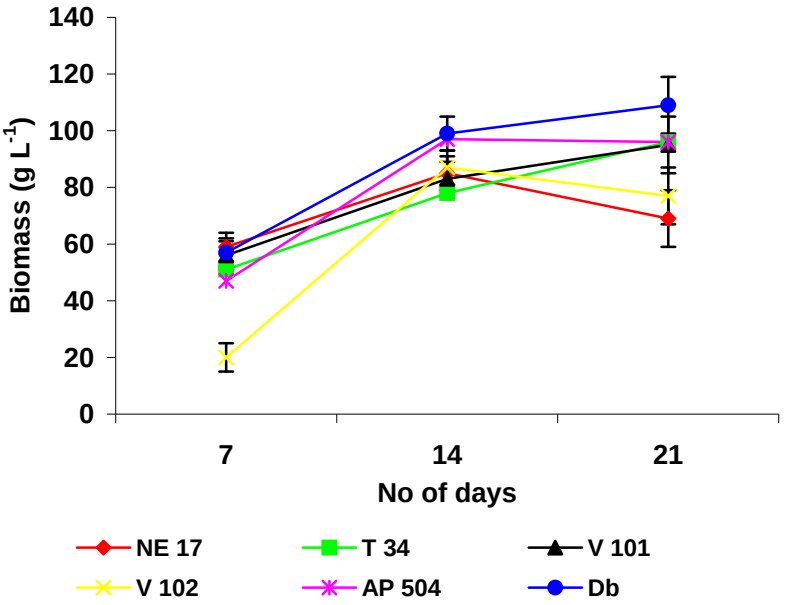



**Fig. 9. Growth (wet weight of biomass) of different *Dunaliella* sp under indoor conditions of 1.5-2 Klux light intensity.**

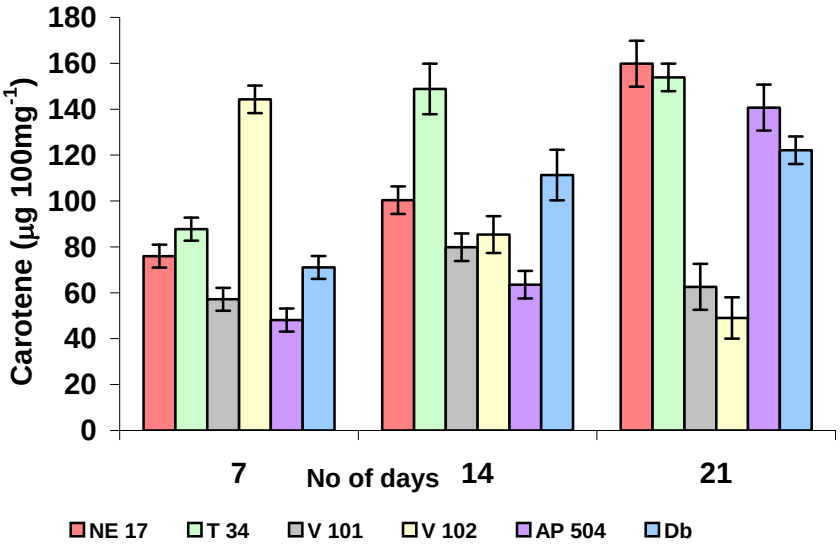



**Fig. 10. Carotenoid content in different *Dunaliella* sp. grown indoor under 1.5-2 Klux light intensity.**

### 3.1.4. Growth and carotenoid production in *D. bardawil* under different culture conditions in indoor culture system

**Background**

In this section, the influence of different physical and media parameters on the growth and carotenogenesis of *D. bardawil* was studied. Algal cells were subjected to various stress conditions like light, salt and different metal ions after a growth period of 7 ± 2 days to study their effect on the growth and carotenogenesis.

#### 3.1.4.1 Growth and carotenoid production under different sodium chloride concentrations

*D. bardawil* cells were grown in different NaCl concentrations ranging from 0.5M to 5.0M. A higher growth rate was observed at a NaCl concentration of 0.5 M to 1.0M (28 and 56 g $L^{-1}$ NaCl). Cell number decreased as NaCl concentration increased from 2.0M to 4.0M (Fig. 11). There was marked growth inhibition at 4.0M initial NaCl concentrations, however growth was observed after 7 days. Initial concentration of NaCl at 5.0M and above resulted in cell death. However the carotene content was found to be high on $15^{th}$ day, in the cells grown at 2.0M NaCl (0.2%) compared to 1.0M (0.1%) NaCl concentration (Fig.12). A two-fold increase in the carotenoid content was observed when the cultures were grown at 2.0M NaCl concentrations.

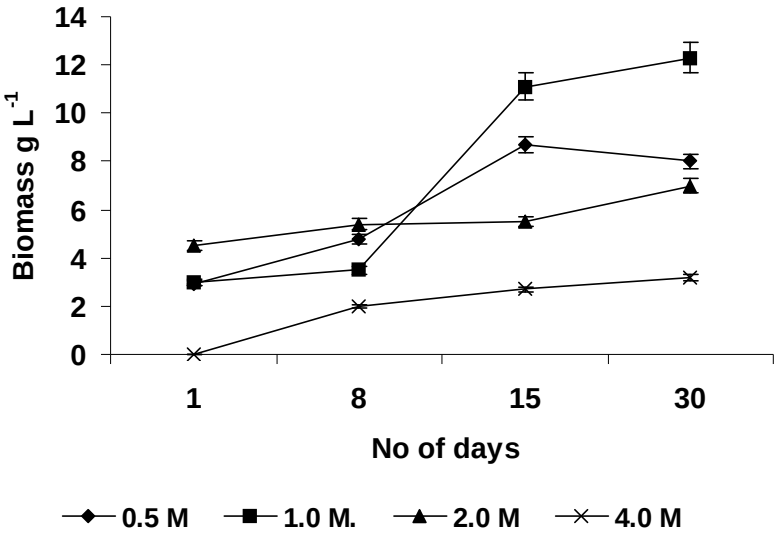



**Fig.11. Growth pattern of *D. bardawil* under different NaCl concentrations ranging from 0.5 M to 4.0 M at indoor laboratory culture conditions.**

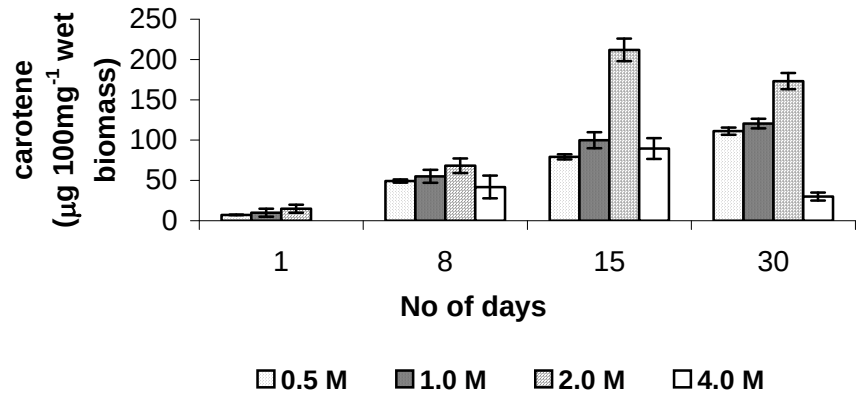



**Fig. 12. Carotene content of *D. bardawil* under different NaCl concentrations ranging from 0.5 M to 4.0 M at indoor laboratory culture conditions.**

### 3.1.4.2. Growth and carotenoid production under various levels of carbon dioxide

*D. bardawil* being photoautotropic is capable of utilizing the external source of carbon dioxide such as bicarbonates or gaseous $CO_2$. Hence the effect of $CO_2$ on growth and carotenogenesis was observed by supplementing either as $NaHCO_3$ or 2% $CO_2$ in gaseous form by two-tier flask method (Fig. 13).

Growth was enhanced when $CO_2$ supplementation was provided in gaseous form rather than supplementing $NaHCO_3$ in the medium (Fig. 14). However no significant difference ($P<0.05$) was observed both in carotene and chlorophyll content (Fig 15 and 16).

**Fig 13. Experimental set up showing the $CO_2$ supplementation for *Dunaliella bardawil* cultures adopting two-tier flask method.**

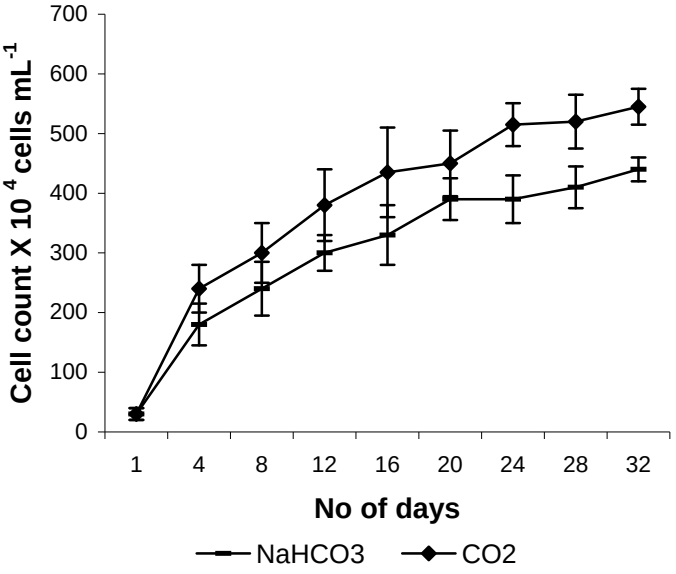



**Fig 14. Growth pattern of *D. bardawil* under $CO_2$ supplementation, by $NaHCO_3$ (4.0g $L^{-1}$) or 2% $CO_2$ in gaseous form by two-tier flask method.**

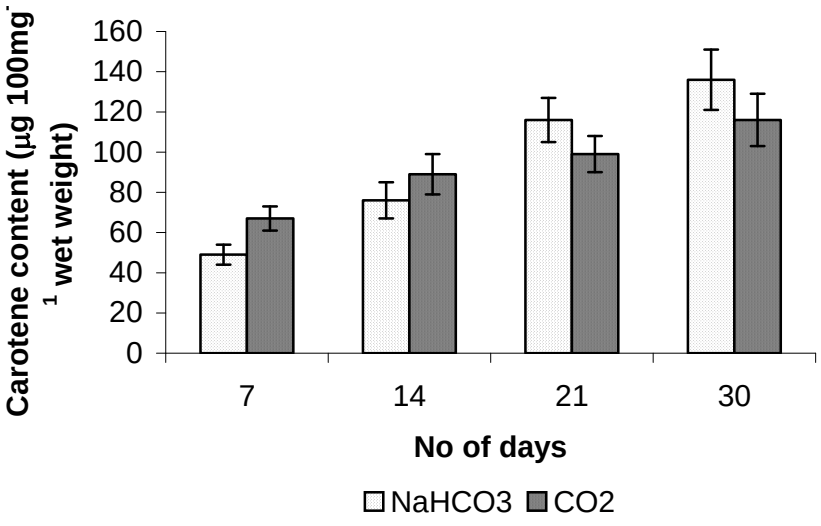



**Fig 15. Carotene content of *D. bardawil* under $CO_2$ supplementation, by supplementing $NaHCO_3$ (4.0g $L^{-1}$) or 2% $CO_2$ in gaseous form by two-tier flask method.**

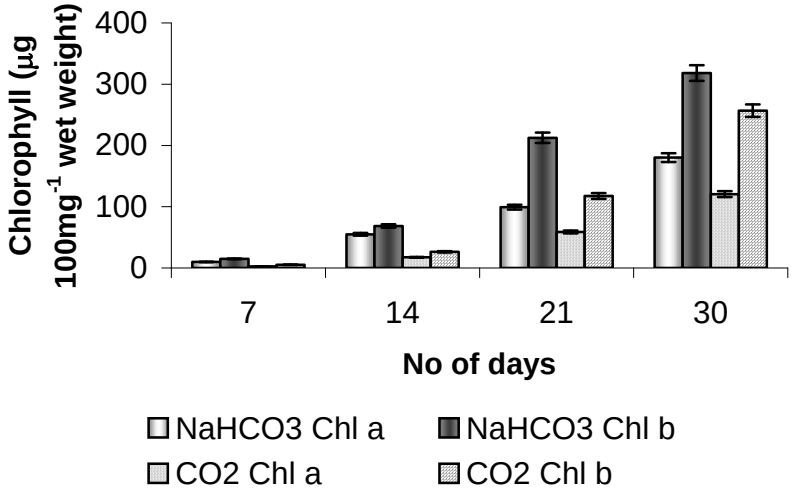



**Fig 16. Chlorophyll a and b content of *D. bardawil* under $CO_2$ supplementation by $NaHCO_3$ (4.0g $L^{-1}$) or 2% $CO_2$ in gaseous form by two-tier flask method.**

### 3.1.4.3. Growth and carotenoid production under the influence of different bivalent metal ions

Metal ions such as iron and zinc are regarded as essential components for algal growth (Matsunaga et al, 1998). They also play a role as cofactor for important enzymes. Hence it is important to know the optimal concentration of these metal ions for better growth and carotenogenesis. Therefore, the influence of selected bivalent metal ions on the growth and carotenogenesis in *D. bardawil* was studied.

#### 3.1.4.3.1 Effect of $Fe^{2+}$ on the growth and carotenogenesis in *D. bardawil*

The growth and carotene content with different concentration of $Fe^{2+}$ ions are given in Fig 17 and 18. A range (0.2 – 2.0mM) of Ferric chloride was used in terms of $Fe^{2+}$ ion concentration. Among the levels used 0.8 mM and 1mM were found to be ideal for growth of *D. bardawil.* However high β-carotene content was observed with increasing concentration of $Fe^{2+}$. The carotene content was 120µg/100mg (0.12%) at 0.8mM $Fe^{2+}$ compared to 150 µg/100mg (0.15%) at 2mM $Fe^{2+}$ levels on 22$^{nd}$ day (Fig 18).

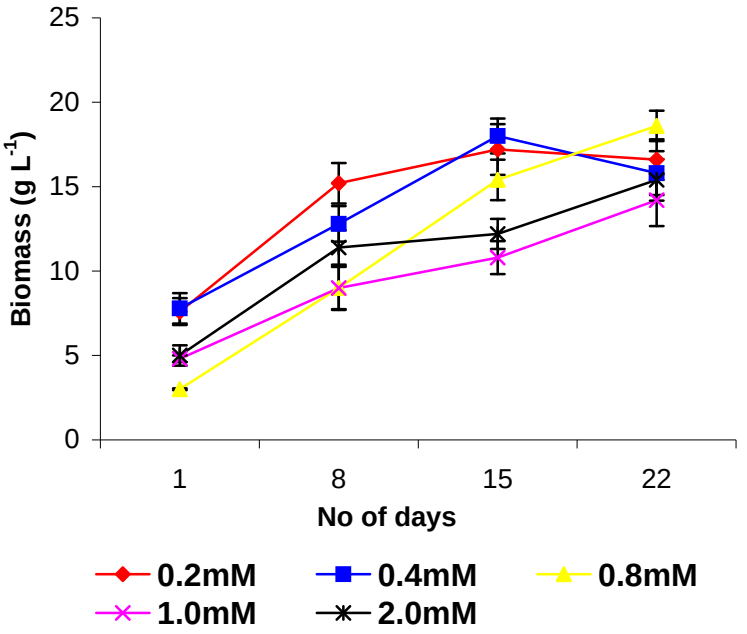



**Fig 17. Growth of *D. bardawil* on different concentrations of $Fe^{2+}$ ions (0.2mM to 2mM) at indoor laboratory culture conditions.**

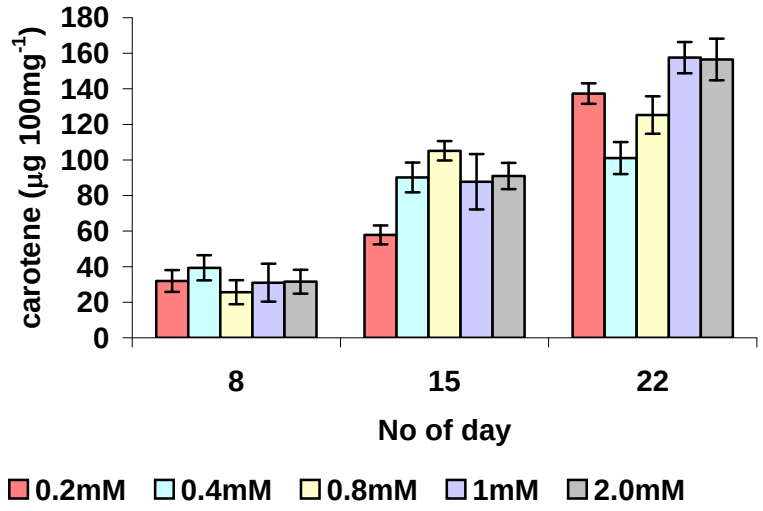



**Fig 18. Carotene content of *D. bardawil* on different concentrations of $Fe^{2+}$ ions (0.2mM to 2mM) at indoor laboratory culture conditions.**

### 3.1.4.3.2 Effect of Zn ion on the growth and carotenogenesis in *D. bardawil*

Different levels of Zinc chloride ($ZnCl_2$) equivalent to 0.2-1.0µM of Zn were used. After 22 days, the cultures grown at 0.4µM of Zn showed high growth in terms of biomass (10.6 ± 1.6g $L^{-1}$) and was found to be ideal for the growth of *D. bardawil* compared to other levels. The increased concentration of Zn up to 1µM did not show

any inhibition to algal growth. The β-carotene content showed a high value at 0.6 µM (180 µg $100mg^{-1}$) $ZnCl_2$ concentration, compared to 0.2µM (120 µg $100mg^{-1}$). The growth and carotene content of *D. bardawil* grown under different concentration of Zinc ion is presented in the Fig 19 and 20.

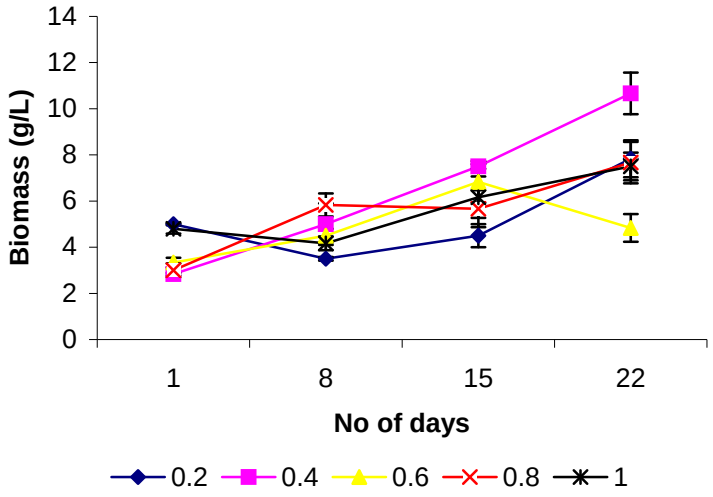



**Fig 19 Growth of *D. bardawil* on different concentrations of Zinc ions (0.2µM to 1µM) grown at indoor laboratory culture conditions.**

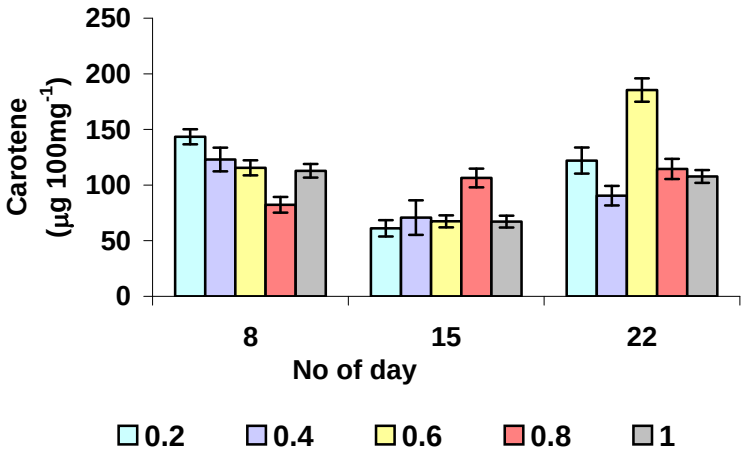



**Fig 20. Carotene content of *D. bardawil* on different concentrations of Zinc ions (0.2 µM to 1µM) grown at indoor laboratory culture conditions.**

### 3.1.4.3.3 Effect of Mn ion on the growth and carotenogenesis in *D. bardawil*

A range of Manganous chloride ($MnCl_2$) was used in terms of Mn ion concentration (2 to 10 µM). Among the different levels used, no significant difference was observed in growth for 22 days (Fig. 21). However the β-carotene content showed a high value

of 190 µg 100mg$^{-1}$ (0.19%) at 6 µM of Mn concentration (Fig. 22). Increasing concentration of $MnCl_2$ up to 10 µM showed an enhancement in carotene content without affecting the growth.

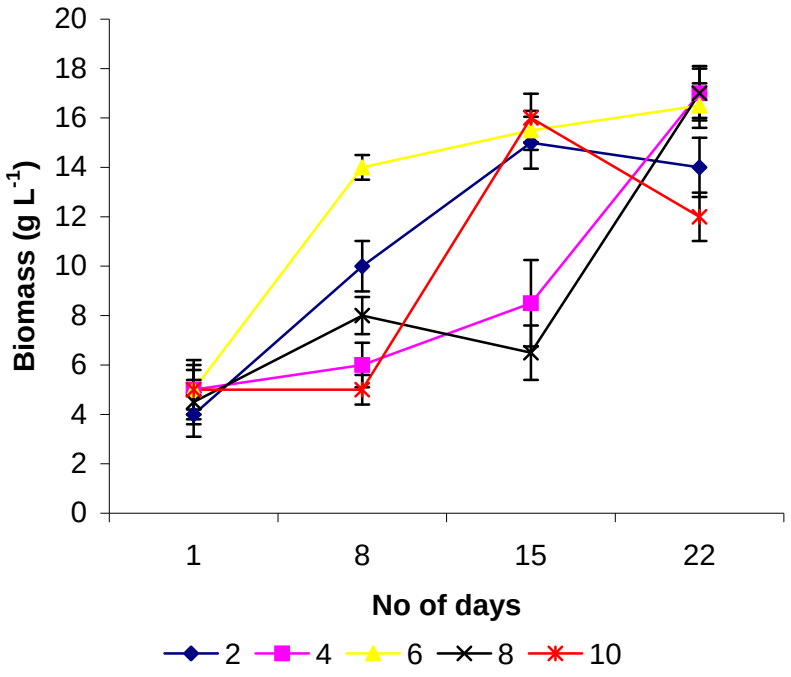



**Fig. 21 Growth of *D. bardawil* on different concentrations of Mn ions (2 to 10µM) grown at indoor laboratory culture conditions.**

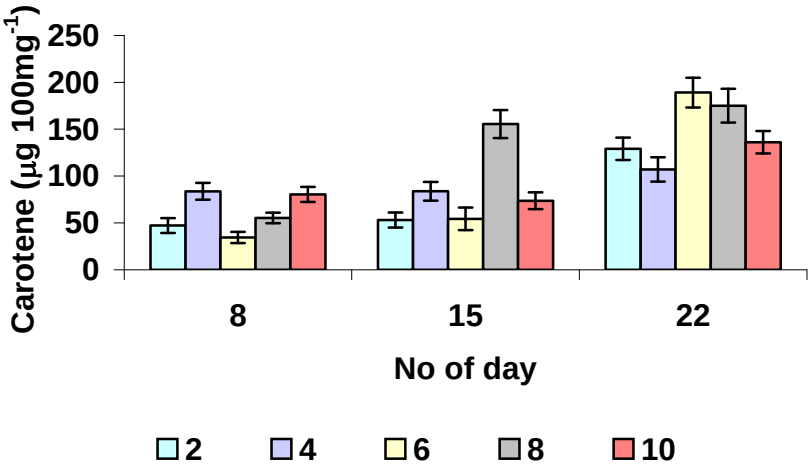



**Fig 22. Carotene content of *D. bardawil* on different concentration of Mn ions (2µM to 10µM) grown at indoor laboratory culture conditions.**

### 3.1.5. Growth and scale up studies in outdoor culture conditions

*D. bardawil* cultures of ~ $10^6$ cells $mL^{-1}$ were sub cultured once in every 30 days with AS 100 media (Table 7, material and methods 2.1.1) in the ratio of 1:6. This provided a culture density of ~40 X $10^4$ cells initially. Further, these cultures were raised in 250mL, 500mL and 1L capacity culture flasks. Subsequently the cultures were transferred from indoor to outdoor culture condition. In outdoor, the cultures were maintained in a glass tank (40cm height X 40cm length X 20cm width) with a culture depth of 10cm with 15±2L culture (Fig 23). In glass tank maximum cell count of 6 ± 0.5 x $10^6$ cells $mL^{-1}$ was attained. *D. bardawil* cultures from the glass tanks were transferred to circular cement tanks of 2m diameters with a total volume of 150-200L cultures (Fig 24). The algae was cultured in these circular tanks for 30 days and then transferred to raceway tanks of 500L capacity (Fig 25). These cultures were protected from the direct sunlight with the help of a green house shade net. This cuts off 50% light intensity and provides a light intensity of 15-20 Klux. The cultures were continuously maintained in the vegetative phase of the alga throughout the year by repeated sub culturing once in a month.

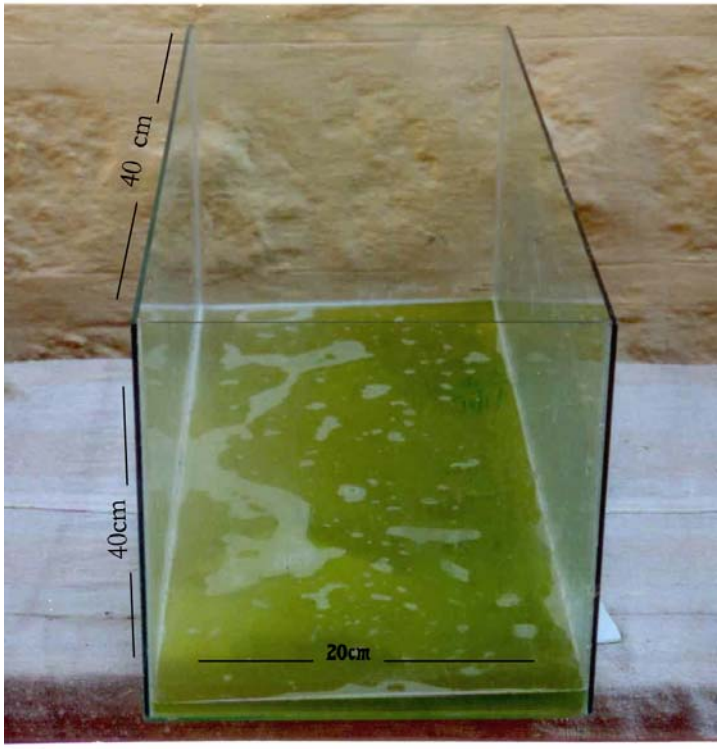



**Fig 23. *D. bardawil* vegetative stage culture in glass tank under outdoor culture (15-20 Klux light intensity).**

**Fig 24. *D. bardawil* vegetative stage culture growing in circular cement tank with a total volume of 150-200L cultures under outdoor condition (bar= 25cm internal diameter).**

**Fig 25. *D. bardawil* vegetative stage culture in raceway tank of 500L capacity under outdoor condition.** The cultures were protected from direct sunlight with the use of green house shade nets (bar = 30cm).

### 3.1.6. Comparison of growth under indoor and outdoor culture conditions

*D. bardawil* cultures were subjected to different light intensities as explained in material and methods (section 2.1.5.1). The growth of *Dunaliella bardawil* was linear for 6-8 days (Fig. 26) under controlled condition of light (1.5-2 Klux), and on 30$^{th}$ day high growth was observed in indoor culture condition compared to outdoor cultures. Comparatively, growth rate was high (0.12 $d^{-1}$) in the indoor cultures than cultures grown at outdoor culture condition of 15-20 Klux (0.04 $d^{-1)}$.

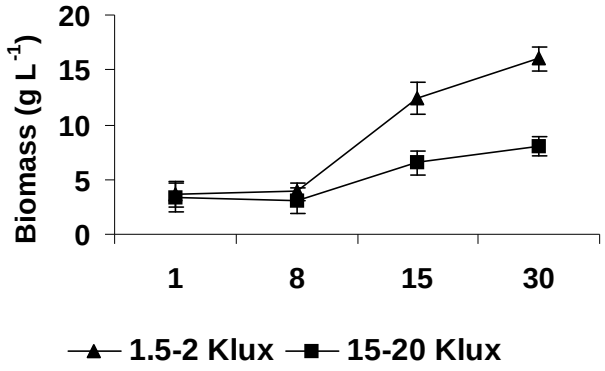
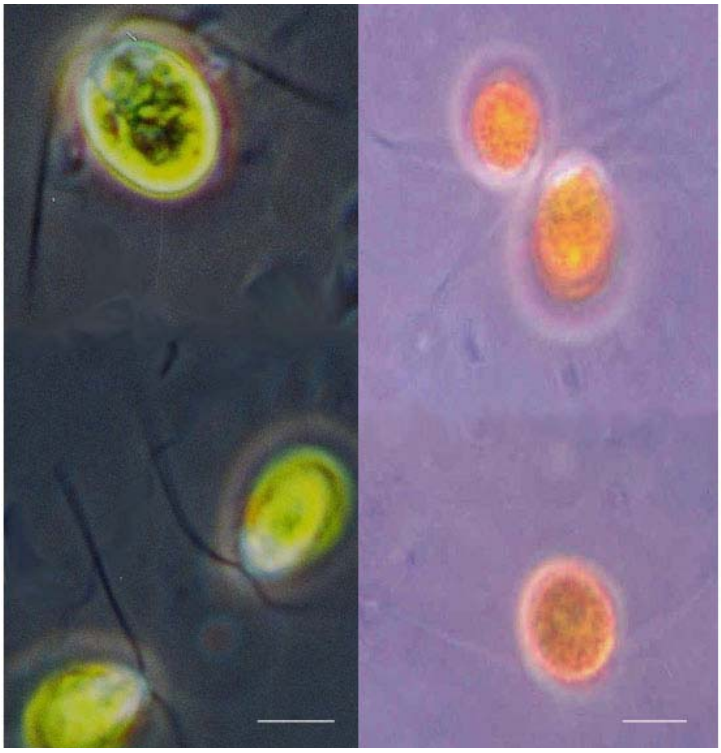



**Fig. 26. Growth pattern of *D. bardawil* under different light intensity.** The cultures were acclimatized for 7 days under indoor condition of 1.5-2 Klux and transferred to outdoor condition of 15- 20 Klux covered with a shade net.

### 3.1.7. Carotenogenesis in *D. bardawil* under outdoor culture conditions

The outdoor grown cultures of *D. bardawil* were exposed to direct sunlight for carotenogenesis by removing the shade nets. Due to high light intensity the cell division ceased and the cells start accumulating carotenoids. The cells turned green to yellow color on 3$^{rd}$ day of exposure to high light of 30- 35 Klux; whereas under low and moderate light condition the cells remained green in color (Fig 27A). After 4-5 days of exposure to high light, these cells completely turned to red (Fig 27B). The cultures also turned green to yellow and deep orange color (Fig. 28).



**A** **B**

**Fig. 27. Photographs showing the A. vegetative green cell and B. carotene accumulated cells of *Dunaliella bardawil* (bar = 5μM)**


**Fig 28. Carotene induced cells of *D. bardawil* in raceway tank under outdoor conditions.**

### 3.1.8. Carotenoid content in indoor and outdoor culture conditions

The vegetative cells of *D. bardawil* contained 0.06% (60± 8 μg $100mg^{-1}$ wet weight) β-carotene and 1.36% (1.36± 0.08mg $100mg^{-1}$ wet weight) lutein under indoor condition of 1.5-2 Klux. When these cells were exposed to a light intensity of 15-20 Klux significant increase in β-carotene was observed (Fig.29). At 30-35 Klux illumination for 48 hrs, the cells attained yellow color and the carotenoid content increased up to 3% (2.8 ± 0.25mg $100mg^{-1}$ wet weight) and after 4 days these cultures showed a carotene content of over 4% (4.21 ± 0.45mg $100mg^{-1}$ wet weight). However significant difference was not observed for lutein content, when the cultures were exposed to high light. Thus exposure of *D. bardawil* cells to increasing light intensities resulted in higher β-carotene accumulation.

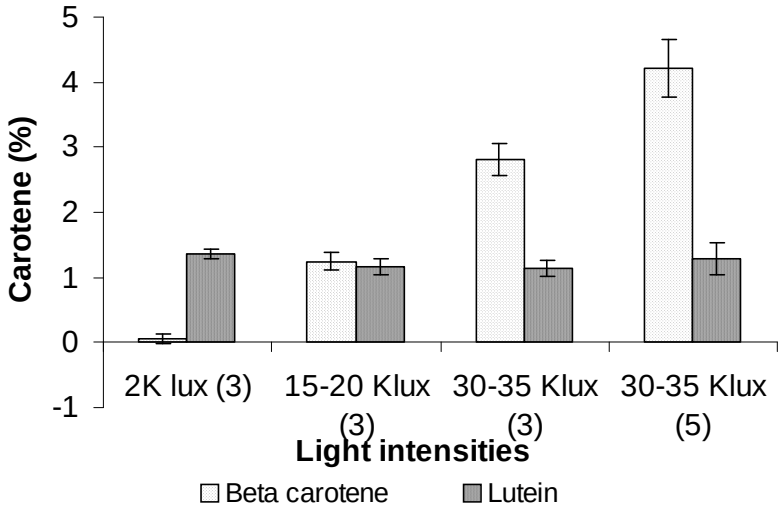



**Fig. 29. Concentration of carotenoids (β-carotene and lutein) under different light intensities**. The number in parenthesis represents the day after illumination to different light intensities.

### 3.1. 9. Harvesting of biomass

Initially, the filtration method was employed to harvest the *Dunaliella* cells. But the results were unsatisfactory due to the fact that these algal cells are very small to pass through the commercial filtration screens. Hence centrifuges were used for the harvesting. In the batch centrifuge (Westfolia, Germany) a culture volume of 50-75L was harvested in 4hrs. In online centrifugation, loading of the culture was at the rate of 100 L $hr^{-1}$. The wet biomass was collected at the inner side of the rotor. In both the cases 90-95% cell harvesting was achieved.

### 3. 1. 10. Drying of biomass

Different drying methods such as shade drying, sun drying, oven drying, spray drying and freeze-drying were employed. The study revealed loss of carotenoids in all drying methods. The loss of carotene was minimum in case of freeze-drying (9-13%). Though the sun/ shade drying was economical, they were not very efficient in retaining the carotenoids. Hence freeze-drying was employed in further studies. The Percentage losses of carotene in each drying method are compiled in Table 10.

**Table 10. Percentage loss of carotenoids in different drying methods used for *D. bardawil***

| Method | % Loss of carotenoids |
|---|---|
| Shade drying | 33-35 |
| Sun drying | 70-77 |
| Oven drying | 63-65 |
| Spray drying | 55-65 |
| Freeze drying | 9-13 |

### 3.1.11. Stability of dried biomass

Stability of carotene in *D. bardawil* cell powder was assessed during storage under dark conditions at different temperatures of $-80^0$, $0^0$, $25^0$ (room temperature) and $37^0$C for 3 months. The change in color intensity was monitored by measuring the color intensity once in two weeks. The β-carotene content in stored sample was analyzed by HPLC (section 2.1.4).

### 3.1.11. 1 Color stability of carotenoid powder during storage

The change in the color intensity of the *D. bardawil* biomass was assessed once in two weeks with color intensity measurement using Hunter 'L', 'a', 'b' values. The changes in Hunter 'L' (brightness), 'a' (red color) and 'b' (yellow color) value of carotenoid powder during storage at various temperatures in dark are shown in Table 11. Hunter L, a and b values decreased with increasing storage time. The Hunter 'L' value showed a significant ($P< 0.05$) decrease after 12 weeks when stored at $37^0$C. The decrease was minimum when the samples were stored at $-80^0$ and $0^0$C. Thus the brightness of the sample remained unchanged when the samples were stored at $-80^0$ and $0^0$C.

The yellow color of the sample (Hunter 'b' value) also showed a significant decrease with increasing temperature and storage time. The hunter 'b' value decreased by 0.38, 5.73 & 7.55 when stored at $0^0$, $25^0$ and $37^0$C respectively.

Hunter 'a' value (red color) showed a decrease in the values and reached to negative, indicating the green color of the powder. As the β-carotene degraded, the powder decolorized from orange to pale green, suggesting that chlorophyll was still present in the sample.

**Table.11. Changes in the Hunter 'L', 'a' and 'b' values of freeze dried *Dunaliella* powder during storage in dark**

Hunter 'L' value (indicates brightness of the powder) *

| Storage time (In weeks) | Storage temperature | | | |
|---|---|---|---|---|
| | $-80^0$ C | $0^0$ C | $25^0$ C | $37^0$ C |
| 0 | 70.83± 0.028 | 70.83 ± 0.004 | 70.83 ± 0.004 | 70.83 ± 0.004 |
| 2 | 70.83 ± 0.024 | 70.82 ± 0.008 | 70.52 ±0.008 | 67.35 ± 0.012 |
| 4 | 70.79 ± 0.021 | 70.78 ± 0.012 | 69.98 ±0.008 | 65.85 ± 0.002 |
| 6 | 70.65 ± 0.004 | 70.68 ± 0.016 | 67.83 ± 0.009 | 64.89 ± 0.017 |
| 8 | 70.35 ± 0.012 | 70.18 ± 0.012 | 67.35 ± 0.012 | 63.25 ± 0.017 |
| 10 | 70.28 ± 0.014 | 69.89 ± 0.008 | 66.95 ± 0.004 | 63.08 ± 0.012 |
| 12 | 70.08 ± 0.012 | 69.35 ± 0.013 | 66.23 ±0.008 | 61.78 ± 0.012 |

Hunter 'a' value (denotes the red color)*

| Storage time (In weeks) | Storage temperature | | | |
|---|---|---|---|---|
| | -80$^0$ C | 0$^0$ C | 25$^0$ C | 37$^0$ C |
| 0 | 2.95 ± 0.004 | 2.95 ± 0.008 | 2.95 ± 0.005 | 2.95 ±0.009 |
| 2 | 2.95 ± 0.004 | 2.95 ±0.008 | 2.57 ±0.008 | -1.11 ± 0.008 |
| 4 | 2.93 ± 0.005 | 2.91 ± 0.009 | 2.17 ± 0.009 | -1.82 ± 0.008 |
| 6 | 2.93 ± 0.008 | 2.88 ± 0.012 | 1.98 ± 0.013 | -2.6 ± 0.012 |
| 8 | 2.92 ± 0.005 | 2.82 ± 0.009 | 1.48 ± 0.017 | -3.28 ± 0.017 |
| 10 | 2.92 ± 0.008 | 2.8 ± 0.004 | 1.18 ± 0.012 | -3.37 ± 0.012 |
| 12 | 2.92 ± 0.008 | 2.79 ±0.002 | -1.28 ± 0.012 | -4.43 ± 0.012 |

Hunter 'b' value (denotes yellow color) *

| Storage time (In weeks) | Storage temperature | | | |
|---|---|---|---|---|
| | -80$^0$ C | 0$^0$ C | 25$^0$ C | 37$^0$ C |
| 0 | 28.27 ± 0.004 | 28.27 ± 0.004 | 28.27 ± 0.004 | 28.27 ± 0.009 |
| 2 | 28.26 ±0.008 | 28.26 ± 0.008 | 26.58 ± 0.017 | 24.29 ± 0.008 |
| 4 | 28.25 ±0.009 | 28.25 ± 0.009 | 26.39 ± 0.009 | 22.68 ± 0.008 |
| 6 | 28.25 ±0.012 | 28.09 ± 0.012 | 25.98 ± 0.013 | 21.58 ± 0.013 |
| 8 | 28.23 ± 0.012 | 27.98 ± 0.005 | 24.59 ± 0.017 | 20.74 ± 0.008 |
| 10 | 28.21 ± 0.009 | 27.91 ± 0.004 | 24.12 ±0.012 | 20.74 ± 0.012 |
| 12 | 28.21 ± 0.008 | 27.89 ± 0.008 | 22.54 ± 0.012 | 20.72 ± 0.009 |

'*' Mean values of triplicate analysis.

### 3.1.11.2. Carotenoid content during storage

The HPLC analysis revealed that β-carotene content decreased with increasing temperature and storage time. In the samples stored at high temperature, the loss of β-carotene increased with increasing time (Fig. 30). There was minimum loss under storage at –80$^0$ and 0$^0$C. However a gradual decrease was observed when the samples were stored at 25$^0$ and 37$^0$C in dark.

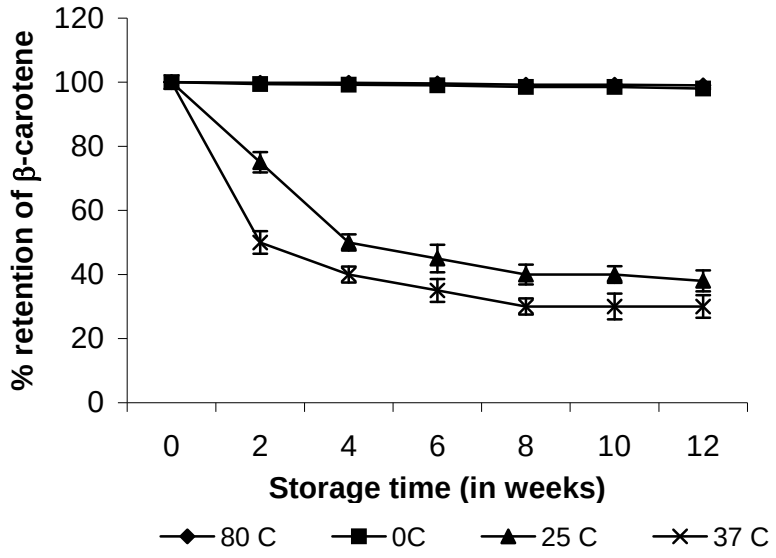



**Fig 30. Degradation pattern of β-carotene during storage under different temperature.**

### 3.1.12. Nutritional compositions of *D. bardawil* dry biomass

Proximate analysis of *D. bardawil* showed the presence of high protein (22%) and carbohydrate (27%) content. Other parameters of algal biomass are given in Table 12.

**Table 12. Proximate composition of *D. bardawil* biomass**

| **Components** | ***D. bardawil (%)*** |
|---|---|
| Protein | 21.51 ± 1.75 |
| Fat | 07.80 ± 0.38 |
| Carbohydrate | 26.70 ± 1.11 |
| Total carotenoids | 02.80 ± 0.30 |
| β- carotene | 03.07 ± 0.70 |
| Ash | 5.80 ± 1.20 |
| Moisture | 06.70 ± 0.90 |

The mineral content of the biomass is presented in Table 13. *D. bardawil* biomass is rich in mineral elements like calcium, iron and potassium. The toxic metals like cadmium and mercury were not detected. Lead and chromium were present in traces and are well below the permitted level.

**Table 13. Mineral composition of *D. bardawil* biomass**

| Mineral content | *D. bardawil* (ppm) |
|---|---|
| Calcium | 8.08 ± 0.6 |
| Magnesium | 4.1 ± 0.03 |
| Potassium | 5.9 ± 0.5 |
| Copper | 0.4 ± 0.04 |
| Zinc | 1.4 ± 0.1 |
| Iron | 9.4 ± 0.4 |
| Chromium | 1.2 ± 0.1 |
| Lead | 0.3 ± 0.002 |
| Mercury | ND |
| Cadmium | ND |

(ND- Not detected)

### 3.1.13. Analysis of fatty acids present in *D. bardawil*

The total fat was ~ 07.80 ± 0.38% in *D. bardawil*. Most of the lipid fractions account to neutral (35.5 ± 2%) and glycolipid (40.5 ± 3%) fractions with a polar lipid of 24.0 ± 2.5%.

The lipids were identified by their mobilities and specific color reactions as explained in material and methods (section 2.1.10.6.3). There were eight spots of neutral lipids and they were visualized on the silica plates developed with iodine vapor. Three glycerolipid fractions were detected and they were visualized by their mobilities as monoglyceride and 1:2 and 1:3 diglycerides (Table 14). In the polar lipid fraction five phospholipids viz., phosphatidyl inositol, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidic acid and phosphatidyl glycerol were identified (Table 15). The fatty acid compositions of *D. bardawil* are given in Table 16. The major fatty acid found was linoleic acid (C18:2; 35%) and the linolenic acid (C18:3; 81%). The saturated fatty acids were observed with C14:0, 16:0, 20:0, 21:0 and 22:0 carbon and unsaturation only in C18 fatty acids. The higher fatty acids, identified were belonging to C20, C21 and C22 (Table 16).

**Table 14. Composition of neutral and glycolipid in *D. bardawil*[*].**

| Component | % |
|---|---|
| 1. Monoglyceride | 9 |
| 2. 1:2 diglyceride | 7 |
| 3. 1:3 diglyceride | 9 |
| 4. Unidentified | 2 |
| 5. Diacyl glycero trimethyl homoserine | 2 |
| 6. Sterols | 12 |
| 7. Free fatty acids | 6 |
| 8. Triglyceride | 8 |
| 9. Sterol esters and hydrocarbons | 22 |
| 10. Polar lipids (remained at the origin) | 23 |

[*]Values are mean of triplicate analysis carried out twice.

**Table 15. Composition of polar lipid in *D. bardawil*[*].**

| Component | % in *D.bardawil* (23% of Total) |
|---|---|
| 1. Phosphotidyl inositol | 2 |
| 2. Phosphatidyl choline | 4 |
| 3. Phosphatidyl ethanolamine | 4 |
| 4. Phosphatidic acid | 5 |
| 5. Phosphatidyl glycerol | 8 |

[*]Values are mean of triplicate analysis carried out twice.

**Table 16. Fatty acid (relative %) composition∗ of *D. bardawil*[*]**

| Fatty Acid | Neutral Lipids | Polar Lipids | Glycolipid Fraction |
|---|---|---|---|
| 11:0 | 0.8 | - | - |
| 12:0 | 0.44 | 1.9 | - |
| !3:0 | 0.8 | - | - |
| 14:0 | 9.2 | 0.8 | 0.17 |
| 14:1 | 0.39 | 0.1 | - |
| 15:0 | 0.3 | 0.1 | 3.5 |
| 15:1 | 2.0 | 3.8 | 1.1 |
| 16:0 | 7.5 | 1.7 | 1.7 |
| 16:1 | 0.9 | 1.3 | - |
| 17:0 | 0.1 | 0.9 | 0.09 |
| 17:1 | 0.2 | 0.14 | - |
| 18:0 | 0.7 | 0.6 | - |
| 18:1 | - | 2.5 | - |
| 18:2 | 35.5 | 2.5 | 0.8 |
| 18:3 | 0.9 | 0.5 | 81.2 |
| 20:0 | 20.8 | 1.0 | - |
| 20:1 | - | 0.6 | - |
| 20:2 | - | 0.98 | - |
| 20:3 | - | 0.1 | - |
| 20:4 | - | 1.5 | - |
| 20:5 | - | 1.02 | 0.07 |
| 21:0 | 0.2 | 4.5 | 0.02 |
| 22:0 | 3.6 | 26.5 | 6.4 |
| 22:1 | 0.18 | 1.6 | - |
| 22:2 | 0.2 | 1.92 | 0.02 |
| 22:6 | 0.07 | - | - |
| 23:0 | 0.15 | 0.9 | 0.3 |
| Unidentified | 15.07 | 42.54 | 4.63 |

*Average value of triplicate analysis carried twice. -Not detected

### 3.1.14. Analysis of carotenoids from *D. bardawil* biomass

#### 3.1.14.1.Thin layer chromatography

In acetone: hexane: acetic acid (7:3:0.1) solvent system carotenoids resolved into eleven spots. The different compounds identified include chlorophylls, lutein, lycopene and β- carotene. The predominant carotenoids were β- carotene and lutein.

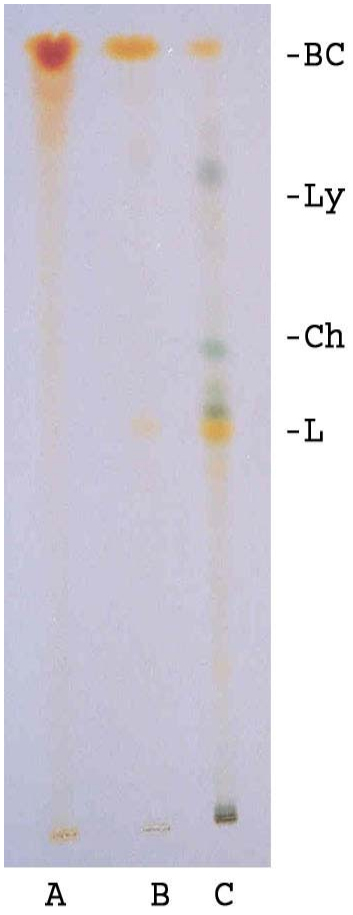



**Fig 31. Thin layer chromatogram of (A) synthetic β-carotene, (B) *D. bardawil* biomass extracted from red cells and (C) green vegetative cells**. The spots identified include; BC-β-carotene, Ly-lycopene, Ch-Chlorophyll and L-lutein.

#### 3.1.14.2. Analysis of carotenoids by HPLC

A clear separation of carotenoids was noticed when methanol: acetonitrile: chloroform (47:47:6) was used as a solvent system (Fig 32). The β-carotene peak was eluted at 19$^{th}$ minute and was identified with standard β-carotene (Sigma). In both the green vegetative and carotene induced cultures the predominant carotenoids include β-carotene, lutein, lycopene, α-carotene and the rest containing chlorophyll and

unidentified peak. The HPLC profiles of both green and red cells are shown in the Figure 32.

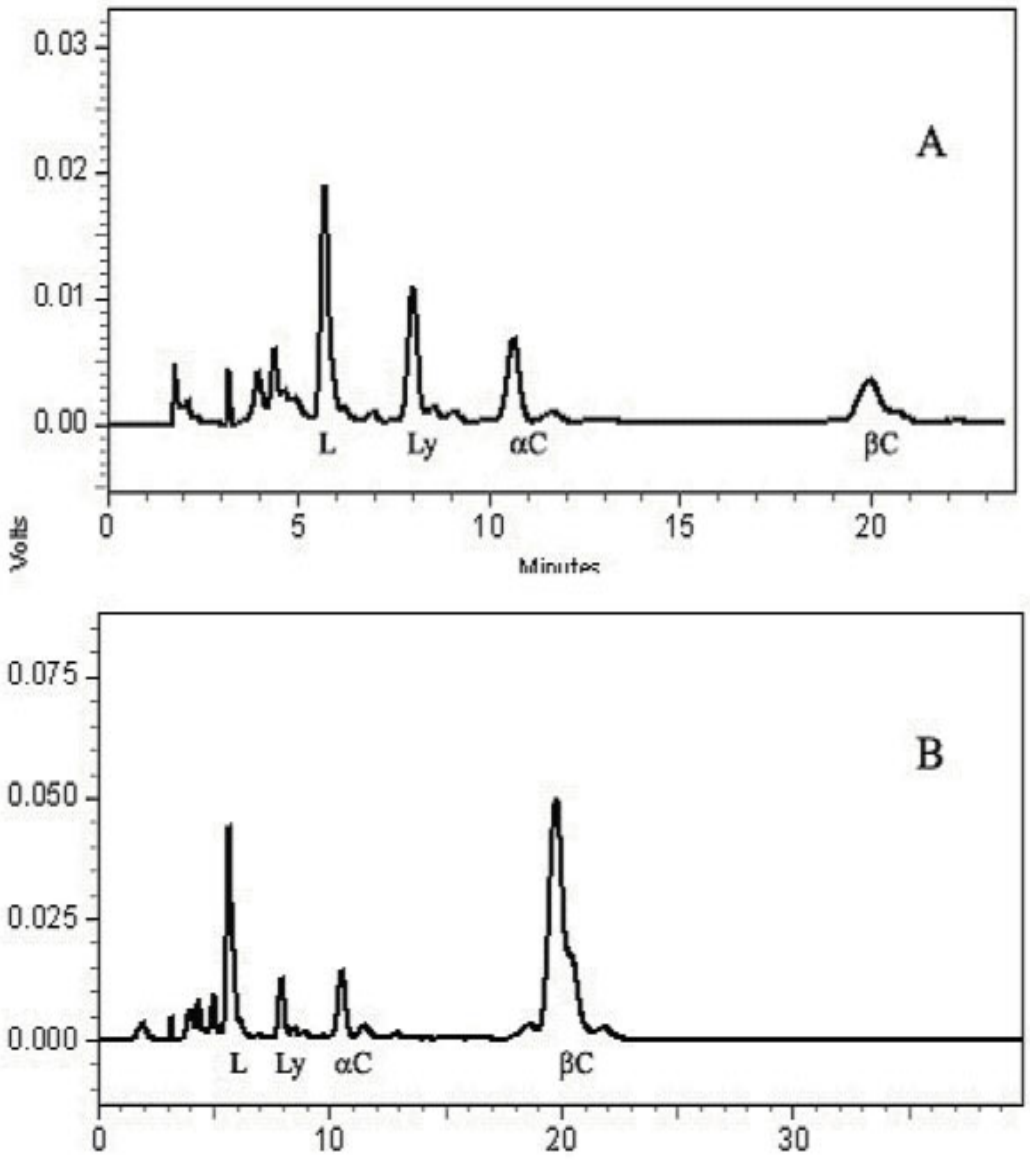



**Fig. 32. HPLC Profile of (A) green vegetative stage and (B) carotene accumulated cells.** The symbols represent L-Lutein, Ly-Lycopene, αC-αcarotene and βC- β-carotene

### 3.1.15. Discussion

*Dunaliella* sp are widely cultivated as a commercial source of natural β-carotene (Borowitzka and Borowitzka, 1988; Ben Amotz and Avron, 1990; Ben-Amotz, 1995). They have two distinct phases in its life cycle, first is the growth or vegetative phase and the next being carotenogenesis phase. Carotenogenesis can be induced physiologically at any stage of the cell cycle (Ben- Amotz, 1995). *Dunaliella* sp are generally found in oceans, salt marshes, and salt water ditches near sea. It can survive in a range of salt (sodium chloride) concentration ranging from 0.1M (less than sea water) to saturation level. Under optimum growth conditions doubling time of *Dunaliella* is 5 hrs, which can go up to 3 days under high salinity conditions (Ginzburg and Ginzburg, 1981). Initially cells can grow in low salt concentration and upon growth they can adapt to 2-3 fold hypertonic and hypotonic change in salt concentrations (Ben-Amotz and Avron, 1983; 1990).

*D. bardawil* cultures were maintained and grown in AS 100 medium both in slant and liquid culture (Fig 5 & 7). The growth pattern of the algae is shown in Fig 6. Our earlier studies (Chidambara Murthy, 2005) showed that *D. salina* grows well at initial pH of 7.0-7.5, initial NaCl concentration of 1.0-1.5M and light intensity of 2-4 Klux. Hence the same parameters were employed in all the studies.

*Dunaliella* sp being halophilic, it is important to study the optimal NaCl concentration for the growth and maximum carotene accumulation. Published reports suggest that optimal concentration of sodium chloride varies depending on the species (Borowitzka and Borowitzka, 1988). In response to high salinity and high irradiances, the microorganisms employ different strategies to maintain the osmotic balance. Oren (2002) suggested that many halophilic bacteria involve accumulation of $Na^+$ and $Cl^-$ ions, but the intracellular salt concentration of *Dunaliella* is low. Such a low concentration of $Na^+$ levels are achieved by the activity of a $Na^+$ pump in the cytoplasmic membrane (Katz and Pick, 2001) and by $Na^+$ extraction coupled with direct electron transport (Ehrenfeld and Cousin, 1984). During this, size alteration takes place in lipid and plastoglobuli, chloroplast, nucleus and other cell organelles (Berube et al, 1990). The low intracellular ionic concentrations and the need for osmotic equilibrium are met by the accumulation of glycerol as a compatible solute (Chitlaru and Pick, 1991). Electron spin resonance studies on *Dunaliella* suggested that physical changes brought by shrinking and swelling act as a sensor for changing

environmental salt concentrations (Curtain et al, 1983). Oren-Shamir et al, (1989) suggested that the activity of plasma membrane is essential for the recovery of *Dunaliella* from a hypertonic shock. In addition to this, the flux of carbon between starch production in chloroplast, synthesis of glycerol in the cytoplasm and accumulation of β-carotene are some of the important physiological responses produced under stress conditions.

According to Borowitzka et al, (1984) the optimum salinity for growth of *D. salina* lies between 18 and 22% NaCl whereas, for the carotenoid production optimum salinity was found to be more than 27%. However, the actual salinity has to be higher than the optimum level to avoid the problems of predatory protozoa and the other algal competitors (Borowitzka and Borowitzka, 1988). Recently a 60-kDa protein known as α-type carbonic anhydrase (dCA I) identified from the surface of *D. salina* was found to be responsible for the salt tolerance. This carbonic anhydrase increases depending upon the salt intensity and retains an active conformation over a broad range of salinity, compared to other carbonic anhydrases, which loose their activity at low salinities (Bageshwar et al, 2004; Fisher et al, 1996).

In this study among the different NaCl concentration studied, results showed that 0.5-1.0M concentration of NaCl was found to be ideal for the growth of *D. bardawil.* The carotene accumulation was favored at 2.0M NaCl concentrations, which showed a two fold increase in the carotene content. There was a marked inhibition of growth initially at 4.0M NaCl, however growth resumed after 7 days (Fig 11 & 12). The initial concentration of NaCl at 5.0M and above did not show any growth of *D. bardawil* and resulted in cell death.

Takagi et al, (2006) studied the effect of NaCl concentration on growth and lipid composition in *Dunaliella tertiolecta*. They observed that initial NaCl concentration higher than 1.5M markedly inhibited cell growth. Increase of initial NaCl concentration from 0.5 (equal to sea water) to 1.0M resulted in a higher intracellular lipid content of 67% compared to 60% at 0.5M. Addition of 0.5 or 1.0M NaCl at mid-log phase or at the end of log phase during cultivation with initial NaCl concentration of 1.0M further increased the lipid content to 70%. They observed that initial NaCl concentration of less than 1.0M was considered to be appropriate to achieve high cell concentration. The same result was observed with this study.

Ginzburg and Ginzburg (1981) reported that the doubling time of algae increases with increase in NaCl concentration. They observed the doubling time for different *Dunaliella* sp at $26^0$C and was found to be 12 to 110 hr at 0.5 to 4.0 M NaCl concentration. However the average of 15-17 hr doubling-time was obtained at 26-$36^0$C between 1.0 and 3.0 M NaC1concentration. The suggested explanations for the varied growth response at different NaCl concentrations are, (a) High salt concentrations decrease the solubility of gases in the medium and thereby reduce the availability of $CO_2$ and $O_2$ and (b) many enzymes are inhibited at high salt concentrations (Loeblich, 1982). Prov (1975) has explained that during different NaCl concentrations, large proportion of the energy goes in maintaining the ion concentrations of the cell rather than to the growth of the algae. Alyabyev et al, (2007) studied the effect of different NaCl concentration on growth and photosynthesis in *Dunaliella* and *Chlorella*. The halotolerant *Dunaliella* readily adapts to very high salinity, whereas *Chlorella* was unable to adapt to concentrations of 1M NaCl and above. They explained that the adaptability of *Dunaliella* cells to high salinity may be due to its ability to decrease the salt toxicity by pumping $Na^+$ ions into the medium by means of proton pump. It was also supposed that $Na^+$ ions decrease the effectivity of the electron transport chain and induce a toxic action on the cells. They also observed that the rate of photosynthesis of *Dunaliella* cells was more in the higher salinity conditions compared with the control. Coa et al, (2001), reported that *Dunaliella* cells had a photosynthesis to respiration activity ratio (*P*/*R*) of about 4:1 at 0.5M NaCl and 22:1 at 1.5M NaCl. Hence several authors discussed the possibility of activation of carbonic anhydrase in $CO_2$ assimilation, which may be an important protective mechanism (Pick, 1998; Katz and Pick, 2001). Pick and colleagues identified two major plasma membrane proteins, which were induced by high salt concentrations in *Dunaliella*. These proteins are transported to the chloroplasts. One of the protein is homologue to the eukaryotic carbonic anhydrase, which is involved in $CO_2$ acquisition.

The supplementation of $CO_2$ is known to favor the growth of algae (Becker and Venkataraman, 1982). Tripati et al, (2001) observed a 5-7 fold increased growth, chlorophyll and carotene content in *Haematococcus pluvialis*, *Scenedesmus obliqus,* and *Chlorella* sp, after 2% $CO_2$ supplementation using a two- tier flask method. However the algae belonging to cyanophyceae group (*Spirulina, Nostoc, Stigonema*)

showed a 1.5-2 fold increase in growth when supplemented with 2% $CO_2$. Herdman (1988) reported that supply of $CO_2$ prolonged the exponential growth phase in *Nostoc.* In accordance with the above results, our study also showed that the growth of *D. bardawil* can be enhanced by supplementation of 2% $CO_2$ in gaseous form. But no significant difference was observed in pigment profile. Giordano and Bowes (1997) cultured *D. salina* cells on 10mM $NH_4^+$ or $NO_3^-$ with or without 5% (v/v) $CO_2$. The growth and doubling time was found to be more when the algae was supplemented with ammonia as a nitrogen source in the presence of 5% carbon dioxide. However the cells contained less protein and starch, 28% more glycerol and the pigment content was unchanged.

In order to achieve optimum growth, nutrients should be adequate in quantity (Borowitzka and Borowitzka, 1988). Nitrate and sulphate starvations are shown to cause the accumulation of β-carotene in *Dunaliella* (Becker, 2004). Nitrogen and phosphorus limitations affect the photosynthetic apparatus of *D. tertiolecta* (Milko, 1962). The ratio of carotene pigments to chlorophyll increased under nitrogen (Geider et al, 1998) and phosphorus limited conditions (Krom et al, 1991). Leon et al, (2003) observed a 33 fold increased carotene: chlorophyll ratio, when *D. salina* was grown in aqueous two-phase systems under $N_2$-starvation conditions and the total carotenoid concentration increased to 8%.

Although different elements namely, Zn, Co, Cu and Mo, are included in the growth medium of *Dunaliella*, there are no reports to show an absolute requirement for most of these elements (Borowitzka and Borowitzka, 1988). It was also observed that high content of magnesium and calcium ions inhibit the growth of *Dunaliella* (Bass-Becking, 1931). Iron is an essential element for most of the living organisms and it acts as a co-factor in major processes like DNA synthesis, respiration and photosynthesis (Schwarz et al, 2003). Kobayashi et al, (1993) have shown that addition of acetate and $Fe^{2+}$ ions enhanced the carotenoids formation in *Haematococcus pluvialis*. Recently Mojaat et al, (2007) studied the effect of $Fe^{2+}$ (alone or in combination with acetate or mevalonate) on the growth and pigmentation of *Dunaliella salina*. They reported that addition of $Fe^{2+}$ in the basic medium at 0.45mM greatly affects the growth, resulting either in a reduction of growth rate without significant β-carotene production or causing the loss of culture after 72 hours. However in presence of acetate or mevalonate the carotene content increased

compared to the control. In our study, the $Fe^{2+}$ concentration of up to 2mM did not show any growth inhibition of the algae.

Zinc is a well-known essential micronutrient for normal growth of algae. Its deficiency leads to poor growth and low dry weight (Shrotri et al, 1981). The growth response and tolerance of different species of algae to $Zn^{2+}$ have been reported by Whitton (1970), who showed that algal growth is stimulated by lower concentrations of the metal and totally inhibited by higher concentrations. Moreover, higher concentrations of $Zn^{2+}$ decreased cell division and growth rate (Bariaud and Mestre, 1984), movement and total chlorophyll content in *Euglena* (De Filippis et al, 1981). Omar et al, (2002) reported that lower concentrations of zinc (0.5 to 1.5ppm) increased dry weight, chlorophyll a, b, carotenoids and total amino acid contents in *Scenedesmus obliquus* and *Scenedesmus quadricauda.* On the other hand, higher concentrations of zinc (above 1.5ppm) were inhibitory for growth and other metabolic activities. In our study, growth reduction was not observed in *D. bardawil* up to 1μM $Zn^{2+}$ level. Another metal ion Mn at 2-10μM did not show any significant increase in either growth or carotenogenesis. Though there was a slight increase in carotene content among the treatments, this was within the normal level of carotene content in *D. bardawil.*

The exposure of *D. bardawil* cultures grown under laboratory conditions when subjected to outdoor condition of 30-35 Klux light intensity induced carotene accumulation. However, no growth of *D. bardawil* was observed at 30-35 Klux light intensity.

Most of the reports in *Dunaliella* cultivation deal with the studies conducted under controlled (indoor) culture conditions. Published reports on the outdoor cultivation of *Dunaliella* sp are very scanty. Ben-Amotz (1995) studied the growth and carotenogenesis in a two-phase study. In the two-phase cultivation methodology, initially the cultures were grown in nitrate rich medium. Once the cells attained sufficient growth, they were transferred to another tank with nitrate depletion media for 5 days to induce carotenogenesis (up to 5%). In our study, subjecting the cultures to high light intensity for a period of 5 days triggered carotenogenesis. The results indicated that over 4% β-carotene content was achieved with this method (Fig 29).

Gonzalez et al, (2005) reported the growth of *D. salina* in a tubular photobioreactor under outdoor condition. To avoid high light stress they covered tubular loops with a

sunshade screen, which helps to avoid the light-induced damage of cells during vegetative phase. In our studies also we observed that it is very essential to maintain low light during vegetative growth phase.

Growth under outdoors, with natural environmental factors is not easy like indoor cultivation (Borowitzka, 1999). Borowitzka (2005) suggested that scale up is the difficult task in outdoor mass culture, because they are more prone to contamination by other algae and bacteria during acclimatization stage. In our study, the diatoms and protozoan predators were eliminated successfully in outdoor culture with the addition of 2% NaCl to the culture.

In general, temperature and light are the two main factors that affect the growth and carotenogenesis of algae under outdoor culture conditions. The quality and quantity of light determines the photosynthesis capacity of algae (Raja et al, 2007). Light plays a vital role in growth of *Dunaliella* as well as in carotenogenesis. The accumulation of β- carotene under high light intensity has been reported by Ben Amotz et al, (1989). Light or irradiance stress is created whenever the incident light intensity is greater than that needed to saturate photosynthesis (Raja et al, 2007). *Dunaliella* produces β-carotene in excess to overcome the irradiance stress (Ben Amotz et al, 1989). When exposed to high intensity of light, the following sequence of events occur: photo inhibition of $O_2$ evolution, photo destruction of carotenoids in the order 9-cis-β-carotene, all-trans-β-carotene, chlorophyll and finally the destruction of the cell (Ben-Amotz et al, 1989). The accumulation of β-carotene (9-cis/all-trans isomers ratio) depends on the integral light intensity to which *Dunaliella* is exposed during a division cycle. This ratio is promoted at low (20-50 μmol $m^{-2}s^{-1}$) rather than high irradiances (200-1250 μmol $m^{-2}s^{-1}$; Orset and Young 2000). It has also been stated that the accumulation of β-carotene and its isomeric ratio are strongly dependent on the light intensity and the quality of light used (Senger et al, 1993). Exposure to low light has shown to favor the synthesis 9-*cis* β,β-carotene in *Dunaliella salina* (Teod.), however production of α-carotene was not favored by this condition.

In addition to the visible wavelength, ultraviolet (UV) radiation was also found to influence the β-carotene production (Jahnke, 1999). When the UV-A was supplemented along with high photosynthetic photon flux density, a doubling of total carotenoids per unit protein was observed without affecting the growth in different *Dunaliella* sp. However UV-B was not effective in carotenoid induction (Jahnke,

1999). Similar studies conducted by Salguero et al, (2005) proved that 84 hrs exposure to UV-A conditions stimulated an increase in the total carotene content with 3– 5 fold increase in lutein and zeaxanthin content.

Ben-Amotz (1996) studied that in *Dunaliella* decreasing the temperature from 30 to $10^0$C caused a 4-fold increase in the 9-cis/ all-trans-β-carotene ratio. In contrast to the above report, Gomez and Gonzalez (2005) have stated that decrease in temperature from 26 to 15°C did not affect these ratios. However the accumulation of β-carotene at low temperatures was stimulated by high irradiances (1,000 μmol $m^{-2}$ $s^{-1}$). A decrease from the optimal temperature for growth (30°C) to sub optimal (I8°C) temperatures induced β-carotene synthesis and increased lipid content (Mendoza et al, 1996) in *D. salina*. In another report Orset and Young (1999) reported a 7.5 fold increase in the levels of α-carotene, when the temperature was decreased from 34 to $17^0$C, while the level of β-carotene was unaltered. Very low temperature has shown to be the limiting factor for the growth of algae (Vonshak et al, 1982; Bedell, 1985). Low temperature reduces growth rate in *D. salina* (Ginzburg and Ginzburg, 1981; Bororwitzka and Bororwitzka, 1988).

Numerous factors have been shown to induce massive carotenoid accumulation and these include high salinity (Ben-Amotz and Avron, 1983; Loeblich, 1982), photosynthetic photon flux density (PPFDs >500 μmol $m^{-1}s^{-1}$; (Brown et al, 1997), low growth temperature (Abdullaev and Semenenko, 1975), far red light and nutrient limitation conditions (Borowitzka and Borowitzka, 1988). Apart from high PPFDs, all the factors are known to accumulate carotenoid content along with a reduction in the growth rate (Radmer, 1996). Thus, an inverse relationship exists between β-carotene content and the specific growth rate (Ben-Amotz et al, 1982). Hence the major factors of significance in cultivation of algae are nutrients, light, temperature and stress of various natures (Becker and Venkataraman, 1982; Raja et al, 2007).

After an efficient growth and carotenogenesis, it is necessary to study the harvesting methodologies for *D. bardawil. D. bardawil* being small and motile and unicellular, it is difficult to harvest the cells by simple techniques of filtration. Some of the other features of *Dunaliella,* which poses difficulty in harvesting, are lack of rigid cell wall, mucous property of the cell layer and high salinity leading to high density of the medium (Ben-Amotz, 1995). Because of these drawbacks, conventional systems like filtration were found unsuitable for harvesting of *Dunaliella*. Hence, high-speed

centrifugation using online and batch centrifuges were used. Ben-Amotz (1995) reported that continuous flow automatic discharging centrifuges are successful in *Dunaliella* harvesting with minor mechanical failures.

After successful harvesting, the biomass was utilized for various analyses either as wet or dry biomass. Drying is an important step in algal cultivation, which helps in storing of biomass for varied time period. Like harvesting, drying methods should also be simple, easy and economical, so that it will reduce the cost of processing. Drying should also help to remove the maximum moisture content without affecting the other nutritional and biochemical parameters of the biomass. Among the drying technologies employed, freeze drying was found to be ideal with minimum loss of carotenoids (Table 10).

Among the natural pigments, the carotenoids serve as one of the most important as a food colorant (Reviewed by Britton, 1995; Delgado-Vergas, 2000). The degradation and isomerization are the common reactions during processing or storage of carotenoids. Illumination and temperature are the important factors to be controlled during storage to have a good quality and stability of carotenoids (Delgado-Vergas, 2000). Hence proper means of storage is important to maintain the stability of carotenoids upon storage. In this study, the stability of carotenoid rich dry biomass was analyzed for a period of three months, in dark condition and varied temperature. The study indicated that storage at $-80^0$ and $0^0$C over a three-month period, resulted in less degradation of β-carotene content. This method was useful during storage without affecting quality. Though it is an expensive method of storage, since the carotenoids are of high value compound, this method can be used for storage of carotenoid rich *D. bardawil* biomass.

TLC and HPLC methods were carried out to identify the different carotenoids in the biomass. Our earlier studies showed that direct extraction of carotenoids from wet biomass using solvent (acetone) is found to be best suited for *D. salina* (Chidambara Murthy, 2005). Hence the same procedure was employed in this study also. Identification of carotenoids by TLC revealed that there were eleven pigments including chlorophyll a and b. Analysis of carotenoids by HPLC revealed that carotene induced cells contained over 4% β-carotene. The other carotenoids identified include lutein (1%) along with trace amounts of lycopene and α-carotene.

Nutritional composition of *D. bardawil* revealed 22% protein, 27% carbohydrate, and 8% fat content on dry weight basis (Table 12). Becker (2007) reported that *D. salina* contained up to 57% protein of dry matter. Carbohydrate content ranged from 40% (green) to 11% (red) in *D. salina* (Borowitzka and Borowitzka, 1988). Protein and carbohydrate content was found to vary under different environmental conditions like high salinity and varying nitrate sources (Borowitzka and Borowitzka, 1988). In this study, the carotene induced dry biomass was used for the nutritional analysis. Thus algal biomass promises a novel source of protein, carbohydrate and fatty acids. This makes the algae equal, sometimes even superior compared to conventional plant proteins (Becker, 2007).

Among the lipids, the unsaturated fatty acids linoleic acid and linolenic acid was found to be the predominant (Table 16). Borowitzka and Borowitzka (1988) reported that the lipid content varied from 6% (low salinity) to 18% (high salinity) in *D. salina.* Mendoza et al, (1996) studied the effect of different temperature on the β-carotene and lipid content of *D. salina*. A decrease from the optimal temperature for growth (30°C) to sub optimal (I8°C) temperatures induced β-carotene synthesis and increased lipid content in *D. salina* cells, thereby promoting the formation of lipid-carotene globules in the chloroplast periphery. The content of polyunsaturated fatty acids was higher in cells cultured at low temperature. They suggested that the induction of carotenogenesis and accumulation of polyunsaturated fatty acids are mechanisms of acclimatization to unfavorable environmental conditions for growth. The fatty acid content increased from 11% to 24.8% during carotenogenesis. The decrease in growth temperature from 30°C to 18°C caused significant change in the fatty acid composition with increased concentrations of palmitic and oleic acids. These results indicate that under unfavorable growth conditions, the cells accumulate storage lipids, a phenomenon observed in many algae from different taxa.

### 3.1.16. Salient features

*D. bardawil* being an important algae of commercial cultivation, an efficient cultivation and harvesting methodology was developed both in indoor and outdoor condition. In indoor condition maximum of 20 ± 5 g $L^{-1}$ wet biomass was obtained. *D. bardawil* cultures grown at 1M NaCl concentration showed good growth, however for carotene accumulation, 2.0 M NaCl was required. Addition of 2% $CO_2$ in gaseous

form did not show any significant enhancement in pigment profile. Increasing the concentration of metal ions Fe and Zn showed increased carotene content in indoor culture conditions.

The β-carotene content was low (0.05-0.1%) when *D. bardawil* was grown under laboratory culture conditions. The carotene content was enhanced when the cultures were subjected to high light intensity of 30-35 Klux. This resulted in 4% β-carotene content along with lutein (1%) and traces of lycopene and α-carotene. The contamination by protozoa and diatoms in outdoor cultivation was successfully eliminated by the addition of 2.0 % NaCl to the medium. Analysis of the carotene induced biomass revealed 22 % protein, 27 % carbohydrate, and 8 % fat content on dry weight basis. Among the lipids, the unsaturated fatty acids linoleic and linolenic acid was found in higher levels. *D. bardawil* biomass is also rich in mineral contents like calcium, iron and potassium. The toxic metals like cadmium, mercury, lead and chromium were either absent or present in trace amounts, which are well below the permitted level.

## SECTION II

## ANALYSIS OF GENES INVOLVED IN CAROTENOID BIOSYNTHESIS PATHWAY DURING LIGHT INDUCED CAROTENOGENESIS

### Background

In plants, changes in carotenoid profile are known to be regulated by light and other stress factors (Reviewed by Cunningham and Gantt, 1998; Cunningham, 2002; Hirschberg, 2001). Several unicellular green algae have been known to accumulate large amounts of carotenoids when exposed to various stress conditions such as nitrogen and phosphate limitations as well as salt stress and high light intensities. Thus, massive accumulation of β-carotene occurs in *Dunaliella* species (Ben Amotz and Avron, 1983), whereas the red ketocarotenoid astaxanthin is accumulated in *Haematococcus pluvialis* (Boussiba, 1992). The expression of carotenoid genes in response to light and other stress has been widely studied in *Haematococcus pluvialis.* In this alga, elevated expression of carotenoid biosynthesis genes plays an important role in the accumulation of secondary carotenoids (Steinbrenner and Linden, 2000; Sun et al, 1998; Grunewald et al, 2000). However, very little is known about the light induced regulation of carotenogenesis in *Dunaliella bardawil* at the molecular level. Further there are no reports on the genes involved in the carotenogenesis in *Dunaliella.* Hence in this section, the expression of carotenoid biosynthesis genes in response to light, during carotene accumulation in *Dunaliella bardawil* was studied. The carotenoid profile and mRNA expression of genes involved in the pathway (Fig. 33), viz., phytoene synthase (*PSY*), phytoene desaturase (*PDS*), lycopene cyclase (*LCY*), and carotenoid hydroxylase (*CH*) in *D. bardawil* cultured under different light intensity has been analyzed.

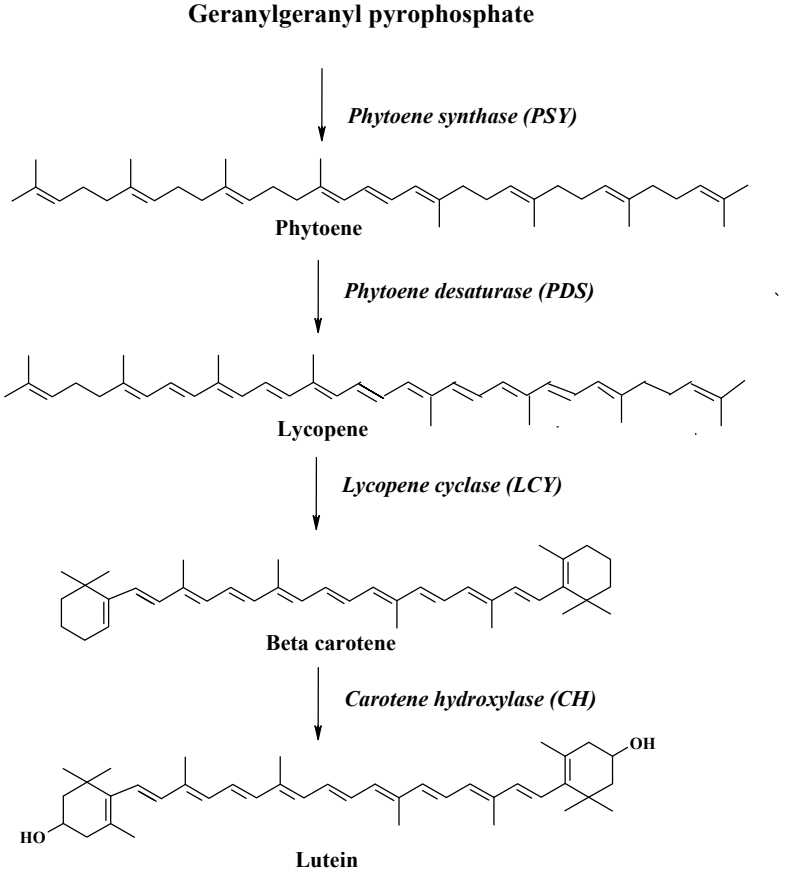



**Fig. 33. Brief description of carotenoid biosynthetic pathway in *Dunaliella bardawil*.** (Re-drawn based on literatures, using ISIS draw) The carotene biosynthesis enzymes phytoene synthase (*PSY*), phytoene desaturase (*PDS*), lycopene cyclase (*LCY*) and carotenoid hydroxylase (*CH*) are known to be the key steps in the carotenogenesis.

The growth and carotenoid content in different light intensities are presented in the section 3.1.8. The growth rate was high (0.12 $d^{-1}$) in the cultures grown at 1.5- 2 Klux compared to cultures grown at 15-20K lux (0.04$d^{-1)}$. When the cultures were subjected to outdoor direct sunlight, the cell ceased its division and tends to accumulate carotenoids (result section 3.1.7). The vegetative cells of *D. bardawil* contained 0.06% β-carotene (60±8 μg 100$mg^{-1}$ wet weight) and 1.36% lutein (1.36±0.08 mg 100$mg^{-1}$ wet weight) under indoor condition of 1.5-2.0 Klux. When these cells were exposed to a light intensity of 15-20 Klux, cells accumulated β-carotene, whereas no significant increase was observed in lutein content (Fig.29). At 30-35 Klux illumination for 48hrs, the cells attained yellow color, and the carotenoid content increased up to 3% (2.8 ± 0.25 mg 100$mg^{-1}$ wet weight) and after 96 hrs, these

cultures showed a carotene content of over 4% (4.21 ± 0.45 mg 100mg$^{-1}$ wet weight). When the cultures were grown under indoor conditions of low light, the lutein content was high, whereas when exposed to high light condition there was no significant change in lutein content, instead only β-carotene was increased. The illumination of *D. bardawil* cells with increasing light intensities resulted in higher β-carotene accumulation.

### 3.2.1.Expression of carotenoid pathway genes in response to high light intensities

*D. bardawil* cells developed red pigments due to carotene accumulation when cultivated under high light intensities. The expression profile of carotenogenesis related genes under various light regimes were determined by semi quantitative reverse transcriptase (RT) -PCR, using gene-specific primers (material and methods section, Table 9) for *PSY, PDS, LCY* and *CH.* Transcript analysis was done on the 3$^{rd}$ day after exposure to different light condition and finally at the complete end of carotenogenesis (red stage cells) on the 5$^{th}$ day of exposure to high light. The illumination of *D. bardawil* cells with 15-20 Klux resulted in a strong up-regulation of phytoene synthase, phytoene desaturase, and lycopene cyclase as compared to low-light conditions (Fig 34 & 35). The up regulations of *PSY, PDS* and *LCY* genes positively correlated with the carotenoid content in the cells. The transcript level of carotene hydroxylase (*CH*) was high in low light condition and remained almost constant after exposure to high light intensities. A considerable decrease in all these transcripts was noticed after the attainment of red cell stage (After 5$^{th}$ day under 30-35 Klux).

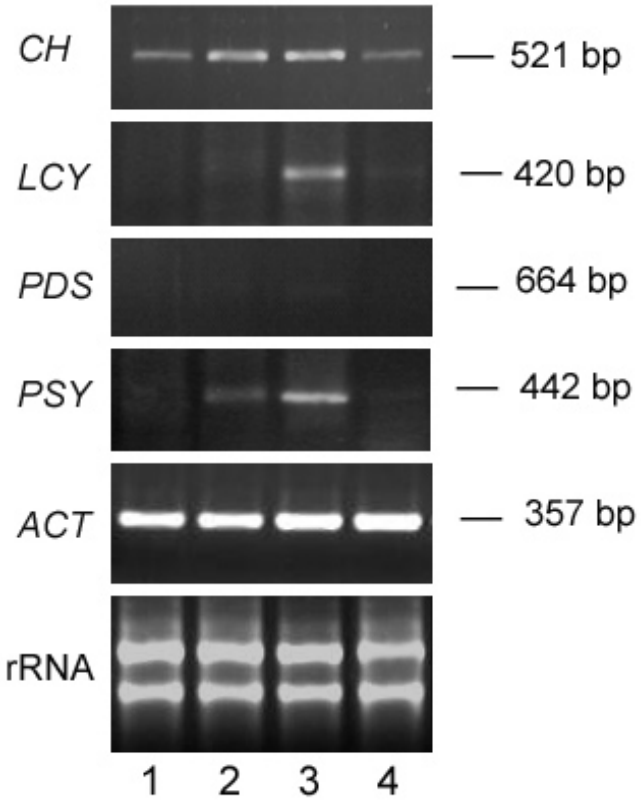



**Fig. 34. Expression of carotenoid biosynthetic genes during carotene accumulation in *D. bardawil* cells under different light intensities.** Lane 1: 1.5 - 2 Klux on 3rd day, Lane2: 15-20Klux on 3rd day, Lane 3: 30-35 Klux on 3rd day, Lane 4: 30-35 Klux on 5th day.

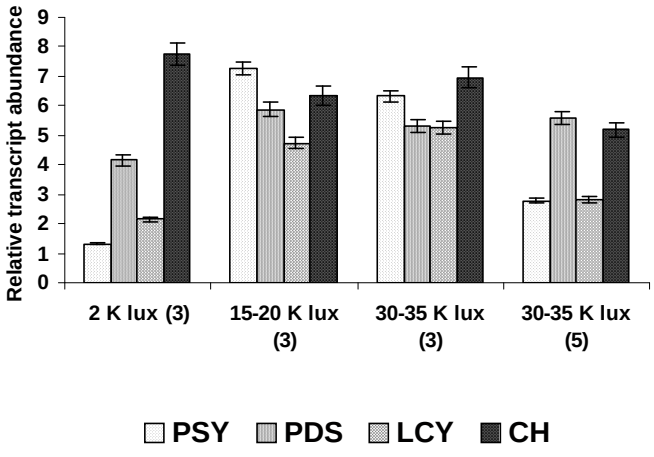



**Fig. 35. Relative abundance of transcripts under different light intensities**. The number in the parenthesis represents the day after illumination from indoor condition of 1.5 2Klux to different light intensities. Values are mean of triplicate analysis carried out twice

### 3.2.2 Discussion

The concentration and composition of leaf xanthophylls are affected by light intensity (Ruban et al, 1994). The synthesis and cyclization of lycopene has been considered as an important branching point of the carotenoid pathway (Hirschberg, 2001). The carotenoid accumulation during fruit ripening in tomato (Fraser et al, 1994), flower development in marigold (Moehs et al, 2001) and *Arabidopsis* plant (Pogson et al, 1996; von-Lintig et al, 1997) has been used as a model system to investigate the regulation of the process. Recently, considerable efforts have been made to understand the molecular regulation of the carotenoid biosynthetic pathway in different algae, especially in *Haematococcus pluvialis* (Sun et al, 1998; Grunewald et al, 2000; Vidhyavathi et al, 2007) and *Chlamydomonas* (Bohne and Linden, 2002). The genes coding for the carotenoid biosynthetic enzymes viz., *PSY* (Yuan et al, 2005) and *PDS* (Yue-Hui et al, 2005) have been isolated and characterized from *D. bardawil.* In *D. bardawil*, during high light intensity to prevent the photo-oxidative damage, cells accumulate massive amounts of carotenoids within the thylakoid in the chloroplast (Levy et al, 1992; 1993; Katz et al, 1995). However there are no reports on the regulation of genes involved during carotenogenesis in *Dunaliella.*

Under high light conditions β-carotene accumulation was predominant and the key factor for this may be the upregulation of mRNA transcripts of phytoene synthase, phytoene desaturase and lycopene cyclase. This up regulation of carotenoid genes may be due to the transcriptional activation of promoters of these genes under high light. Similar phenomenon was observed in *Chlamydomonas* (Bohne and Linden, 2002), *Haematococcus pluvialis* (Sun et al, 1998; Grunewald et al, 2000; Vidhyavathi et al, 2007), *Arabidopsis* (Pogson et al, 1996; von-Lintig et al, 1997) and Marigold flowers (Moehs et al, 2001) upon light stress.

Rabbani et al, (1998) reported that enhanced β-carotene biosynthesis is linked to lipid accumulation and was not correlated to the induction of the carotene biosynthetic pathway. They suggested both mRNA and protein level of *PDS* remained constant during the massive β-carotene synthesis on highlight conditions. However other reports say that accumulation of carotene in *D. bardawil* also involves the changes in the expression of carotenoid-associated genes (Levy et al, 1992; Katz et al, 1995). For example, in response to light stress, the *D. bardawil* gene *cbr*, encoding a 19 kDa thylakoid protein, was activated in parallel to the accelerated accumulation of

xanthophylls, preferentially zeaxanthin (Levy et al, 1992). In addition to accumulation of large amount of β-carotene in plastoglobules, neutral lipids and a 38 kDa protein, Carotene globule protein (Cgp), was also reported to increase under exposure to high light condition (Katz et al, 1995). Our results are in correlation with the latter reports that β-carotene accumulation correlates with the transcript levels of the enzymes involved in the carotenogenesis pathway. Up regulation of transcript levels of *PSY*, *PDS* and *LCY* was evident during the exposure of *D. bardawil* cultures to high light intensity.

### 3.2.3 Salient features

*D. bardawil* cultures when exposed to high light intensity of 30-35 Klux induced carotenogenesis . Among the carotenoids accumulated, β-carotene was predominant along with other carotenoids viz., lutein, lycopene and α-carotene. When the *D. bardawil* cells were exposed to high light intensity for 3 days under controlled (indoor) conditions, a significant elevation in the β-carotene content was observed where as the lutein content remained constant. This elevation was correlated with the up-regulation of the genes involved in the carotene biosynthetic pathway. The transcript analysis of *PSY, PDS* and *LCY* revealed an up-regulation when the cells were subjected to high light intensity. However there was no significant elevation of transcript level of *CH* during carotenogenesis, indicating that lutein remained constant during high light stress.

## SECTION III

## SAFETY AND TOXICITY EVALUATION OF *D. BARDAWIL* BIOMASS IN ALBINO RATS

**Background**

The increasing interest on carotenoid production by microalgae is due to the important commercial applications of these natural compounds, especially for pharmaceutical and nutritional applications. Carotenoids have been used as food additives including colorants, antioxidants and vitamins (Borowitzka and Borowitzka, 1988). Their protective ability against oxygen free radicals seems to be responsible for the therapeutic applications of carotenoids as degenerative disease preventives, anti-cancer agents and immune-system stimulators as claimed by several studies (Reviewed by Fraser and Bramley, 2004; Krinsky 1989, Krinsky and Johnson, 2005). Algae are rich sources of different minerals, vitamins, enzymes and nutrients, which demonstrate a wide range of biochemical and pharmacological activities in humans and animals. Thus a recent upsurge of interest in algae has resulted in isolation of numerous natural products as health enhancing agents with a broad range of biological activities, of which carotenoids have immense popularity. However their safety and toxicity studies are limited and newer sources need to be evaluated from the safety angle.

The present investigation was carried out to ascertain the safety of *D. bardawil* biomass after oral administration to rats. The criteria selected to ascertain the safety of *D. bardawil* biomass are alterations in body weight, biochemical indices of serum, haematological profile and liver parameters.

### 3.3.1. Acute oral toxicity study in rats

In the acute oral toxicity study single dose of test compound was administered at the maximum level (5g $Kg^{-1}$ b.w) and any toxicity symptoms arising was monitored for 15 days. This study is an initial step in establishing a dosage regime of *D.bardawil* biomass for further studies. This will provide information on the health hazards likely to arise from a short-term exposure by the oral route.

### 3.3.1.1 Mortality and toxic signs

No mortality was observed in test as well as control group of animals. No toxic sign and symptoms were noticed in the *D. bardawil* treated group when compared to control group.

### 3.3.1.2. Body weight and Organ weight

No significant differences ($p<0.05$) were observed in the body weight (gain/loss) pattern of all the test groups when compared to control (Table 17). No significant changes ($p<0.05$) were noted in absolute organ weight and relative weight of organ weight (Table 18)

**Table 17. Mean body weight* of rats of acute oral toxicity studies**

| **Groups** | **Body weight in grams** | | | | | |
|---|---|---|---|---|---|---|
| | **Day 1** | | **Day 8** | | **Day 15** | |
| | Male | Female | Male | Female | Male | Female |
| Control | 173.8±4.32 | 167.6±4.66 | 179.0±5.09 | 172.8±5.84 | 184.2±5.80 | 177.8±5.06 |
| *D.bardawil* | 172.4±6.06 | 168.4±4.03 | 172.2±6.05 | 172.8±4.14 | 181.4±6.26 | 177.4±3.78 |

**Table. 18. Average organ weight* of acute oral toxicity studies**

| **Organs** | **Weight (g)** | | | |
|---|---|---|---|---|
| | **Control** | | ***D. bardawil* biomass** | |
| | Male | Female | Male | Female |
| Lung | 1.52 ±0.05 | 1.60 ±0.05 | 1.54 ± 0.05 | 1.56 ± 0.09 |
| Liver | 8.15 ±0.35 | 7.94 ± 0.34 | 7.55 ± 0.63 | 7.44 ± 0.62 |
| Kidney | 1.59 ± 0.03 | 1.42 ± 0.10 | 1.63 ± 0.03 | 1.49 ± 0.09 |
| Gonads | 2.44 ± 0.09 | 0.10 ± 0.01 | 2.60 ± 0.07 | 0.10 ± 0.01 |
| Adrenal | 0.06 ± 0.008 | 0.05 ± 0.008 | 0.05 ± 0.01 | 0.06 ± 0.01 |
| Heart | 0.70 ± 0.04 | 0.71 ± 0.05 | 0.76 ± 0.06 | 0.71 ± 0.07 |
| Spleen | 0.45 ± 0.08 | 0.47 ± 0.05 | 0.43 ± 0.04 | 0.47 ± 0.05 |
| Brain | 1.82 ± 0.09 | 1.67 ± 0.06 | 1.78 ± 0.05 | 1.68 ± 0.06 |
| Uterus | ---- | 0.40 ± 0.03 | ---- | 0.44 ± 0.01 |

*Group of rats (n=5) were administered with single dose of *D. bardawil* biomass (5g $kg^{-1}$ b.w) and toxicity symptoms were monitored for 15 days. The values are expressed as mean ± standard deviation.

### 3.3.1.3. Haematological evaluations

There was no significant difference ($p< 0.05$) in the haematological parameters (Table 19) as well as biochemical parameters of the test and control animals. The values fell within the accepted limits of the normal variations in albino rats.

Hence the single dose administration of *D. bardawil* biomass at the dose of 5g $kg^{-1}$ b.w. to albino rats did not induce any treatment related observable toxic effects, when compared to control group of animals.

**Table.19. Mean hematological data of rats after acute oral toxicity studies**

| Parameters | Control | | *D. bardawil* biomass | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| WBC ($10^3$) | 8.82 ±0.42 | 8.72 ± 0.17 | 8.62 ± 0.27 | 8.50 ± 0.38 |
| Lymphocytes (%) | 82.20±0.83 | 81.00 ± 1.00 | 78.60 ± 1.14 | 78.40 ± 1.14 |
| Neutrophils (%) | 17.80 ±0.83 | 18.80 ± 0.83 | 20.00 ± 0.70 | 21.00 ± 1.00 |
| Eosinophils (%) | 0.00 ± 0.00 | 0.20 ± 0.44 | 0.60 ± 0.54 | 0.40 ± 0.54 |
| Monocytes (%) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.80 ± 0.83 | 0.20 ± 0.44 |
| Basophils (%) | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| RBC ($10^6$) | 7.24 ± 0.26 | 7.26 ± 0.28 | 7.18 ± 0.10 | 7.32 ± 0.23 |
| Hb (%) | 11.30 ± 0.50 | 10.70 ± 0.51 | 10.48 ± 0.48 | 10.74 ± 0.23 |
| Platelets ($10^5$) | 9.12 ± 0.30 | 8.86 ± 0.27 | 8.66 ± 0.36 | 8.62 ± 0.44 |

Group of rats (n=5) were administered with single dose of *D. bardawil* biomass (5g $Kg^{-1}$ b.w) and toxicity symptoms were monitored for 15 days. The values are expressed as mean ± standard deviation. WBC- White blood cell count, RBC-Red Blood cell count, Hb-haemoglobin.

### 3.3.1.4. Biochemical analysis of liver parameters

Liver parameter analysis revealed no significant difference ($p < 0.05$) between the *D. bardawil* treated and control group (Table 20).

**Table 20. Mean data of biochemical parameters after acute oral toxicity study**

| Parameters | Control | | *D. bardawil* biomass (5g $kg^{-1}$ b.w) | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| AST (Units $mL^{-1}$) | 35.10±1.80 | 36.50±3.56 | 35.20±1.71 | 33.80±1.19 |
| ALT (Units $mL^{-1}$) | 35.80 ±1.17 | 35.10±2.81 | 35.50±1.46 | 35.76±2.11 |
| Albumin (g $dL^{-1}$) | 3.32±0.08 | 3.44± 0.16 | 3.16±0.15 | 3.40±0.07 |
| ALP (KA units) | 181.70±12.52 | 137.90± 10.87 | 135.54±4.63 | 125.20±9.84 |
| Cholesterol (mg $dL^{-1}$) | 124.80±13.66 | 134.60±10.50 | 103.10±15.04 | 123.40±13.75 |

Group of rats (n=5) were administered with single dose of *D. bardawil* biomass (5g $Kg^{-1}$ b.w) and toxicity symptoms were monitored for 15 days. The values are expressed as mean ± standard deviation. AST-serum aspartate aminotransferase, ALT-serum alanine aminotransferase, ALP-serum alkaline phosphatase.

### 3.3.2. Sub-chronic (90 days repeated) oral toxicity study in rats

The toxicological effects of 90 days oral administration of *D. bardawil* biomass was assessed and compared with the control rats. This will provide information on the possible health implication arising from repeated exposure over a limited period of time. Two doses of *D. bardawil* biomass (100 and 1000mg $Kg^{-1}$ b.w) were studied.

#### 3.3.2.1. Mortality and toxic signs

No mortality was observed in any of the test groups and control group of animals. No toxic sign and symptoms were noticed in the *D. bardawil* treated group when compared to control group.

#### 3.3.2.2. Body weight and Organ weight

No significant differences ($p<0.05$) were observed in the body weight (gain/loss) pattern of all the test groups when compared to control (Table 21). No significant difference ($p < 0.05$) was noted in average organ weight (Table 22) after 90 days of *D. bardawil* supplementation.

**Table 21. Average body weight after 90 days of *D. bardawil* biomass supplementation**

| Groups | Body weight (g) | | | |
|---|---|---|---|---|
| | Male | | Female | |
| | Initial | Final | Initial | Final |
| Control | 101 ± 7 | 206 ± 5 | 102 ± 4 | 211 ± 5 |
| *D. bardawil* (100 mg $kg^{-1}$ b.w) | 102 ± 4 | 208 ± 5 | 104 ± 7 | 211 ± 7 |
| *D. bardawil* (1000 mg $kg^{-1}$ b.w) | 103 ± 8 | 210 ± 8 | 102 ± 8 | 209 ± 8 |

Group of rats (n=5) were administered with repeated doses of *D. bardawil* biomass for 90 days. The toxicity symptoms were monitored for 90 days. The values are expressed as mean ± standard deviation.

**Table 22. Average organ weight (in grams) of rats after 90 days of repeated oral toxicity**

| Organs | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Control | *D.bardawil* 100 $mgkg^{-1}$ | *D.bardawil* 1000 mg $kg^{-1}$ | Control | *D.bardawil* 100 $mgkg^{-1}$ | *D.bardawil* 1000 $mgkg^{-1}$ |
| Lung | 1.55±0.08 | 1.63±0.09 | 1.70±0.06 | 1.63±0.09 | 1.56±0.07 | 1.79±0.06 |
| Liver | 8.48±0.14 | 8.73±0.09 | 9.01±0.18 | 8.65±0.15 | 7.54±0.14 | 8.66±0.04 |
| Kidney | 1.62±0.08 | 1.78±0.09 | 1.63±0.08 | 1.71±0.06 | 1.48±0.08 | 1.76±0.06 |
| Testis | 2.47±0.09 | 2.56±0.10 | 2.63±0.10 | ----- | ----- | ----- |
| Ovaries | ----- | ----- | ----- | 0.12±0.02 | 0.09±0.01 | 0.10±0.01 |
| Uterus | ----- | ----- | ----- | 0.37±0.06 | 0.33±0.07 | 0.35±0.03 |
| Adrenal | 0.06±0.008 | 0.06±0.009 | 0.06±0.01 | 0.06±0.009 | 0.06±0.009 | 0.06±0.009 |
| Heart | 0.74±0.06 | 0.86±0.05 | 0.74±0.09 | 0.68±0.05 | 0.73±0.06 | 0.83±0.06 |
| Spleen | 0.42±0.007 | 0.53±0.03 | 0.49±0.04 | 0.51±0.04 | 0.45±0.07 | 0.45±0.04 |
| Brain | 1.79±0.06 | 1.73±0.01 | 1.71±0.07 | 1.74±0.06 | 1.72±0.07 | 1.77±0.04 |

Group of rats (n=5) were administered with repeated doses of *D. bardawil* biomass for 90 days. The Toxicity symptoms were monitored for 90 days. The values are expressed as mean ± standard deviation.

### 3.3.2.3. Effect of *D. bardawil* biomass on water and diet intake

The diet intake is given in Table 23. The rats consumed an average of 20-30mL water per day. There were no significant difference was observed in the water intake among the test groups and control.

**Table 23. Average data on feed consumption during 90 days of repeated oral toxicity study**

| Groups | Average feed consumption (g/ week) | |
|---|---|---|
| | Male | Female |
| Control | 136.84 ± 11.03 | 134.60 ± 10.39 |
| *D. bardawil* 100mg $kg^{-1}$ b.w | 134.61 ± 13.70 | 133.80 ± 16.27 |
| *D. bardawil* 1000mg $kg^{-1}$ b.w | 136.87 ± 7.88 | 134.30 ± 7.01 |

Group of rats (n=5) were administered with daily doses of *D. bardawil* biomass for 90 days. The Toxicity symptoms were monitored for 90 days. The values are expressed as mean and ± standard deviation

### 3.3.2.4. Haematological evaluations

There were no significant changes ($p< 0.05$) on haematological parameters as well as biochemical parameters in the test and control groups (Table 24). The parameters fell within the accepted limits of the normal variations in albino rats.

### 3.3.2.5. Clinical biochemistry evaluations

Serum biochemistry evaluations disclosed no significant differences ($p<0.05$) in the test as well as in the control group of animals (Table 25) and all the parameters fell within the limits of normal variations.

Hence, the 90 days repeated oral administration of *D. bardawil* biomass at the dose of 100 and 1000 mg $kg^{-1}$ b.w. to albino rats did not induce any observable toxic effects, when compared to the control group of animals. Hence the level of 1000mg $kg^{-1}$ b.w biomass can be taken as No Observable Effect Level (NOAEL).

**Table 24. Haematological data of rats after 90 days repeated treatment of *D. bardawil* biomass**

| Parameters | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Control | *D. bardawil* 100 mg $kg^{-1}$ | *D.bardawil* 1000mg$kg^{-1}$ | Control | *D. bardawil* 100 mg$kg^{-1}$ | *D. bardawil* 1000mg$kg^{-1}$ |
| WBC ($10^3$) | 9.04±0.25 | 8.43±0.42 | 8.6±0.69 | 8.92±0.16 | 8.22±0.31 | 8.65±0.50 |
| L% | 76.9±1.91 | 75.0±1.82 | 76.3±2.11 | 76.9±2.37 | 74.7±1.7 | 76.0±1.63 |
| N% | 22.6±1.71 | 24.4±1.26 | 23.3±2.0 | 22.6±2.41 | 24.7±1.41 | 23.6±1.57 |
| E% | 0.2±0.42 | 0.2±0.42 | 0.3±0.48 | 0.3±0.48 | 0.3±0.67 | 0.3±0.48 |
| M% | 0.3±0.48 | 0.3±0.48 | 0.1±0.31 | 0.2±0.42 | 0.3±0.67 | 0.1±0.31 |
| RBC ($10^6$) | 7.64±0.11 | 7.42±0.12 | 7.42±0.14 | 7.58±0.31 | 7.39±0.11 | 7.34±0.13 |
| Hb (%) | 11.71±0.51 | 11.5±0.48 | 12.1±0.33 | 11.66±0.3 | 11.55±0.48 | 12.01±0.31 |
| Platelets count | 9.01±0.21 | 8.21±0.28 | 8.52±0.30 | 8.75±0.23 | 8.15±0.35 | 8.38±0.45 |

Group of rats (n=5) were administered with daily doses of *D. bardawil* biomass for 90 days. The Toxicity symptoms were monitored for 90 days. The values are expressed as mean ± standard deviation. WBC- White blood cell count, RBC-Red Blood cell count, Hb-haemoglobin. L- Lymphocytes, N- Neutrophils, E- Eosinophils, M- Monocytes.

**Table. 25. Mean data of biochemical parameters after 90 days of repeated oral dose of *D. bardawil* biomass**

| Parameters | Male | | | Female | | |
|---|---|---|---|---|---|---|
| | Control | *D. bardawil* 100mgkg$^{-1}$ | *D. bardawil* 1000mgkg$^{-1}$ | Control | *D.bardawil* 100mgkg$^{-1}$ | *D. bardawil* 1000mgkg$^{-1}$ |
| AST (Units mL$^{-1}$) | 37.39±1.45 | 35.95±2.99 | 34.25±2.90 | 35.26±3.49 | 36.67±1.32 | 35.56±2.51 |
| ALT (Units mL$^{-1}$) | 33.54±1.85 | 33.81±1.37 | 35.46±2.25 | 36.63±1.29 | 35.3±1.17 | 37.03±1.65 |
| Albumin (g dL$^{-1}$) | 3.77±0.25 | 3.58±0.37 | 3.73±0.27 | 3.80±0.18 | 3.58±0.20 | 3.67±0.18 |
| Cholesterol (mg dL$^{-1}$)) | 96.14±4.96 | 90.68±9.16 | 94.35±7.09 | 97.74±2.45 | 84.78±5.92 | 91.47±3.78 |
| ALP (KA units) | 120.42±5.97 | 122.17±3.30 | 116.85±5.56 | 119.7±7.73 | 119.1±4.86 | 119.92±3.88 |

Group of rats (n=5) were administered with daily doses of *D. bardawil* biomass for 90 days. The Toxicity symptoms were monitored for 15 days. The values are expressed as mean ± standard deviation. AST-serum aspartate aminotransferase, ALT-serum alanine aminotransferase, ALP-serum alkaline phosphatase.

### 3.3.2.6. Discussion

In order to explore the utilization of *D. bardawil* biomass as nutritional and nutraceutical supplement, safety evaluations of the freeze dried biomass was studied. There are two reports (Mokady et al, 1989; Kuroiwa et al, 2006) that studied the safety of *D. bardawil* whole biomass and carotene extract in rats. Mokady et al, (1989), studied the safety of the alga *D. bardawil*, in a four-generation reproductive study with rats. Four generations were raised on diets containing 0, 5 or 10% dehydrated *D. bardawil* biomass. The blood chemistry and hematology of the first generation animals, after one year on the diets, showed no appreciable differences between the experimental and control animals. During the experimental period no mortality and no-observable-adverse-effect levels (NOAELs) of the carotene were recorded. The differences in histopathology observed were a decrease in some chronic inflammations and an increased frequency of bronchopneumonia in rats fed with 10% algae compared with the controls. This multigenerational feeding study revealed the

safety of *D. bardawil* for human consumption. Recently, Kuroiwa et al, (2006) evaluated the toxicity of carotene extract in a single dose and 90 days repeated study and reported no toxicity symptoms. In the present study, an indigenous strain of *D. bardawil,* isolated from Indian lakes was analyzed for its safety using rat as an animal model. The study revealed that administration of either single dose (5g $kg^{-1}$ b.w) or repeated oral doses (90 days at 100 and 1000mg $kg^{-1}$ b.w.) of *D. bardawil* biomass to albino rats did not induce any observable toxic effects, when compared to control animals. The safety of blue green algae, *Spirulina* (Becker and Venkataraman, 1982; Krishnakumari et al, 1981) and its coloring pigment, the phycocyanin has been well established in rats (Naidu et al, 1999). Krishnakumari et al, (1981) showed that *Spirulina* and *Scenedesmus* were safe up to 800mg $Kg^{-1}$ b.w in albino rats. *Spirulina* when supplemented in the diet as 10% protein supplement showed less growth and body weight compared to rats treated with 10% casein. However the relative organ weights of vital organs was higher compared to casein treated groups (Becker and Venkataraman, 1982). Concerning the toxin of nucleic acids, the safe level of algae is known to be 20g of algae per day or 300mg $kg^{-1}$ body weight (Becker 2007). In our study a single oral dose of 5g $kg^{-1}$ body weight and a repeated 90 days oral dosage of 100 and 1000mg $kg^{-1}$ b.w did not reveal any toxicity symptoms.

**3.3.2.7. Salient features**

The safety of *D. bardawil* biomass was studied using single and repeated dose oral administration in rats. The single dose oral administration of *D. bardawil* biomass at 5g $kg^{-1}$ b.w and the 90 days repeated oral administration of *D. bardawil* biomass at the dose of 100 and 1000mg $kg^{-1}$ b.w. to albino rats, revealed no treatment related observable toxic effects, when compared to control group of animals devoid of biomass.

## SECTION IV

## BIOACCESSIBILITY AND BIOCONVERSION OF CAROTENOIDS FROM *D. BARDAWIL - IN VITRO, INVIVO* AND CELL LINE STUDIES

### Background

Epidemiological studies reveal that consumption of carotenoid rich fruits and vegetables is associated with a reduced risk of developing chronic diseases (Krinsky, 1994a; Krinsky and Johnson, 2005; Stahl and Sies, 2005). It has been estimated that over 3 million children worldwide suffer from xerophthalmia and 140-250 million children go blind annually due to vitamin A deficiency (Food and Nutrition Board, 2001; Underwood, 2004). This situation occurs most commonly in Indonesia, India, Africa, Latin America and the Caribbean (Food and Nutrition Board, 2001). Grains such as rice and wheat are the predominant foods in these regions, and these grains are milled to remove the aleurone layer prior to further processing into foods. This milling results in a food product that is deficient in provitamin A carotenoids.

Humans are unable to biosynthesize carotenoids and must acquire these essential compounds through the diet. Research to understand and enhance the carotenoid bioavailability is also important. Hence in this section, the importance of *D. bardawil* biomass as a vitamin A supplement is dealt with both *in vitro* and *in vivo* model studies.

Existing *in vivo* methods to estimate the carotenoid bioavailability include both short and long term studies in humans (Micozzi et al, 1992; De Pee et al, 1995) as well as in appropriate animal models such as ferret (White et al, 1993), rats (Parvin et al, 2000) and preruminant calves (Poor et al, 1993). The most commonly followed method includes measuring the increase in plasma concentrations of carotenoids or retinol following administration of an acute or chronic dose of an isolated carotenoid or a carotenoid containing food (Brown et al, 1989; Micozzi et al, 1992; van het Hof et al, 1999).

However, studies based on humans or animals are tedious and costly and are not useful to screen a large number of food samples. Hence an *in vitro* model has been adopted here.

β-carotene, α-carotene and β-cryptoxanthin are the three important carotenoids, which are known as precursors of vitamin A, a nutrient essential for human health.

However, little is known about the bioavailability of these carotenoids from whole foods. In this regard the present section is aimed at developing an *in vitro* digestion method to assess the bioaccessibility of carotenoids from the whole cells of *D. bardawil* and compared with the carotenoids of carrot, a known carotene rich vegetable. Subsequently the bioavailability of the whole cell of *D. bardawil* is also analyzed by *in vivo* method and compared with synthetic β-carotene in rat model.

### 3.4.1. Bioaccessibility of *D. bardawil* biomass by *in vitro* method

#### 3.4.1.1 Percentage bioavailability of lutein from *D. bardawil* biomass

The lutein bioavailability was higher in carrot than *D. bardawil* biomass both in gastric as well as in intestinal phase (Fig. 36). In gastric phase, the percent bioaccessibility of lutein from carrot was significantly ($P<0.05$) higher than *D. bardawil* biomass. In intestine, the availability of lutein was higher in carrot than *D. bardawil* biomass.

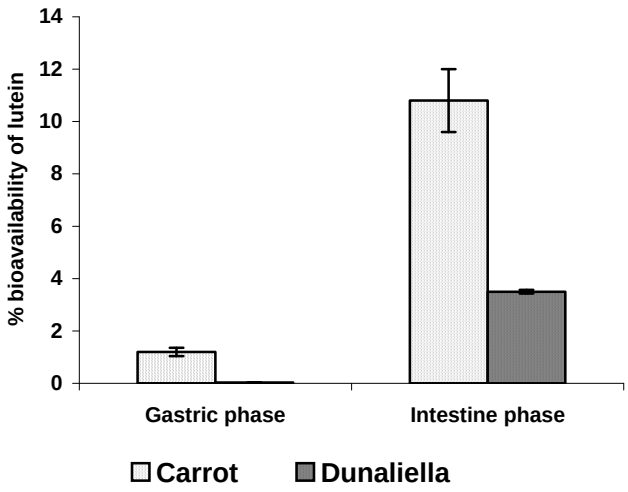



**Fig. 36. Percentage bioaccessibility of lutein from carrot and *D. bardawil* (Dunaliella) biomass after simulated digestion in gastric and intestinal phase**.

### 3.4.1.2. Percentage bioavailability of β-carotene from *D. bardawil* biomass

The bioavailability of β-carotene was higher in *D. bardawil* biomass compared to carrot. In gastric phase, the percent bioaccessibility of β-carotene from *D. bardawil* biomass (8.1) was significantly higher than ($P<0.05$) carrot (0.5). In intestine also the availability of β-carotene was higher (Fig. 37) in *D. bardawil* biomass (22.0) than carrot (5.5).

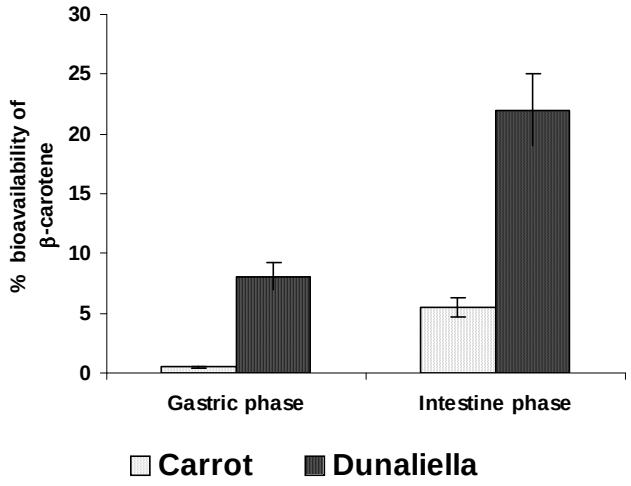



**Fig. 37. Percentage bioaccessibility of β-carotene from carrot and *D. bardawil* (Dunaliella) biomass after simulated digestion in gastric and intestinal phase**.

### 3.4.1.3. Discussion

The bioavailability of lutein was higher in carrot compared to *D. bardawil* biomass in both the gastric and intestinal phase. The bioavailability of β-carotene was higher in the intestinal phase of digestion in *D. bardawil* biomass. Garrett et al, (1999b) studied the carotene bioavailability from the carotene rich meal and vegetables. They observed a greater percentage of available lutein when the digestion was stopped at gastric phase compared to intestinal phase of digestion. However in their study, the gastric phase did not contribute to the availability of other carotenoids i.e. lycopene, α and β-carotene. Chitchumrooncholechai et al, (2004) also reported that micellarization of lutein and zeaxanthin is more efficient than β-carotene, during simulated digestion of spinach puree. Gartner et al, (1996) reported that efficiency of absorption of lutein and zeaxanthin into triglyceride rich fraction was higher than β-carotene, after a single dose of Betatene formulation (a formulation of *D. salina* containing 0.5% lutein, 0.75% zeaxanthin, 3.6% α-carotene, 70.3% all-trans β-carotene, 22.7% cis β-

carotene, 2.1% unidentified carotenoids and no lycopene). The reason for the differential uptake of this, may be due to the location of carotene and xanthophylls. Borel et al, (1996) reports that carotene and lycopene are located deep within the lipid droplet, whereas xanthophylls reside at the surface and are kinetically active molecules. In the study by Garrett et al, (1999b) upto 35% lutein and 20% β-carotene was transferred to the digesta during the complete digestion procedure. Hedren et al, (2002) also observed a 22% bioavailability of β-carotene from carrot during *in vitro* digestion. In our study after intestinal phase of digestion, 5% lutein and 20% β-carotene (Fig. 37) was found to be bioavailable from *D. bardawil* biomass. The results also demonstrated that carotenoid bioavailability from *D. bardawil* biomass was higher in comparison with carrot. As vitamin A malnutrition being widely prevalent, understanding the bioaccessibility of dietary β-carotene from any food source is of utmost importance. Such information may lead to optimization of dietary approaches to increase the bioaccessibility of dietary β-carotene.

### 3.4.2. *In vivo* bioavailability of carotenoids from *D. bardawil* biomass

#### 3.4.2.1. Bioconversion of *D. bardawil* carotenoids to vitamin A by intestinal perfusion method

The main aim of this study was to understand the time required for the conversion of *D. bardawil* carotenoids to vitamin A in the intestine. The intestinal perfusion method of Wang et al, (1993) was employed in this study.

In the perfusate after 30 min, very less amount of vitamin A content was detected. However a three-fold increase in the vitamin A was observed in the intestine after 30 min, compared to control. This indicated that vitamin A conversion takes place within 30min in the intestine. After 60 min, a significant increase in vitamin A was observed in the perfusate (Fig 38), indicating the conversion of carotenoids to vitamin A in the intestinal walls and its secretion to the lumen of the intestine. In the intestinal wall, the vitamin A content was high at 30 min, and thereafter it declined. In contrast to this, the vitamin A content was found to increase in the perfusate upto 2hrs.

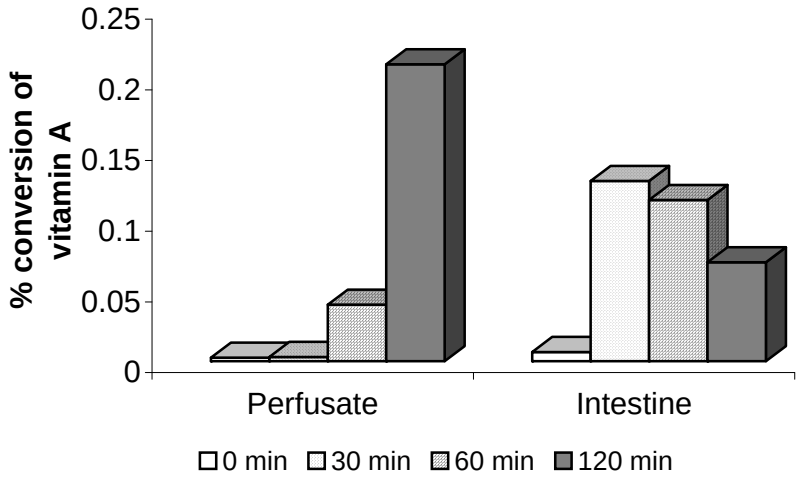



**Fig. 38. Vitamin A conversion by intestinal perfusion method**. The intestinal perfusion was carried out by the midline abdominal incision. The proximal end of intestine was filled with the known volume of carotenoid rich solution of *D. bardawil*. To prevent the perfusate from washing back into the stomach or continuing to large intestine, both distal and proximal ends were tied immediately. The water soluble carotenoid extract was perfused and the whole intestine was removed from group of rats in a definite interval of 30,60 and 120min and analyzed by HPLC. Value are average of 3 animals.

### 3.4.2.1.2. Discussion

The study was aimed to know the time required for the conversion of carotenoids to vitamin A in the intestine. In the study it was confirmed that the vitamin A conversion takes place within 30min in the intestine (Fig. 38), and there observed a three-fold increase in the vitamin A content within 30min, compared to control. Richard and Olson (1965) studied the vitamin A conversion using isotope labeled study in isolated perfused rat liver. After 1 hr of perfusion, 40-50% of perfusate contained retinol in addition to retinol esters (20-30%), and about 10% of the original perfusate radioactivity appeared in the bile as a group of water-soluble metabolites.

Rosenberg and Sobel (1953) studied the *in vitro* conversion of carotene to vitamin A in isolated small intestine of rats and found that it is the major site of conversion. They further stated that, vitamin A first appears in the intestinal wall and contents, and much later in the liver, and observed a 2% conversion of vitamin A in the intestine after 2 hrs. In comparison, our study revealed upto 0.25% of vitamin A conversion in the perfusate after 2 hrs. Wang et al, (1993) reported that in Ferret,

about 28% of the total administered radioactivity from β-carotene was taken up in the intestine during 4 hr perfusion.

### 3.4.2.2. Bioavailability of carotenoids from *D. bardawil* biomass using feeding trials

This study was intended to know the bioavailability of *D. bardawil* carotenoids and its bioconversion to vitamin A after single and multiple oral doses (7 days) of *D. bardawil* biomass to rats. The influence of synthetic β-carotene and *D. bardawil* biomass on liver and serum response of β-carotene and retinol has been studied in rats.

#### 3.4.2.2. 1.Single dose studies

The postprandial response of retinol in serum and liver after a single oral dose of β-carotene was studied from 0 to 8 hr (Fig 39 and 40). The serum vitamin A content increased and reached a maximum after 4 hours of intubation and thereafter a gradual decrease was observed for both the synthetic β-carotene and *D. bardawil* biomass treated groups. Maximum serum retinol content after 4hrs of intubation was observed in case of synthetic β-carotene group (5.3 μg $dL^{-1}$) compared to *D. bardawil* fed group (4.7 μg $dL^{-1}$) at the same time point (Fig 39). The enhanced level was found to be 7.4 and 6.5 fold respectively for the synthetic β-carotene and *D. bardawil* biomass fed groups compared to the control. However there was no significant difference ($P < 0.05$) between the area under curve of retinol in synthetic β-carotene (14.73 μg/dL/h) and *D. bardawil* biomass (14.36 μg/dL/h) fed group. After a single dose of carotene supplementation, liver stores of vitamin A was unaffected between the synthetic β-carotene and *D. bardawil* biomass fed groups (Fig. 40).

β-carotene was absent in serum of control group. The β-carotene was detected at very low level in the serum after single oral dose of either *D. bardawil* biomass or synthetic β-carotene. However accumulation of β-carotene was significant ($P < 0.05$) in liver. The β-carotene content was very low (0.35 μg $100g^{-1}$) in the liver at zero-time in control group. After 8 hrs of intubation, the liver β-carotene levels increased to 1.5 μg $100g^{-1}$ wet tissue and 1.26 μg $100g^{-1}$ wet tissue respectively (Fig. 41) in the

synthetic and *D. bardawil* fed groups. It was clear from the study that the accumulation of β-carotene in liver was observed after 8 hrs of oral dosage.

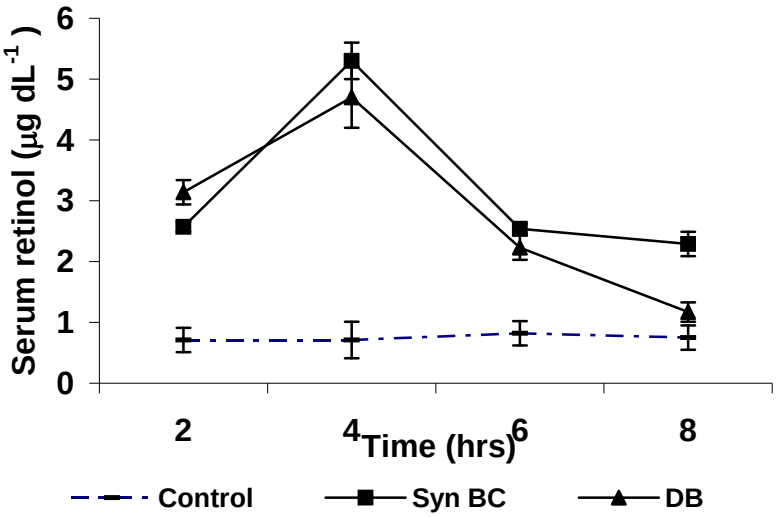



**Fig. 39. Postprandial response of serum retinol after single oral dose of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass**. Rats were fed with single dose of 100µg $kg^{-1}$ b.w. β-carotene equivalent and sacrificed after various time intervals. Data represents the mean ± SE.

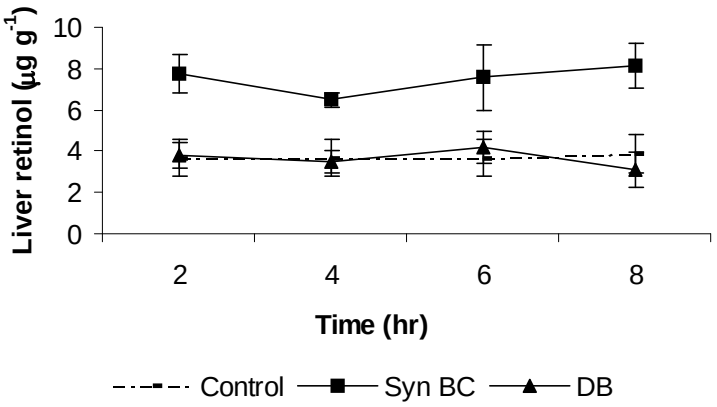



**Fig. 40. Liver retinol content after single oral dose of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass**. Rats were fed with single dose of 100 µg $kg^{-1}$ b.w. β-carotene equivalent and sacrificed after various time interval. Data represents the mean ± SE.

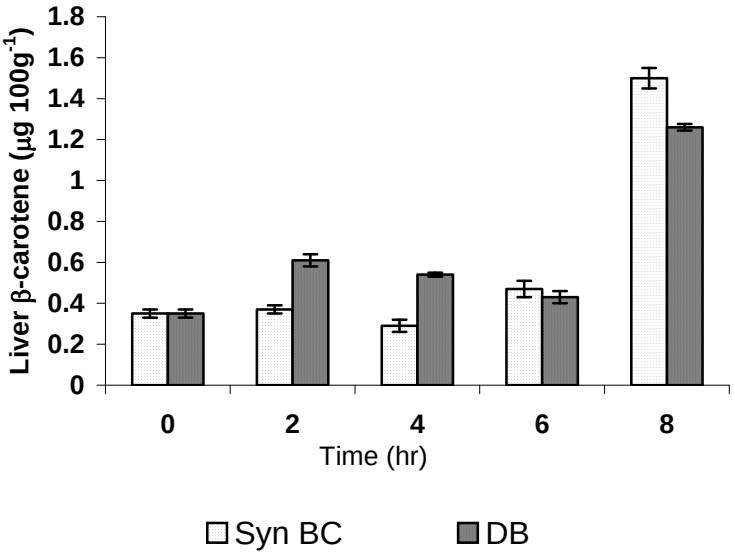



**Fig. 41. Liver β-carotene levels after single oral dose of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass**. Rats were fed with single dose of 100µg $kg^{-1}$ b.w. β-carotene equivalent and sacrificed after various time intervals. Data represents the mean ± SE.

### 3.4.2.2. 2. Multiple dose study

Differences in animal growth were not observed between the control and experimental groups during the 7 days feeding trial. Rats were weighed initially and after 7 days. The food intake was measured daily and was found to be 61, 58 and 61 g $day^{-1}$ for the synthetic β-carotene, *D. bardawil* biomass and control groups respectively (Table 26). The serum retinol content was 4 and 2.78 µg $dL^{-1}$ respectively in synthetic β-carotene and *D. bardawil* fed groups after 7 days of carotene dosage (Fig. 42).

Hepatic analysis of the experimental groups revealed a very high liver store of retinol and β-carotene (Fig 43). A 3-4 fold increased accumulation of retinol was observed in the experimental groups compared to that of the control group. The liver retinol (Fig. 43) content was higher in the synthetic β-carotene (16.8 µg $g^{-1}$ wet tissue) fed group compared to *D. bardawil* fed group (12 µg $g^{-1}$ wet tissue).

The reverse was observed in case of β-carotene accumulation in liver, where the animals fed with *D. bardawil* biomass (2.4 µg $100g^{-1}$ wet tissue) accumulated higher β-carotene than the synthetic β-carotene (1.25 µg $100g^{-1}$ wet tissue) fed animals (Fig.44).

The percentage accumulation of β-carotene and retinol in serum and liver of synthetic β-carotene and *D. bardawil* biomass treated groups over control was given in Fig. 45. The retinol accumulation in serum and liver was 81 and 78% for synthetic β-carotene fed group and it was 74 and 69% for *D. bardawil* fed group respectively. The liver β-carotene accumulation was 85 and 72% over control for *D. bardawil* biomass and synthetic β-carotene fed animals.

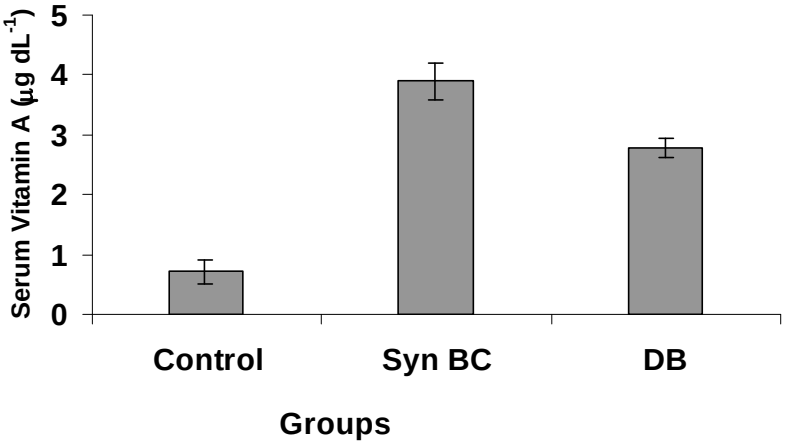



**Fig. 42. Serum retinol level after multiple doses of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass**. Rats were received a daily dose of 100µg $kg^{-1}$ b.w. β-carotene equivalent either from synthetic or *D. bardawil* biomass for 7 days and were sacrificed 24 hrs after the last dose. Values represent the mean ± SE.

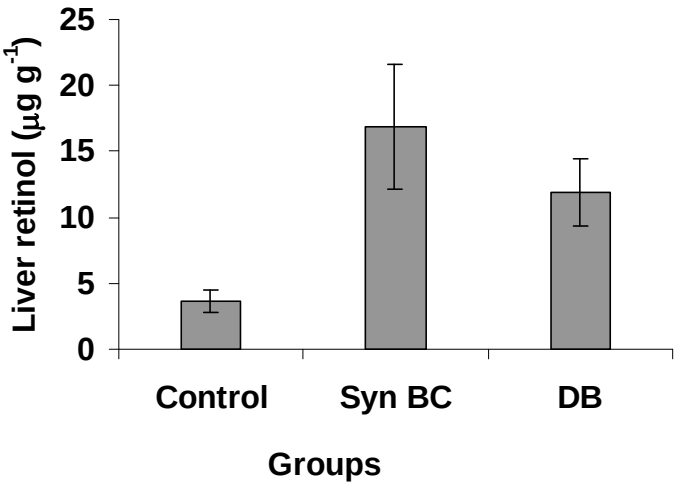



**Fig. 43. Liver retinol content after multiple doses of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass**. Rats were received a daily dose of 100µg $kg^{-1}$ b.w. β-carotene equivalent either from synthetic or *Dunaliella* biomass for 7 days and were sacrificed 24 h after the last dose. Values represent the mean ± SE.

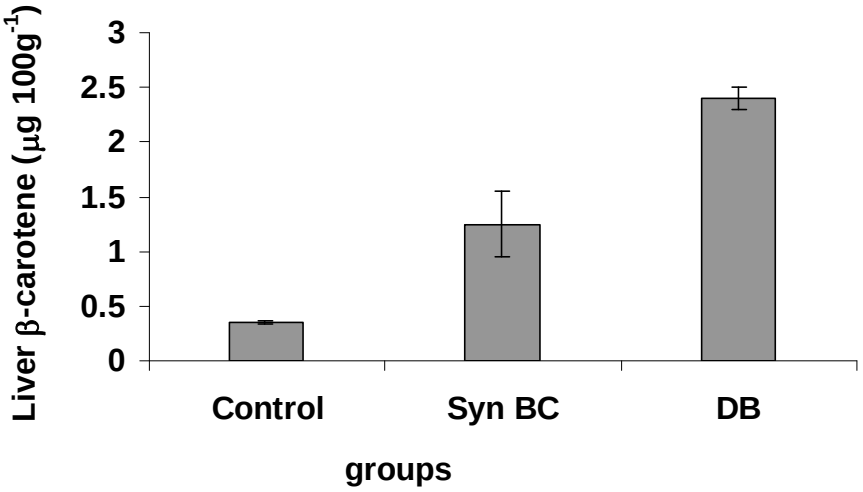



**Fig. 44. Liver β-carotene content after multiple doses of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass**. Rats were received a daily dose of 100μg $kg^{-1}$ b.w. β-carotene equivalent either from synthetic or from *Dunaliella* biomass for 7 days and were sacrificed 24 hr after the last dose. Values represent the mean ± SE.

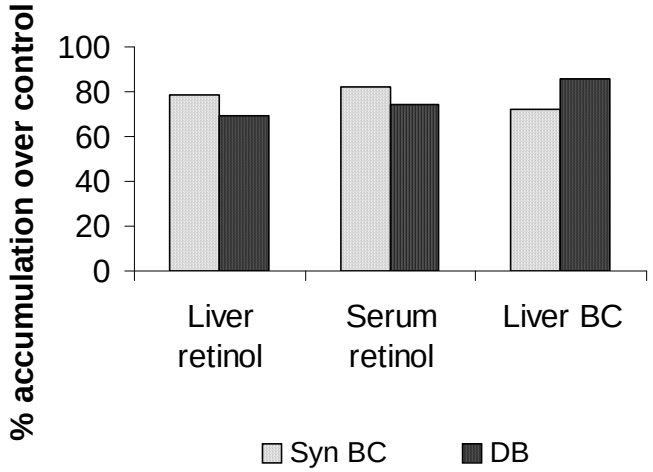



**Fig. 45. Percentage accumulation of β-carotene and retinol in liver and serum of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) fed groups over control after multiple dosage**. Rats were received a daily dose of 100μg $kg^{-1}$ b.w.β-carotene equivalent either from synthetic β-carotene or from *D. bardawil* (DB) biomass for 7 days and were sacrificed 24 h after the last dose.

The level of β-carotene excreted in the feces of different treatment groups are shown in Table 26. β-Carotene excreted was higher (0.73 μg/g/day) in *D. bardawil* biomass fed group compared to synthetic β-carotene fed group (0.34 μg /g/day).

**Table 26. Food intake and fecal β-carotene level after 7 days of carotene supplementation in rats***

| Group | Food intake (g/ day) | Feces (g/day) | Feces BC (μg /g/day) |
|---|---|---|---|
| Control | 61± 5 | 8.08 ± 0.2 | $0.086 \pm 0.02^{a}$ |
| Synthetic β-carotene | 61± 6 | 8.3 ± 1.0 | $0.34 \pm 0.21^{b}$ |
| | 58± 4 | 8.2 ± 1.8 | $0.73 \pm 0.14^{c}$ |

*Values are mean ± SE of 4 analysis; Values with different superscripts are significantly different at $p<0.05$.

### 3.4.2.2.3. Serum triglycerides

Single oral dose of synthetic β- carotene and *D. bardawil* biomass did not reveal any significant ($P<0.05$) increase in the serum triglyceride levels (Fig. 46), where as multiple oral doses showed significant ($P<0.05$) increase in mean serum triglyceride levels (Fig. 47) were noticed after 7 days of feeding of multiple dose of either *D. bardawil* or synthetic β- carotene.

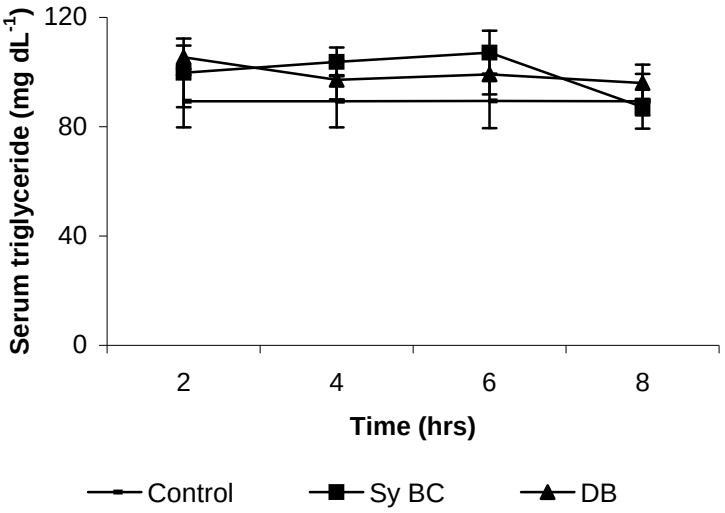



**Fig. 46. Serum triglyceride level after single oral dose of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass.**

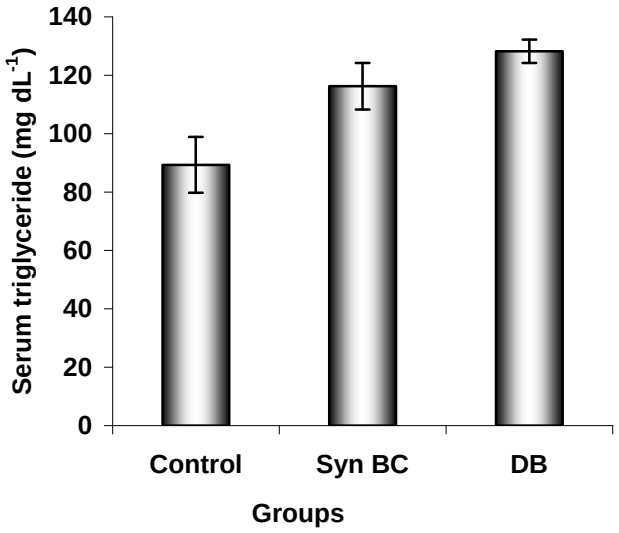



**Fig. 47. Serum triglyceride levels after multiple doses of synthetic β-carotene (Syn BC) and *D. bardawil* (DB) biomass.** Rats were received a daily dose of 100μg $kg^{-1}$ b.w. β-carotene equivalent either from synthetic or from *Dunaliella* biomass for 7 days and were sacrificed 24 h after the last dose. Values represent the mean ± SE of 4 analyses.

### 3.4.2.2.4 Discussion

In this study, the possibility of *D. bardawil* biomass as a vitamin A precursor in food supplementation was elucidated. Since *Dunaliella* accumulates huge amounts of β-carotene (Ben-Amotz et al, 1989) it can be utilized as a potential food supplement. Rats have been extensively used to evaluate the efficiency of β-carotene conversion to vitamin A by monitoring changes in serum and liver vitamin A stores (Parvin et al, 2000; Mittal, 1983). Hence the same model was used in the present study to evaluate the carotenoids for its biological efficacy from *D. bardawil* biomass. In human beings, for maintenance of the body requirement of serum vitamin A levels, administration of 100μg $kg^{-1}$ body weight of β-carotene is essential (Parvin et al, 2000). Therefore, 100 μg β-carotene either in the form of *D. bardawil* biomass or synthetic β–carotene in water-soluble form was administered to rats. The present study investigated the *in vivo* bioavailability of carotenoids after a single and repeated dose of administration.

The postprandial appearance of retinol and β-carotene in serum is measured as an indicator of its absorption after single and repeated dosages. In case of single dose

study, the result showed that retinol and β–carotene are higher in both the experimental groups than the control group. The retinol content in serum was maximum after 4 hrs of intubation (Fig. 39). Similar to this result, Krinsky et al, (1990) observed that a peak in plasma vitamin A occurs 4 hrs after administration of isotope labeled β–carotene in rats. Though the liver retinol level was lower after a single dose of carotene administration, the results of the repeated dose study showed that levels were significantly ($P < 0.05$) elevated compared to control. The decline in the serum retinol level after reaching maxima in single dose study (Fig. 39) reflects its transport to other tissues. It is further confirmed by the accumulation in liver after 8 hours of single oral dose (Fig 41).

In the multiple oral dosage study, the β-carotene accumulation in liver was higher in *D. bardawil* treated group compared to synthetic β-carotene (Fig 44). However more of vitamin A conversion and its accumulation were observed in the synthetic β-carotene treated group (Fig 42 & 43). At the end of multiple dose study, the percent accumulation of serum retinol was 82 and 75% over control in synthetic β-carotene and *D. bardawil* fed groups respectively. However, the accumulation of β-carotene in liver was more (85.4%) in *D. bardawil* biomass fed group than synthetic β-carotene (72%) fed group (Fig 45). Plausibly, the lower retinol conversion of β-carotene from *D. bardawil* compared to synthetic β-carotene may be explained in two ways. One is its complex nature with the matrix protein and other may be due to the mixture of carotenoids present in the algae. Earlier reports have shown that the bioavailability of carotenoids was lower from vegetables such as carrots, broccoli and spinach (Brown et al, 1989; Micozzi et al, 1992) compared to its pure form. In a study on school children in Indonesia, Dee Pee et al, (1998) observed that the carotene from orange colored fruits is more effective and bioavailable than dark-green leafy vegetables. Recently, Tang et al, (2005) also supported the same result by observing that the vitamin A conversion from carrot carotene is comparatively higher to that of spinach. The possible assumptions underlying this hypothesis were that the food matrices associated with the carotenoids are contributing to the bioavailability. Carotenoids could be more easily freed from their matrices in fruit than from their matrix in leafy vegetables and carrots. In spinach leaves carotene is in the form of pigment proteins located in chloroplasts, whereas the carotene in carrot is in the form of carotene crystals in chromoplasts. Thus in line with the above research findings and owing to

the association of carotenes with the chloroplasts in *D. bardawil* the bioavailability of β-carotene from *D. bardawil* biomass was lesser than that of synthetic β-carotene.

Higher levels of serum triglyceride observed in the *Dunaliella* treated group (Fig.47) further supports the higher level of β-carotene in *D. bardawil* treated group. This is because the ingested carotene molecules are absorbed by the intestinal mucosa and gets packaged in to the triacylglycerol-rich chylomicrons and later carried along for its delivery to blood stream and tissues (Parker, 1996). The increase in serum triglyceride levels were observed in the CARET participants during active treatment, however a decrease was observed when the intervention studies were discontinued (Redlich et al, 1999). Jackson et al, (2002) also observed higher level of plasma triacylglycerol chylomicron response after a single and repeated dosage of olive oil rich meal.

Ben Amotz et al, (1986) carried out a study by feeding dry algal powder of *D. bardawil* as a retinol supplement in chick diet over a period of two months. The normal and retinol deficient chicks were used in the study. The result showed that β-carotene from dry powder of *Dunaliella* was efficiently absorbed and converted to vitamin A in the animals. Serum and liver analyses revealed a normal content of retinol and a 10-fold increase in β-carotene content in all chicks except those grown on the retinol deficient diet, when supplemented with *Dunaliella* biomass. This study demonstrated the possibility of using dry powder of *D. bardawil* as a dietary supplement that can fully satisfy the retinol requirement.

In further experiments, they extended this study to rats, (Ben Amotz et al, 1988) where the rats were fed with retinol deficient diet for 60 days and thereafter they were supplemented with either algae as a dry powder or as oil extract or synthetic retinol in a diet and compared with synthetic β-carotene. Results revealed a comparable amount of liver retinol in the rats treated with *Dunaliella* and the presence of 9 cis-retinol, exclusively obtained from the algae. It was concluded that the algae can serve as a dietary retinol supplement in rats and can satisfy the total requirement of retinol in the diet.

There are also reports on the bioavailability of *D. bardawil* carotenoids on human beings. After 14 days of *D. bardawil* dosage, Ben-Amotz and Levy (1996) reported that the serum mainly showed oxycarotenoids, and less all-trans β-carotene and α-carotene, and 9-cis-β-carotene was absent. Jensen et al, (1987) studied the

bioavailability of cis- and trans-β- carotenes on 16 healthy adults, who had been on a low-carotene diet for ten days. They were fed with either β-carotene extracted from *D. salina* alga, containing approximately equal amounts of all-trans-β-carotene and 9-cis-β-carotene, or β-carotene in the form of fresh carrots containing predominantly trans-β-carotene, or avocado oil-placebo capsules. Subjects were randomly divided into three groups: they consumed daily in a single dose either β-carotene capsules or 207.3 g carrots containing 24 mg β-carotene in each; or β-carotene free placebo capsules for seven days. The serum analyses showed trans β-carotene to be the predominant isomer in serum before and during all treatments. Serum trans-β-carotene concentrations were significantly increased in the β-carotene capsules and carrot fed groups. Increased concentrations of cis β-carotene were observed in the carrot and placebo groups. These data demonstrated a predominant absorption of intact trans-β-carotene over intact cis-β-carotene into human serum even when approximately equivalent amounts of these isomers were ingested (Jensen et al, 1987). Tamai et al, (1995) studied the bioavailability of *D. bardawil* on thirty male volunteers. They were given daily either 60 mg of synthetic all-trans β-carotene or β-carotene derived from *D. bardawil* or a placebo for 44 weeks. The plasma levels of β-carotene reached a maximum after two weeks of administration and plateaued thereafter in the subjects who took the β-carotene preparations. The serum all-trans β-carotene level in the synthetic carotene treated group was almost twice than for the *Dunaliella* preparation. The plasma 9-cis carotene levels were found to be higher in the all-trans β-carotene group than in the *Dunaliella* group, despite no intake of the 9-cis forms in the all-trans group and the higher intake of the 9-cis forms in the *Dunaliella* group. This finding suggests that isomerization of the all-trans form to the 9-cis form may occur in the body either during or after absorption.

In our study it was confirmed that *D. bardawil* β-carotene could be used as an efficient source of retinol. In *D. bardawil* biomass fed rats, the β-carotene accumulation was significantly higher compared to the synthetic β-carotene fed rats.

### 3.4.3. Biological activity of *D. bardawil* carotenoids on primary cell lines

**Background**

Carotenoid absorption and conversion takes place in the small intestine. Hence the primary epithelial cells of small intestine have been widely used for pharmaceutical, biochemical and toxicological studies as well as studies on absorption and availability of micronutrients such as carotene and iron (Zodl, 2005; Garret et al, 1999a). Hence in this study the primary intestinal epithelial cells were used as a cell line model to study the uptake of β-carotene, its accumulation and vitamin A conversion.

#### 3.4.3.1. Cell viability

More than 95% of the intestinal epithelial cells were viable; cell count was $5x10^7$ cells $mL^{-1}$ of cell suspension.

#### 3.4.3.2. Uptake of carotene and conversion to vitamin A in primary cell lines

Two concentrations (5 and 10μM) of *D. bardawil* carotene extract and synthetic β-carotene were used to study the uptake of β-carotene and its conversion to vitamin A in the primary intestinal cell lines. In the cell lines treated with *D. bardawil* extract, traces of β-carotene accumulation were observed during the initial 90 min (Fig. A & B). However the vitamin A content was found to be maximum up to 90 min. The possible reason may be the absorbed carotenoids are immediately converted to retinol. After 60 min, the β-carotene accumulation was increased with a gradual decrease in the retinol content. The same pattern was observed for both the concentrations of *D. bardawil*.

In contrast to this, the cell line treated with synthetic β-carotene (5 and 10μM), revealed initial accumulation of β-carotene upto 60 min and thereafter an increase in the vitamin A content indicating the conversion of absorbed carotenoids to vitamin A (Fig. 48 C & D).

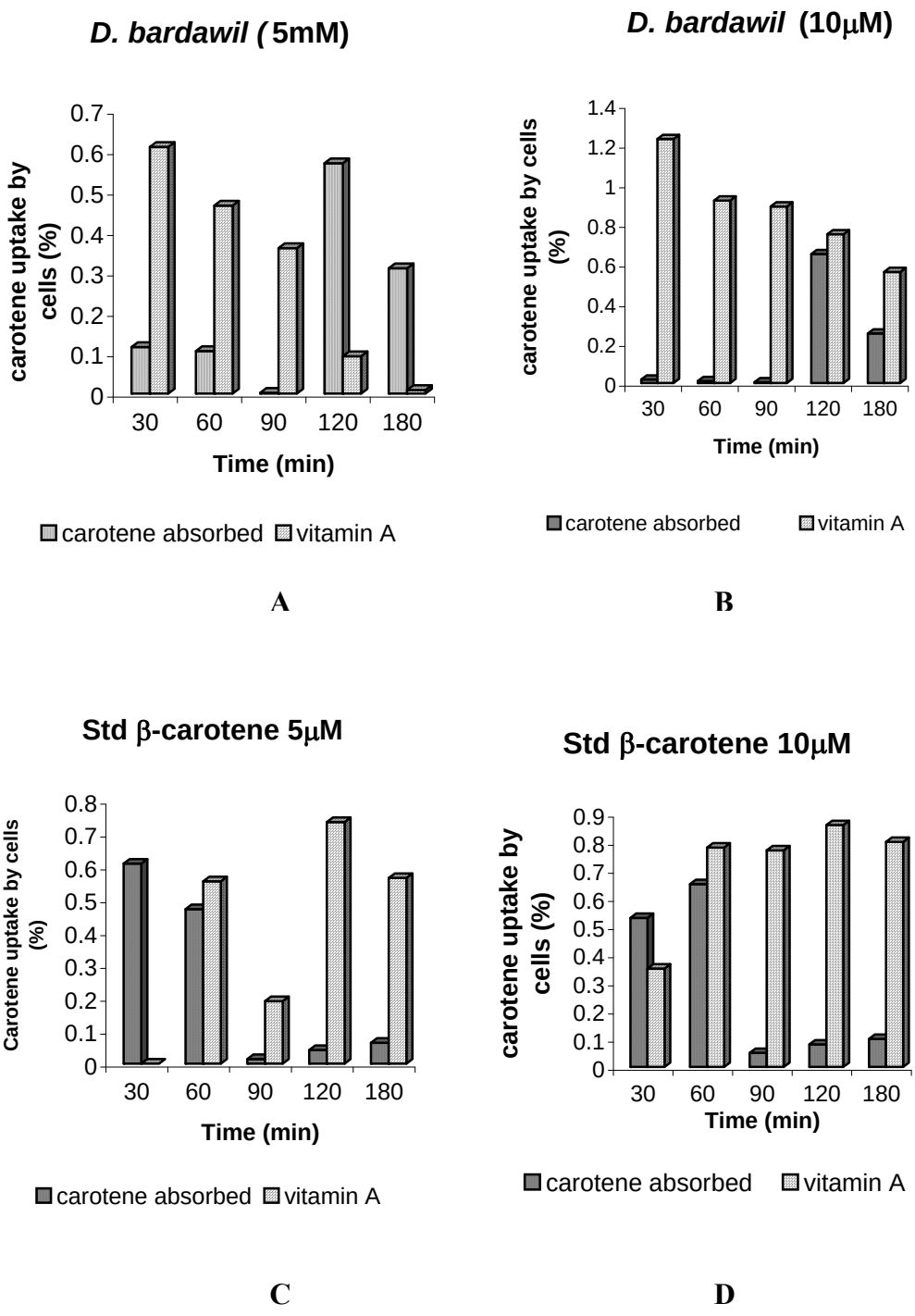



**Fig. 48. Percentage of cellular uptake of β-carotene and vitamin A conversion in primary intestinal cell cultures of rat.** Intestinal primary epithelial cells were incubated with *D. bardawil* carotenoid extract and synthetic β-carotene (5 and 10 μM) at $37^0$C. At different time periods (30, 60, 90, 120 and 180 min) an aliquot of the cells were pelleted and extracted with acetone and analyzed by HPLC.

### 3.4.3.3. Discussion

The *in vitro* cell culture models offer a new experimental tool to study the carotene retention and its conversion to vitamin A. In this experiment, the carotene retention and its conversion to vitamin A was studied in primary intestinal epithelial cells. Vitamin A conversion takes place within 30min of incubation in both *D. bardawil* and synthetic β-carotene treated cell lines. Garrett et al, (1999a) observed that uptake of β-carotene and its cellular accumulation in Caco-2 cells was proportional to the media content of carotenoids, and 72% of the initial load of β-carotene was observed after incubation for a period of 36hrs. In their study, retinol was not detected in either in cells or in the medium after exposure to micellar β-carotene, because the cell line (HTB-37) they studied was lacking in dioxygenase activity required for the conversion of β-carotene to retinol. However During et al, (2001) observed the conversion of β-carotene to retinol in Caco-2 cell lines, PF11 and TC7 clones. Beta-carotene uptake and conversion to retinol has also been reported in non-intestinal cell lines too. The cultures of mouse embryonic fibroblasts, rabbit corneal epithelial cells, human skin fibroblasts (Wei et al, 1998), hepatocytes (Blaner and Olson, 1994), human lung cancer cells (Prakash et al, 1999), human prostrate cancer cell lines (Williams et al, 2000) and human skin melanocytes and keratinocytes (Anderson et al, 2001) has also shown the conversion of β-carotene to retinol. Torres et al, (2004) studied the β-carotene accumulation and conversion to vitamin A in human breast carcinoma cells (MCF-7). In our study also, we observed the conversion of β-carotene to retinol in the primary intestinal cells from both *D. bardawil* extract and synthetic β-carotene (Fig. 48). No significant difference was observed for the two different concentrations either for carotene retention or for retinol accumulation in both the treatments of synthetic β-carotene and *D. bardawil* carotene extract.

The *in vitro* cell culture models can be exploited to study the conversion of β-carotene to retinol, other retinoids and also to study the effect of carotenoid metabolites at the cellular level. A combination of several *in vitro* and *in vivo* systems are recommended to provide the best possible data for characterizing how provitamin A carotenoids may influence the cellular properties (During et al, 2004). Hence the *in vitro* cell culture studies can be used as a simple model to screen the vitamin A availability from carotene rich diet or food sources.

### 3.4.3.4. Salient features

The bioaccessibility and bioconversion of *D. bardawil* biomass was assessed by *in vitro, in vivo* and cell line models. The *in vitro* bioaccessibility of carotenoids was studied by simulated digestion method. The study revealed that the percent bioavailability of β-carotene and lutein from *D. bardawil* biomass was 22 and 12% respectively after complete (gastric and intestine) digestion.

The uptake and conversion of synthetic β-carotene and *D. bardawil* carotenoids to retinol was studied in primary intestinal cell lines. The study revealed that the cell lines could be efficiently used as an alternative model to study the availability of carotenoids.

The intestinal perfusion study revealed that the retinol conversion starts within 30 min in the intestine, and a three-fold increase in vitamin A content was observed at the same time.

Further, the bioavailability of *D. bardawil* carotenoids and vitamin A conversion was studied in rats using single and multiple oral doses (7 days) and the same was compared with the synthetic β-carotene treated rats. The study showed that maximum retinol conversion takes place within 4 hrs after single oral dosage. Detectable level of β-carotene was observed in the serum after single oral dose of either *D. bardawil* biomass or synthetic β-carotene. The study also revealed that the accumulation of β-carotene in liver was observed at 8 hrs after oral dosage

Hepatic analysis of the experimental groups after multiple doses, revealed a very high liver stores of retinol and β-carotene. The liver retinol content of both the experimental groups was significantly higher compared to the control. A 3-4 fold enhanced accumulation of retinol in liver was observed in the experimental groups than control group. The liver retinol content was higher in the synthetic β-carotene (78%) fed group compared to *D. bardawil* fed group (69%). In contrast to this, β-carotene accumulation in the liver of *D. bardawil* biomass fed group was higher (85%) than synthetic β-carotene (72%) fed group. This study clearly reveals that *D. bardawil* biomass is nearly efficient as synthetic β-carotene in terms of β-carotene accumulation, retinol formation and its accumulation in the liver.

## SECTION V

## BIOLOGICAL ACTIVITY OF *D. BARDAWIL BIOMASS* ON $CCl_4$ INDUCED TOXICITY: BENEFICIAL ATTRIBUTES OF *D. BARDAWIL* AND ITS POTENTIAL TO MODULATE EXPERIMENTALLY INDUCED DISEASE CONDITIONS

### Background

In recent years, more attention is given towards the role of diet in human health. Epidemiological evidences say that there is a correlation between the high fruits and vegetable intake with decreased risk of several diseases such as cancer, atherosclerosis etc (Reviewed by Fraser and Bramley, 2004; Krinsky, 1994a; Krinsky and Johnson, 2005). Algae are known as good nutritional supplements. Published data on biological activity of whole cell biomass or carotenoids of *D. bardawil* in protecting against carbon tetrachloride ($CCl_4$) induced hepatotoxicity and nephrotoxicity are scanty. In this connection, the possible protective effects of *D. bardawil* biomass against $CCl_4$ induced toxicity were studied by monitoring changes in serum enzyme levels, serum bilirubin, creatinine content and lipid peroxidation in hepatic and renal tissues. The $CCl_4$ was used as toxic agent because it is one of the best models to study the hepatic and renal damage (Slater, 1987; Recknagel et al, 1989).

### 3.5.1. Effect of algal feeding on body weight and organ weight

Administration of *D. bardawil* biomass did not show significant variation in body weight (Table 27) when compared to controls with normal diet. Clinical signs of any toxicity as well as notable changes in the behavior of the animals were not observed among rats fed with 2.5 or 5.0 g $kg^{-1}$ b.w biomass. Further, no mortality occurred during the experimental period. No significant variation in the relative weight of vital organs (Table 28) were observed, however increase in relative weight of liver was observed in the groups treated with $CCl_4$, indicating the symptoms of fatty liver.

**Table. 27. Body weight of the experimental rats* fed with synthetic *β*-carotene and *D. bardawil* biomass compared with control group devoid of carotenoid source during $CCl_4$ induced intoxication**

| Groups | | Body weight (g) | |
|---|---|---|---|
| | | Initial | Final |
| Group 1 | Normal | 128±3 | 175±6 |
| Group 2 | Control ($CCl_4$ 2mL $Kg^{-1}$ b.w.) | 147±6 | 180±10 |
| Group 3 | *D. bardawil* 5g $kg^{-1}$ b.w. | 133±13 | 167±20 |
| Group 4 | *D. bardawil* 2.5g $kg^{-1}$ b.w. + $CCl_4$ | 128±5 | 155±4 |
| Group 5 | *D. bardawil* 5g $kg^{-1}$ b.w. + $CCl_4$ | 135±10 | 170±15 |
| Group 6 | Synthetic β-carotene + $CCl_4$ | 128±14 | 166±7 |

**Table 28. Relative weights (g $100g^{-1}$)* of different organs of the experimental rats fed with synthetic *β*-carotene and *D. bardawil* biomass compared with control group devoid of carotenoid source during $CCl_4$-induced intoxication**

| Organs | Group 1 | Group 2 | Group 3 | Group 4 | Group 5 | Group 6 |
|---|---|---|---|---|---|---|
| **Liver** | 3.8±0.3 | 4.4±0.9 | 3.4±0.1 | 4.4±1.4 | 4.0±1.4 | 4.0±0.9 |
| **Kidney** | 0.8±0.07 | 0.75±0.1 | 0.77±0.07 | 0.78±0.13 | 0.67±0.11 | 0.72±0.07 |
| **Heart** | 0.34±0.07 | 0.4±0.08 | 0.35±0.1 | 0.33±0.1 | 0.3±0.03 | 0.34±0.04 |
| **Lung** | 0.52±0.1 | 0.5±0.12 | 0.47±0.07 | 0.55±0.09 | 0.55±0.09 | 0.54±0.1 |
| **Spleen** | 0.37±0.08 | 0.27±0.07 | 0.37±0.04 | 0.29±0.15 | 0.25±0.13 | 0.25±0.1 |
| **Testis** | 1.35±0.14 | 1.4±0.15 | 1.42±0.2 | 1.5±0.2 | 1.37±0.04 | 1.35±0.05 |
| **Brain** | 0.8±0.14 | 0.9±0.14 | 0.94±0.12 | 1.0±0.09 | 0.8±0.07 | 0.9±0.1 |

*Average organ weight from 6 animals per group, values is expressed as mean ± SE.

The carotenoid rich biomass of *D. bardawil* (~50 and 100mg carotene respectively) or synthetic β-carotene (~50 mg carotene) was fed for 14 days.

Group 1: Normal (receiving normal diet without toxin treatment);

Group 2: Control (receiving normal diet with toxin treatment);

Group 3: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight;

Group 4: *D. bardawil* dry biomass was fed at 2.5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 5: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 6: fed with synthetic *β*-carotene at 50 mg $kg^{-1}$ body weight along with $CCl_4$.

### 3.5.2. Effect of $CCl_4$ on liver parameters and its amelioration by *D. bardawil* biomass and synthetic *β*-carotene

#### 3.5.2.1. Histological observation

In case of control group (without $CCl_4$ treatment), hepatocytes with normal architecture were noticed (Fig. 49a). Similar characteristics were noticed in groups fed with *D. bardawil* (without $CCl_4$ treatment, Fig. 49c). Histopathological analysis of $CCl_4$ treated animals (Fig. 49b) showed fatty liver symptoms, total loss of hepatic architecture, acute liver injury, centrilobular necrosis with hemorrhages and degenerated hepatic cords and hepatocytes. Experimental groups fed with *D. bardawil* followed by $CCl_4$ treatment have retained better hepatic architecture compared to toxin treated group. The level of protection against liver injury and necrosis was higher in case of groups fed with *D. bardawil* whole cells (Fig. 49d and e) when compared to synthetic β-carotene fed group (Fig. 49f). No significant variations were noticed between male and female rats in terms of hepatic architecture in different groups.

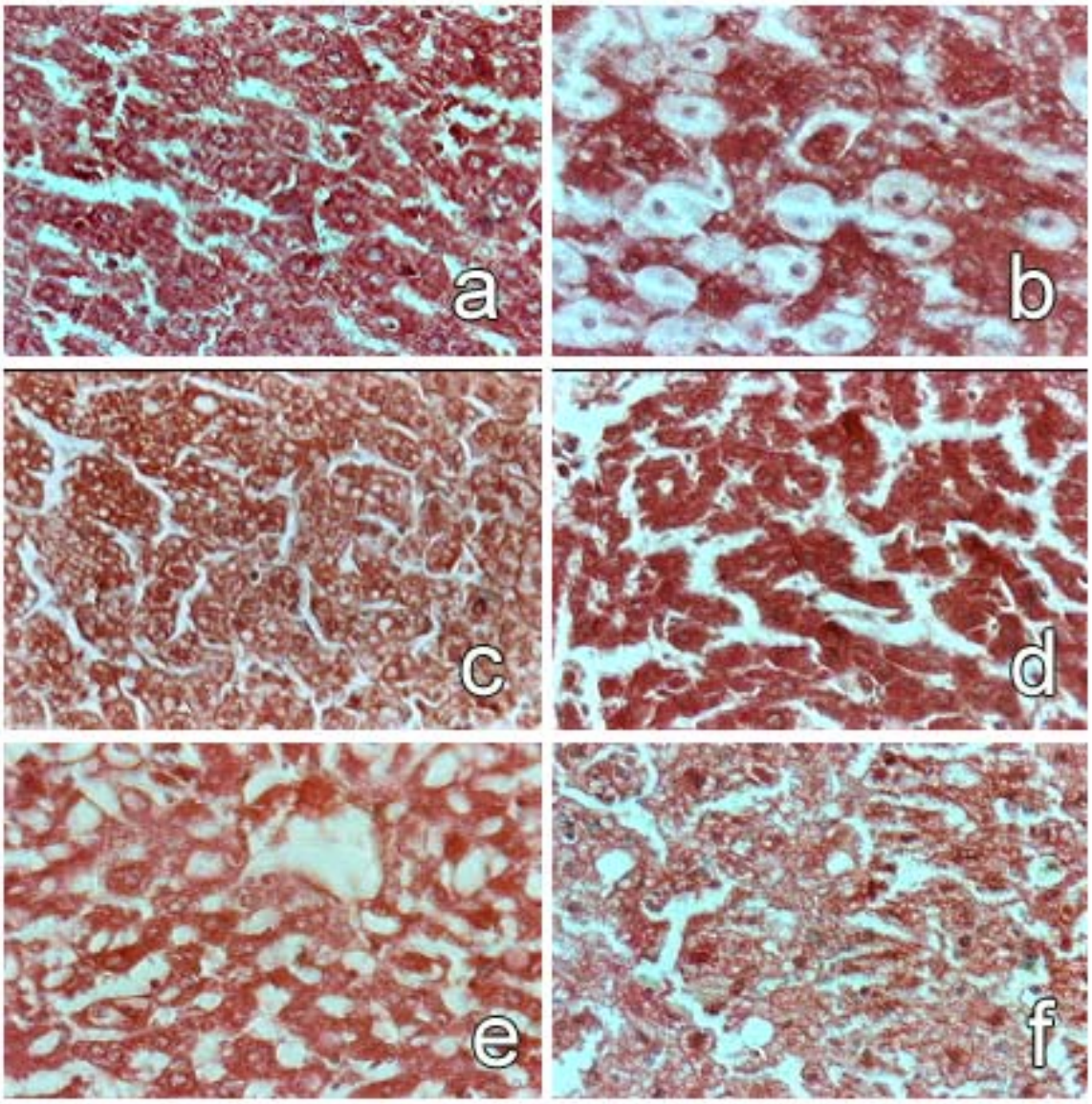



**Fig. 49. Photomicrographs of liver tissue of rats of different groups examined for hepatoprotective effect of whole cells of *D. bardawil* stained with hematoxylin and eosin** (magnification 100×). (a) Normal; (b) control (treated with $CCl_4$); (c) *D. bardawil* biomass (5.0 g $kg^{-1}$ b.w.); (d) 2.5 g $kg^{-1}$ b.w. *D. bardawil* biomass treated along with $CCl_4$; (e) 5.0 g $kg^{-1}$ b.w. *D. bardawil* biomass treated along with $CCl_4$; and (f) synthetic $\beta$-carotene (50 mg $kg^{-1}$) along with $CCl_4$.

### 3.5.2.2. Effect of $CCl_4$ on liver marker enzymes

Feeding of *D. bardawil* (5g $kg^{-1}$ b.w) alone did not alter the activity of liver marker enzymes. Rats treated with a single dose of $CCl_4$ produced a marked increase in the activities of serum aspartate aminotransferase (AST, Fig. 50), serum alanine aminotransferase (ALT, Fig. 51) and serum alkaline phosphatase (ALP, Fig. 52) indicating the hepatic damage. Administration of *D. bardawil* biomass at 2.5, 5.0 g $kg^{-1}$ b.w. and synthetic β-carotene (50 mg $kg^{-1}$ b.w.) decreased the deleterious effect of $CCl_4$ as evident by the decreased level of liver marker enzymes

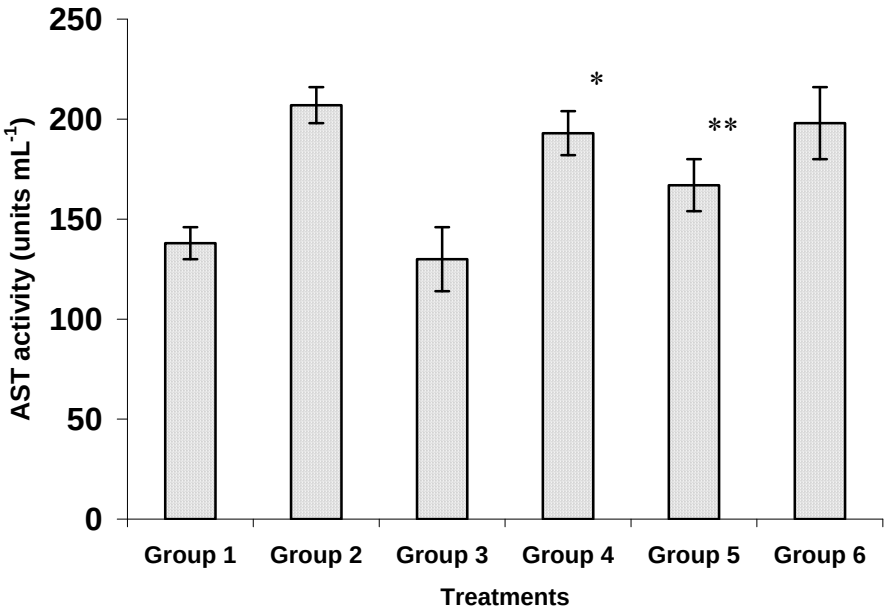



**Fig. 50. Effect of synthetic *β*-carotene and *D. bardawil* biomass on serum aspartate aminotransferase (AST) activity**

Values are expressed as mean ± SE.

*, ** Statistically significant compared to $CCl_4$-treated group at $p < .05$ and $p < .01$, respectively.

Group 1: Normal (receiving normal diet without toxin treatment);

Group 2: Control (receiving normal diet with toxin treatment);

Group 3: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight;

Group 4: *D. bardawil* dry biomass was fed at 2.5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 5: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 6: fed with synthetic *β*-carotene at 50 mg $kg^{-1}$ body weight along with $CCl_4$.

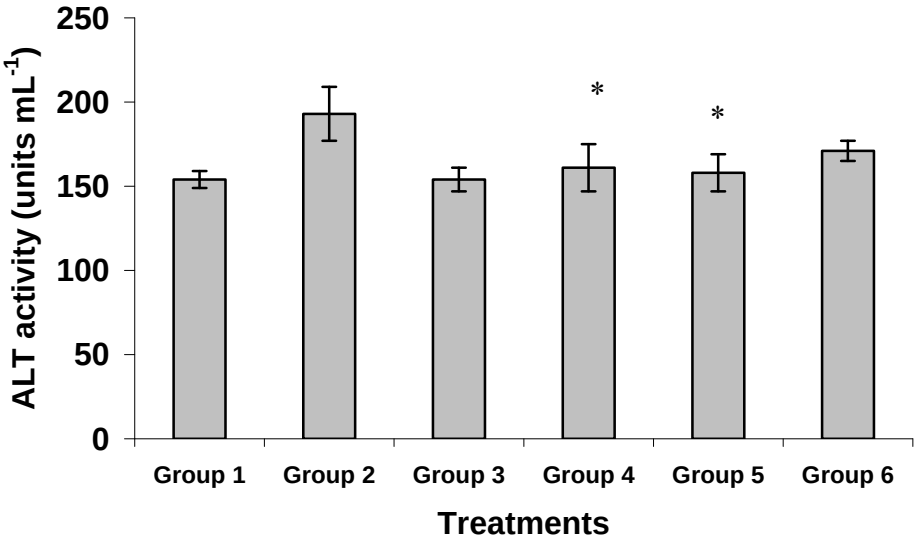



**Fig. 51. Effect of synthetic *β*-carotene and *D. bardawil* biomass on serum alanine aminotransferase (ALT) activity**

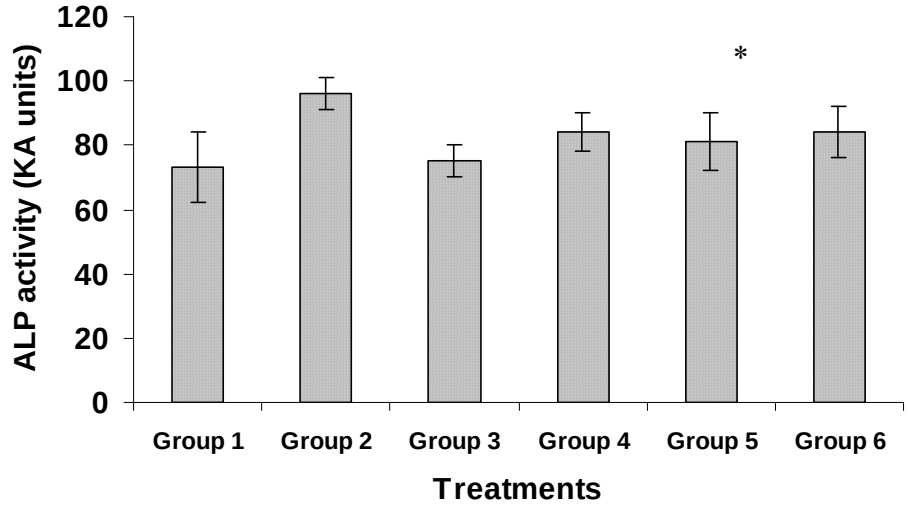



**Fig. 52. Effect of synthetic *β*-carotene and *D. bardawil* biomass on serum alkaline phosphatase (ALP) activity**

Values are expressed as mean ± SE.

* Statistically significant compared to $CCl_4$-treated group at p<0 .05.

Group 1: Normal (receiving normal diet without toxin treatment);

Group 2: Control (receiving normal diet with toxin treatment);

Group 3: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight;

Group 4: *D. bardawil* dry biomass was fed at 2.5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 5: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 6: fed with synthetic *β*-carotene at 50 mg $kg^{-1}$ body weight along with $CCl_4$.

### 3.5.2.3. Effect on the hepatic TBARS levels

Lipid peroxidation was measured in terms of TBARS activity and expressed as nmols $mg^{-1}$ protein. A marked increase in lipid peroxidation in liver tissues was observed in group of rats treated with $CCl_4$ alone. Administration of *D. bardawil* or synthetic *β*-carotene for a period of 14 days significantly reduced the enhanced level of lipid peroxidation (Fig. 53).

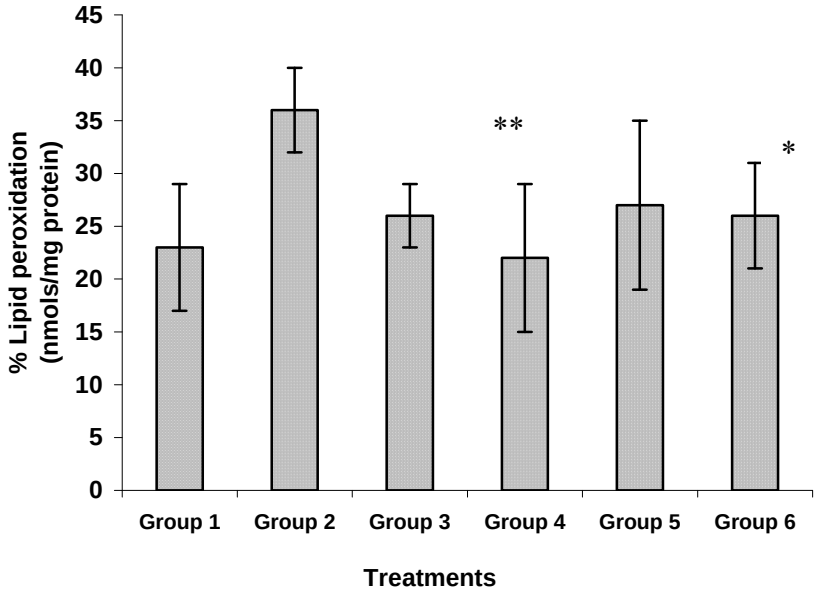



**Fig. 53. Effect of synthetic *β*-carotene and *D. bardawil* biomass on $CCl_4$-induced lipid peroxidation in hepatic tissues**.

Values are expressed as mean ± SE.

*, **Statistically significant compared to $CCl_4$-treated group at $p<0.05$ and $p<0.01$ respectively.

Group 1: Normal (receiving normal diet without toxin treatment);

Group 2: Control (receiving normal diet with toxin treatment);

Group 3: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight;

Group 4: *D. bardawil* dry biomass was fed at 2.5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 5: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 6: fed with synthetic *β*-carotene at 50 mg $kg^{-1}$ body weight along with $CCl_4$.

### 3.5.2.4. Effect on Serum Bilirubin levels

The serum bilirubin content was increased in $CCl_4$ treated group indicating the injury to liver. Both the doses of *D. bardawil* and synthetic β-carotene attenuated the $CCl_4$ induced elevated levels of serum bilirubin (Figure 54).

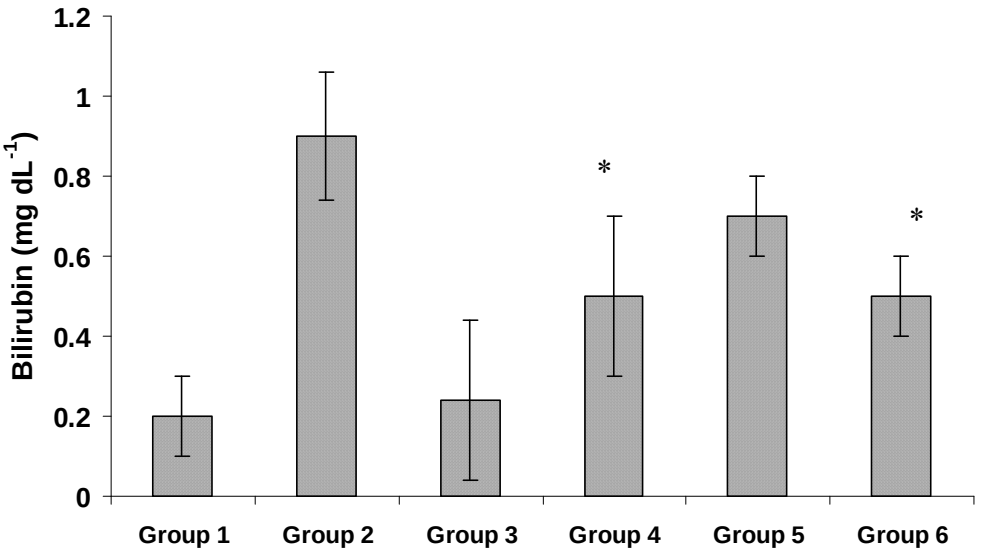



**Fig. 54. Effect of synthetic *β*-carotene and carotenoid-rich whole-cell biomass of *D. bardawil* on $CCl_4$-induced increase in serum bilirubin content**.

Values are expressed as mean ± SE.

*, **Statistically significant compared to $CCl_4$-treated group at $p < .05$ and $p < .01$, respectively.

Group 1: Normal (receiving normal diet without toxin treatment);

Group 2: Control (receiving normal diet with toxin treatment);

Group 3: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight;

Group 4: *D. bardawil* dry biomass was fed at 2.5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 5: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 6: fed with synthetic *β*-carotene at 50 mg $kg^{-1}$ body weight along with $CCl_4$.

### 3.5.3. Effect of $CCl_4$ on Kidney parameter and its amelioration by *D. bardawil* biomass and synthetic β-carotene

#### 3.5.3.1. Effect on renal TBARS level

Lipid peroxidation was measured in terms of TBARS activity and expressed as nmols $mg^{-1}$ protein. A higher value of lipid peroxidation was observed in renal tissues in the $CCl_4$ treated group. The elevated level of lipid peroxidation was restored in the groups administered with *D. bardawil* biomass and synthetic *β*-carotene (Fig. 55).

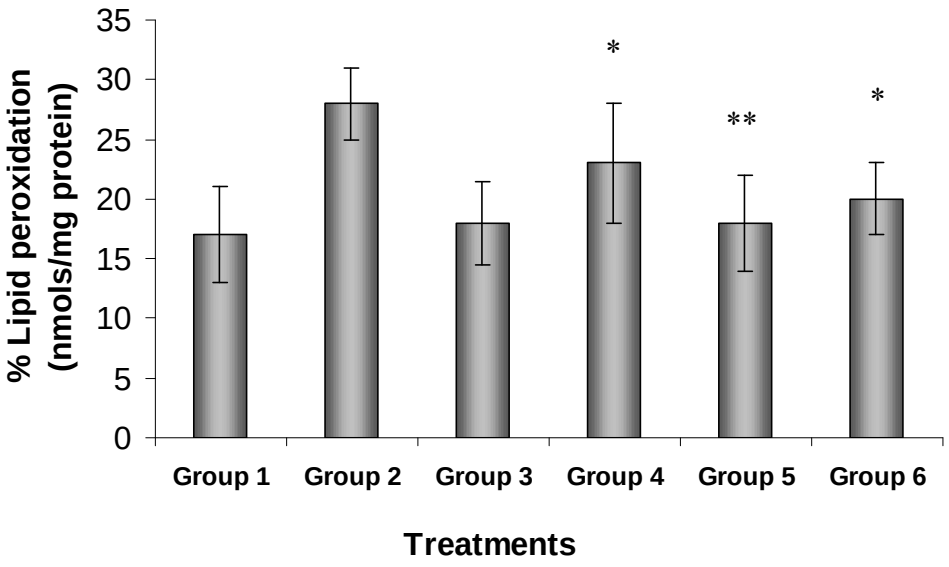



**Fig. 55. Effect of synthetic *β*-carotene and *D. bardawil* biomass on $CCl_4$-induced lipid peroxidation renal tissues.**

Values are expressed as mean ± SE.

*, **Statistically significant compared to $CCl_4$-treated group at $p < .05$ and $p < .01$, respectively.

Group 1: Normal (receiving normal diet without toxin treatment);

Group 2: Control (receiving normal diet with toxin treatment);

Group 3: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight;

Group 4: *D. bardawil* dry biomass was fed at 2.5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 5: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 6: fed with synthetic *β*-carotene at 50 mg $kg^{-1}$ body weight along with $CCl_4$.

### 3.5.3.2. Effect on Serum creatinine

The serum creatinine content was increased in $CCl_4$ treated group indicating the injury to kidney (Fig. 56). Restoration in the level of serum creatinine was observed in groups fed with *D. bardawil* biomass and Synthetic β-carotene.

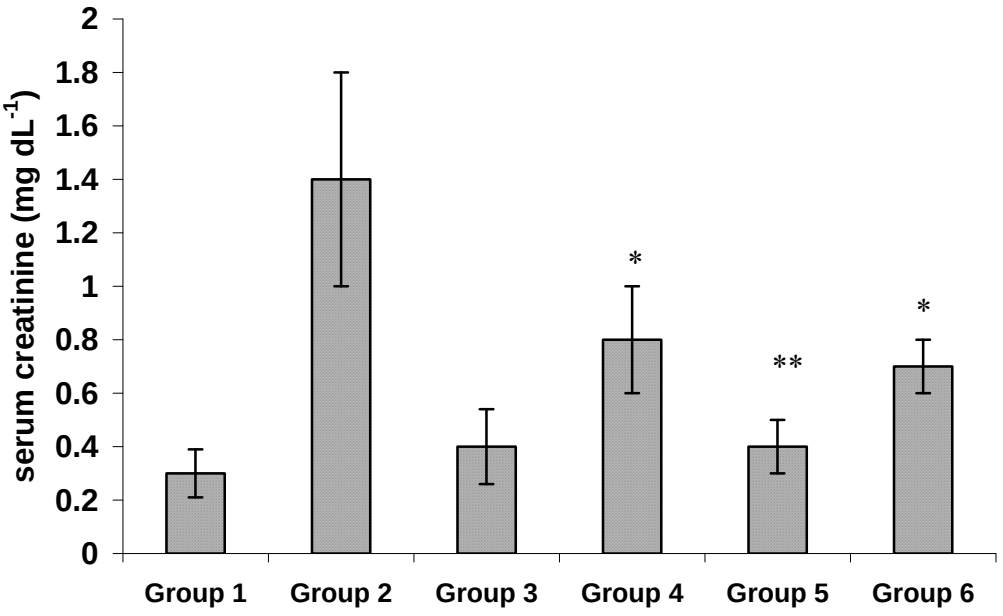



**Fig. 56 Effect of synthetic *β*-carotene and *D. bardawil* biomass on $CCl_4$-induced increase in serum creatinine levels.**

Values are expressed as mean ± SE.

*, **Statistically significant compared to $CCl_4$-treated group at $p < .05$ and $p < .01$, respectively.

Group 1: Normal (receiving normal diet without toxin treatment);

Group 2: Control (receiving normal diet with toxin treatment);

Group 3: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight;

Group 4: *D. bardawil* dry biomass was fed at 2.5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 5: *D. bardawil* dry biomass was fed at 5 g $kg^{-1}$ body weight along with $CCl_4$;

Group 6: fed with synthetic *β*-carotene at 50 mg $kg^{-1}$ body weight along with $CCl_4$.

### 3.5.4. Discussion

In this section the hepatoprotection and renal protective activity of *D. bardawil* biomass was studied under $CCl_4$ intoxication. The hepatotoxicity of $CCl_4$ is widely known (Slater, 1987; Recknagel et al, 1989). Carbon tetrachloride ($CCl_4$) is an industrial toxicant, known to cause hepatic necrosis in addition to free radical generation in kidney, heart, lung, testis, brain and blood (Ahmad et al, 1987; Ohta et al, 1997; Ozturk et al, 2003). The toxic effects of $CCl_4$ are the consequences of production of free radicals, which initiate cell damage (Slater, 1984; Kadiiskaa et al, 2005). In this study, $CCl_4$ was used to induce oxidative stress in rat liver and kidney. The resulting damage was measured using biochemical markers of serum and liver. The damage caused by $CCl_4$ was evident by the increased levels of serum enzymes and lipid peroxidation after $CCl_4$ treatment. This elevation could be explained on the basis that $CCl_4$ causes necrosis of liver and renal cells. As a result of necrosis caused by acute infection or chronic liver disease, the marker enzymes are known to be released into the circulation with consequent rise in the serum levels (Wroblewski and La Due 1955). A persistent rise in serum transaminase level is an indication of liver cell damage. The elevation of the serum transaminases, especially AST and ALP (Fig 50 & 51), is due to the hepatic damage caused by $CCl_4$. When the rats were treated with $CCl_4$, it also causes increase in liver weight, mainly due to fatty liver symptoms. The development of fatty liver in $CCl_4$ treated rats occurs due to a block in the release of hepatic triglycerides to the plasma. The liver rapidly converts the free fatty acids to triglycerides, but fails to release it into the plasma. As a result, triglycerides accumulate within the liver and decrease in the plasma (Lombard and Ugazio, 1965).

Hepatic damage also increases the serum bilirubin levels due to the leakage of cellular contents from hepatocytes to the serum (Recknagel et al, 1989). The enhanced level of tissue lipid peroxidation in the $CCl_4$ treated group expressed in terms of thiobarbituric acid reactive substances (TBARS) indicates the damage to liver and kidney (Fig 53 & 55). Among the parameters studied in the kidney, marked restoration of renal TBARS and serum creatinine was observed in the synthetic $\beta$-carotene and *D. bardawil* biomass treated groups. This suggests that upon $CCl_4$ administration there is damage to the renal tissues too. Fadhel and Amran (2002) had reported increased levels of renal TBARS in rats after $CCl_4$ exposure, which could be restored by black tea extract.

In this study *D. bardawil* biomass as well as synthetic *β*-carotene showed restoration of the elevated levels of AST, ALT, ALP, bilirubin and creatinine content that are coupled with hepatic and renal oxidative stress. Chidambara Murthy et al, (2005a) studied the hepatoprotective activity of *D. salina* using the same model and there observed a marked protection for the damage caused by $CCl_4$.

The hepatoprotective activity of *D. bardawil* may be attributed to the presence of different carotenoids, mainly *β*-carotene. Carotenoids are known to possess high radical scavenging activity and thereby provide adequate protection against free radical induced liver damage. Many of the antioxidants present in plants are known for their hepatoprotective activity against $CCl_4$-induced hepatic damage (Chidambara Murthy et al, 2002; 2005). The results of this study indicate that repeated daily administration of *D. bardawil* ameliorates $CCl_4$ induced liver toxicity in rats. Because algae contain a good amount of protein and carotenoids of biological significance, it can be utilized as a food supplement for the prevention of free radical mediated hepatic and renal damages that might be caused by various drugs and natural products. This also supports the use of *D. bardawil* biomass as nutraceuticals claiming antioxidant properties.

### 3.5.5. Salient features

In this section, the possible protective effects of synthetic β-carotene and *Dunaliella bardawil* biomass against carbon tetrachloride induced toxicity was studied by monitoring the changes in serum enzyme levels, serum bilirubin, creatinine content and lipid peroxidation in hepatic and renal tissues. The damage caused by $CCl_4$ was evident by the increased levels of serum enzymes and lipid peroxidation due to necrosis of liver and renal cells. Increase in relative weight of liver in the groups treated with $CCl_4$, has been observed indicating the symptoms of fatty liver. Histopathological analysis of $CCl_4$ treated animals also confirmed this, and there was loss of hepatic architecture and necrosis was observed. Experimental groups fed with either synthetic β-carotene or *D. bardawil* biomass, followed by $CCl_4$ treatment have retained better hepatic architecture compared to toxin treated group.

The hepatic damage in the $CCl_4$ treated rats indicated by the marked increase in the activities of serum enzymes viz., serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) and serum alkaline phosphatase (ALP) and lipid

peroxidation and serum bilirubin content were restored by the intake of *D. bardawil* biomass and synthetic β-carotene. Similarly the increased levels of serum creatinine and lipid peroxidation in the renal tissues were also restored by the intake of D. *bardawil* biomass and synthetic β-carotene in the experimental rats.

The carotenoids of *D. bardawil* biomass were as efficient as synthetic β-carotene in protecting against $CCl_4$ induced hepatic and renal damage. Thus the study clearly demonstrated the beneficial effect of *D. bardawil* biomass in comparison to synthetic β-carotene in experimentally induced disease conditions.

# *SUMMARY & CONCLUSION*

## 4.1. Brief background:

*Dunaliella* is a unicellular green alga, generally ovoid in shape 4-10 μm wide and 6-15 μm long (Ben-Amotz and Avron, 1983; 1990). The cells are motile due to the presence of two equal, long flagella with a single chloroplast in the center. Chloroplast contains a large pyrenoid surrounded by polysaccharide granules (a storage product). The chief morphological character of *Dunaliella*, in contrast to other members of chlorophyta is that it lacks a rigid polysaccharide cell wall. Cell is a natural protoplast, enclosed by thin elastic membrane. This makes the *Dunaliella* cells responsible for its rapid change in shape and response to osmotic changes (Ben-Amotz and Avron 1983; Ben-Amotz et al, 1982; Borowitzka and Siva, 2007). There are two commercially important species of *Dunaliella* i.e*., D. salina* and *D. bardawil.* In early years they were valued for the production of glycerol and protein but now the focus is on carotenoid especially the β-carotene and lutein.

The halotolerant alga *D. bardawil* possesses the unique ability to accumulate a very high content of β-carotene. The amount of β-carotene in *D. bardawil* under controlled conditions can be manipulated by different growth conditions, to vary from about 3 to 8% (Ben-Amotz and Avron, 1983). The β-carotene found in *D. bardawil* contains almost equal amount of cis and trans β-carotene owing to 90% of total carotenoids, with the rest composed mostly of lutein and other carotenes (Ben-Amotz et al., 1982).

The carotene pigments are usually associated in lipid globules located in the interthylakoid space of the chloroplasts (Ben-Amotz and Avron, 1983). *Dunaliella* produces carotenoid during all stages of growth, and can be manipulated at any stage of physiology (Yong and Lee 1991). Commercial production of *Dunaliella* sp as a source of β-carotene is the major micro algal industry in many parts of the world. Numerous factors have been shown to induce massive carotenoid accumulation in *Dunaliella.* The important ones are high salinity and high light intensity (Ben-Amotz and Avron, 1983; Loeblich, 1982). It has observed that, an inverse relationship exists related to β-carotene content and the specific growth rate (Ben-Amotz et al. 1982). Hence the major factors of significance in cultivation of algae are nutrients, light, temperature and stress of various natures (Becker and Venkataraman 1982; Raja et al, 2007).

The important function of carotenoid is their potential to prevent chronic disease and vitamin A deficiency (VAD) (Von-Lintig et al, 2005). In humans, VAD leads to night

blindness in milder forms, while more severe progression results in corneal malformations, e.g., xerophthalmia. Besides visual defects, this deficiency affects the immune system, leads to infertility or causes malformations during embryogenesis. Being essential for vision, in vertebrates the vitamin A derivative retinoic acid (RA) is a major signal-controlling molecule in a wide range of biological processes (Underwood, 2004). VAD is a major problem particularly in developing countries. Vitamin A demand can be met either by supplementing vitamin A or carotenoids with provitamin A activity. All naturally occurring vitamin A in the food chain derives from provitamin A conversion and that the world's population mainly relies on carotenoids from staple food sources to meet vitamin A requirements. The central cleavage mechanism splits β-carotene at the central double bond (Castenmiller and West, 1998) by a specific enzyme, β-carotene 15,15'-oxygenase found to yield two molecule of retinal in intestinal cell and liver cytosol (Goodman and Olsen, 1969).

*Dunaliella* is the only organism which produces massive amount of carotenoids and feasible for commercial production. The cultivation of this alga for the carotenoid rich biomass and its biological significance and safety aspects are the main focus of the present work. This study was undertaken in view of β- carotene need and possibility of utilization of algal source for large-scale carotenoid production.

**Objectives of the study:**

- To optimize a cultivation process of *D. bardawil*.
- Analysis of genes involved in light regulated synthesis of carotenoids in *D. bardawil.*
- Studies on the involvement of selected metal ions in the regulation of carotenoid biosynthesis in *D. bardawil.*
- Biological activity of *Dunaliella* carotenoids in *in vitro, in vivo* and cell culture models.

## 4.2. SUMMARY OF RESULTS

### 4.2.1.Cultivation, growth, carotenogenesis and nutritional composition of *D. bardawil* biomass:

An indigenous culture of *D. bardawil* was established in both indoor and outdoor culture conditions. The cultures were grown in AS100 medium. During vegetative phase of *D. bardawil* showed a maximum of 20g $L^{-1}$ wet biomass on $30^{th}$ day. On $30^{th}$ day the chlorophyll a, b and carotene content was 200mg, 75mg and 136µg /100mg biomass respectively under laboratory culture conditions.

*D. bardawil* cultures grown at 1M NaCl concentration showed good vegetative growth, however for carotene accumulation, 2.0 M NaCl was required. Addition of 2% $CO_2$ in gaseous to the vegetative phase of *D. bardawil* did not show any significant enhancement in pigment profile. However, feeding of metal ions/micronutrients Fe and Zn to the culture during vegetative growth stage, showed enhanced carotene content in indoor culture condition.

Scale up of cultures up to 2000L in outdoor culture conditions was achieved. During the vegetative phase in outdoor growth condition, *D. bardawil* cultures required a light intensity of < 20 Klux. Hence, the cultures were protected by green house shade nets to obtain optimal light intensity. This helps to maintain the cultures through out the year. The carotenogenesis was achieved in *D. bardawil* by subjecting the culture to high light intensity of 30-35 Klux under outdoor raceway tanks. A maximum of 4% (w/w) β-carotene and 1% (w/w) lutein was observed on wet weight basis in the carotene induced *D. bardawil* biomass.

After successful mass cultivation of *D. bardawil,* the harvesting, drying and storage conditions were studied. The online centrifuges were used for efficient harvesting of *D. bardawil* cultures. The carotene induced *D. bardawil* cultures were harvested using online centrifuges. In this 100L cultures were harvested per hour, containing 90-95% cell harvesting. Among the different drying methodologies employed, freeze drying showed a minimum loss of carotenoids during drying. Hence further freeze drying was employed to get a dry biomass. The storage under different temperatures at dark conditions revealed that the dry biomass must be stored at ultra low temperature to retain the carotenoids upon storage.

Analysis of the biomass revealed 22% protein, 27% carbohydrate and 8% fat content on dry weight basis. The unsaturated fatty acids linoleic (35%) and linolenic acid

(81%) was observed among the neutral and glycolipid fractions respectively. In *D. bardawil* biomass, the heavy metals were found to be below the permitted level.

### 4.2.2. Analysis of genes involved in carotenoid biosynthesis pathway during light induced carotenogenesis:

The carotenogenesis in *D. bardawil* cultures was induced by exposing the cultures to high light intensity of 30-35 Klux. During this, the major carotene that accumulated was β-carotene (up to 4%). During this, the transcript analysis of Phytoene synthase (*PSY),* Phytoene desaturase (*PDS)* and Lycopene cyclase (*LCY)* revealed an upregulation of the genes for these enzymes, when the cells were subjected to high light intensity. This upregulation in the genes were positively correlated with the carotenoid production in the cells. In indoor cultures lutein content was high (up to 1%) and an exposure to high light did not exhibit impact on lutein level. In addition to this, there was no significant elevation of transcript level of gene responsible for the conversion of β-carotene to lutein (carotene hydroxylase, *CH)*, during carotenogenesis.

### 4.2.3. Safety and toxicity evaluation of *D. bardawil* biomass in albino rats:

The safety of *D. bardawil* biomass after oral administration to short and long period was studied in rats. In short term study (acute oral toxicity), by administration of single dose of *D. bardawil* at the maximum level (5g biomass containing 2% β-carotene $Kg^{-1}$ b.w) and toxicity symptoms, if any, were monitored for 15 days. In long term toxicological study, the effects of 90 days oral administration of *D. bardawil* biomass (100 and 1000mg biomass $Kg^{-1}$ b.w) was assessed and compared with the control rats. In both the study, *D. bardawil* biomass at the given doses did not induce any treatment related observable toxic effects, when compared to control group of animals devoid of biomass**.** Hence *D. bardawil* biomass was found to be safe at the given doses in animal models.

### 4.2.4. Bioaccessibility and bioconversion of carotenoids from *D. bardawil - in vitro, in vivo* and cell line models

The bioaccessibility and bioconversion of *D. bardawil* biomass was assessed by *in vitro* and *in vivo* methods. The *in vitro* bioaccessibility of carotenoids was studied by

simulated digestion method. The study revealed that the percent bioavailability of β-carotene and lutein from *D. bardawil* biomass was 22 and 12% respectively after complete (gastric and intestine) digestion.

The uptake and conversion of *D. bardawil* carotenoids to retinol was studied in primary intestinal cells. The study revealed carotene uptake and retinol conversion in primary intestinal cells; hence the primary intestinal cell lines could be efficiently used as an alternative model to study the availability of carotenoids.

The intestinal perfusion study revealed that the retinol conversion, starts within 30 min in the intestine, and there observed a three-fold increase in vitamin A content within 30 min.

Further, the bioavailability of *D. bardawil* carotenoids and vitamin A conversion was studied in experimental rats using single oral dose and multiple doses (7 days), and compared with the synthetic β-carotene treated group. The postprandial response of retinol in serum and liver was studied for 0 to 8hr after single oral dose. The study showed that maximum retinol conversion takes place within 4 hrs after single oral dosage. β-carotene was absent in serum of control group of animals, and found at detectable level in the serum after single oral dose of either *D. bardawil* biomass or synthetic β-carotene. In liver significant ($P < 0.05$) accumulation of β-carotene was observed. After 8 hrs of intubation, the liver β-carotene levels increased to 1.5μg $g^{-1}$ and 1.26μg $g^{-1}$ respectively in the synthetic and *D. bardawil* fed groups compared to initial liver β-carotene (0.35μg $g^{-1}$). The study also revealed that the accumulation of β-carotene in liver was observed at 8hrs after oral dosage.

Hepatic analysis of the experimental group, after multiple doses, revealed a very high liver store of retinol and β-carotene. The liver retinol content of both the experimental groups was significantly higher compared to control. Enhanced accumulation of retinol upto 3-4 fold was observed in liver among the experimental groups compared to control group. The liver retinol content was higher in the synthetic β-carotene (78%) fed group compared to *D. bardawil* fed group (69%). In contrast to this, β-carotene accumulation in the liver of *D. bardawil* biomass fed group was higher (85%) than synthetic β-carotene (72%) fed group. The study clearly revealed that *D. bardawil* carotenoids are effective in terms of carotene accumulation and retinol formation in the liver.

### 4.2.5. Biological activity of *D. bardawil* biomass on $CCl_4$ induced toxicity: Beneficial attributes of *D. bardawil* and its potential to modulate experimentally induced disease conditions

The hepatoprotection and renal protection activity of *D. bardawil* biomass was studied under $CCl_4$ intoxication. The damage caused by $CCl_4$ was monitored by parameters of toxicity in serum, liver and kidney. The elevated levels of serum enzymes (serum alanine aminotransferase and serum aspartate aminotransferase) were found to restore in the *D. bardawil* biomass and synthetic β-carotene treated groups. The lipid peroxidation observed in hepatic and renal tissues of $CCl_4$ treated rats were restored in the rats pretreated with *D. bardawil* biomass and synthetic β-carotene. The elevated levels of serum creatinine (1.4mg $dL^{-1}$) indicated the renal damage. In *D. bardawil* and synthetic β-carotene treated groups the serum creatinine levels after $CCl_4$ intoxication was found to be 0.4 and 0.7mg $dL^{-1}$ respectively. Hence the study indicated the possible benefit of *D. bardawil* biomass under experimentally induced disease conditions.

## 4.3. Conclusions

A series of experiments were carried out in order to develop a mass cultivation method for *D. bardawil*. The available literature suggests that *D. bardawil* can be successfully cultivated by optimizing conditions for nutrients, salinity, along with other culture conditions such as light and temperature. During our experiment it has been observed that *D. bardawil* has two distinct phases in its life cycle. The first being vegetative growth, which is highly efficient at 0.5M-1.0M NaCl concentration at indoor culture conditions. The next stage being carotenogenesis stage, which occurs at, high light and salt conditions, wherein the algae showed arrest in the growth rate and begin to accumulate carotenoids. In these studies, a maximum of 20g $l^{-1}$ wet biomass was obtained on $30^{th}$ day at indoor culture condition. When exposed to high light (30-35 Klux) *D. bardawil* accumulates over 4% carotene content. This is on par with the published reports available for *Dunaliella* sp. Thus it has been possible to establish large scale cultivation methodology, which can be adapted at an industrial scale.

Different parameters were tested such as varied concentration of $CO_2$ from different sources, NaCl levels during vegetative phase and its influence on the carotene production. An attempt has been made to identify the influence of metal ions in the form of micronutrients, which is expected to regulate carotene accumulation. In this study, Fe and Zn showed increase in carotene content without affecting the vegetative growth.

After successful mass cultivation, it was important to find an efficient method of harvesting, drying and storage conditions. It was demonstrated that online centrifugation is highly efficient for harvesting, followed by freeze drying and storage at ultra low temperature.

Based on the available literatures it is known that carotenogenesis is greatly affected by the quantity of light. It was speculated to be due to transcriptional regulation of key regulatory genes involved in the carotenogenesis pathway by light. Phytoene synthase, phytoene desaturase, lycopene cyclase and β-carotene hydroxylase were the candidate genes selected to study the role of light in its transcriptional activation. The cultures were subjected to different light intensity for a period of 5 days to accumulate carotenoids. The results indicated that high light induced up regulation of phytoene synthase, phytoene desaturase and lycopene cyclase genes. However the gene

involved in conversion β-carotene to lutein (β-carotene hydroxylase) did not show any significant change in transcript levels at different lights. Therefore, it was evident that light plays a major role in regulating carotenoid pathway.

The safety of *D. bardawil* biomass was assessed in albino rats by oral administration of *D. bardawil* biomass. Results indicated that at the given dose, *D. bardawil* biomass did not induce any treatment related observable toxic effects, when compared to control group of animals receiving normal diet. The result revealed that *D. bardawil* is safe and can be exploited as a potential health supplement after human trials.

*In vitro* and *in vivo* models were used to study the bioavailability of *D. bardawil* carotenoids. The *in vitro* study revealed that the percent bioavailability of β-carotene and lutein was 22 and 12% respectively after gastric and intestinal digestion. Further, the bioavailability of *D. bardawil* carotenoids and vitamin A conversion was studied in experimental rats using single oral dose and multiple doses (7 days), compared with the synthetic β-carotene treated group. The study revealed that maximum retinol conversion takes place within 4 hrs after single oral dosage. The β-carotene was present at detectable level in the serum after single oral dose of either *D. bardawil* biomass or synthetic β-carotene. The study also revealed that the accumulation of β-carotene in liver was observed at 8 hrs after oral dosage.

A high liver store of retinol and β-carotene was observed after 7day feeding trial. Enhanced accumulation of retinol and β-carotene in liver by 3-4 fold was observed in the experimental groups over the control group. The overall study indicated that, the liver retinol content (69-78%) and liver β-carotene (72-85%) was higher in both synthetic β-carotene and *D. bardawil* treated groups when compared to animals devoid of any carotene supplementation. *D. bardawil* biomass was efficient in terms of retinol formation and its accumulation in the liver. The bioaccessibility and bioavailability studies indicated that *D. bardawil* carotenoids are nearly effective in terms of carotene accumulation and retinol conversion in both serum and liver, when compared to synthetic β-carotene.

The protective effect of *D. bardawil* biomass was assessed using $CCl_4$ induced toxicity in rats. The study revealed that the carotene rich *D. bardawil* biomass ameliorated the toxic effects of $CCl_4$, indicating that it can be useful in scavenging free radicals. This study also reflected the beneficial attributes of *D. bardawil* and its

potential to modulate experimentally induced disease conditions during liver and kidney damage.

The investigation embodied in this thesis has largely addressed aspects of cultivation of indigenous strain of *D. bardawil,* nature of carotenoids, bioefficacy of *Dunaliella* biomass from the angle of utility as a source of carotenoid and safety aspects.

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## Appendices

### Publications

1. **Vanitha A**, Chidambara Murthy KN, Sakthivelu G, Ravishankar GA. 2007. Distribution of carotenoids, vitamin A in vital organs of rats fed with *Dunaliella bardawil* whole cells and synthetic β- carotene ***(International Journal of Biomedical and Pharmaceutical Sciences:*** 1(2): 168-171.

2. **Vanitha A,** Chidambara Murthy KN, Vinod Kumar, Sakthivelu G, Jyothi M Veigas, Saibaba P, Ravishankar GA. 2007. Effect of the carotenoid – producing alga, *Dunaliella bardawil*, on $CCl_4$ induced toxicity in rats. ***International Journal of Toxicology.*** 26:159–167.

*3.* **Vanitha A,** Narayan MS, Murthy KNC, Ravishankar GA. 2007. Comparative study of lipid composition of two halotolerant alga *Dunaliella bardawil* and *Dunaliella salina*. ***International Journal of Food Sciences and Nutrition,*** 58(5): 373-382.

### Papers under communication

1. **Vanitha A,** Sakthivelu G, Bhaskaran V and Ravishankar G.A. Uptake of natural β-carotene from *Dunaliella bardawil* biomass vis-à-vis Synthetic β-carotene and their conversion to vitamin A in rats.

2. **Vanitha A** and Ravishankar GA. Carotenoid biosynthetic gene expression and associated pigment profile during light response in the green alga *Dunaliella bardawil*.

3. **Vanitha A** and Ravishankar GA. *Dunaliella*: Biological significance and Nutraceutical applications (Review).

**Papers presented in symposia / conferences:**

1. **Vanitha A,** Murthy KNC, Sarada R, Mahadevaswamy M, Ravishankar GA. Antioxidant activity of carotenoids fractions of *Dunaliella salina.* **Poster presented at ICFOST 2002 held at CFTRI Mysore during 12th and 13th December 2002. C29, P-30.**

2. **Vanitha A,** Murthy K.N.C, Narayan M.S. Sarada R, Mahadevaswamy M and Ravishankar G.A. "Isolation, purification and characterization of carotenoids from *Dunaliella salina*" **Poster presented at IFCON-2003 held at CFTRI, Mysore during 5th to 8th December 2003, BC- 02.**

3. Murthy KNC, Sowmya PR, **Vanitha A**, Mahadevaswamy M, Sarada R., Ravishankar G.A**.** Flocculation technique- a simple process for harvesting halotolerent micro algae *Dunaliella.* **" Poster presented at National Symposium on Microalgal Biotechnology" held at Bharathidasan University, Thiruchanapalli during 11th to 13th March 2004, PP- 31.**

4. Murthy KNC, Mahadevaswamy M, **Vanitha A**, Sowmya PR, Sarada R, Ravishankar G.A. *Dunaliella* marine microalga: A source of protein and β-Carotene. **Poster presented at Colloquium on "Plant Proteins and National Nutrition Security" held at CFTRI, Mysore on 30th March 2004.**

5. **Vanitha A,** Sarada R and Ravishankar G.A. Mass cultivation of *Dunaliella bardawil* and evaluation of safety and efficacy of biomass as a rich source of natural β-carotene. Poster presented at **"The National Academy of Sciences, India 77th annual session and symposia on novel approaches for food and nutritional security" held at CFTRI, Mysore during December 06-08, 2007. FSN 149.**