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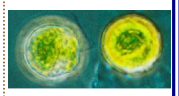

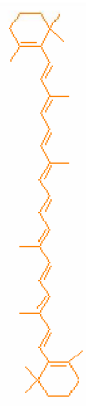

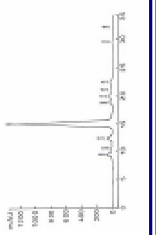

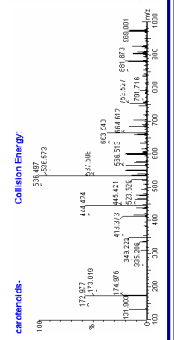

Thesis submitted to the
University of Mysore

for the award of
Doctor of Philosophy
in
BIOTECHNOLOGY

By

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DECLARATION

I hereby declare that this thesis entitled **"PRODUCTION OF β - CAROTENE FROM CULTURED *DUNALIELLA* sp. AND EVALUATION OF BIOLOGICAL ACTIVITIES"** submitted 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 me in **Plant Cell Biotechnology Department,** Central Food Technological Research Institute, Mysore, under the guidance of **Dr. G.A.Ravishankar** during the period January 2002 - December 2005.

I further declare that the results of this work are not previously submitted for any degree or fellowship.

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CERTIFICATE

I hereby certify that the thesis entitled **"PRODUCTION OF β - CAROTENE FROM CULTURED *DUNALIELLA* sp. AND EVALUATION OF BIOLOGICAL ACTIVITIES"** submitted by **Mr. K.N.Chidambara Murthy** 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 him in Plant Cell Biotechnology Department, Central Food Technological Research Institute**,** Mysore under my guidance during the period January 2002 - December 2005.

Date: **G. A. Ravishankar**

Place: **Mysore**-570 020 Research Supervisor

ACKNOWLEDGEMENT

I consider myself to be very lucky to be associated with my mentor Dr. Gokare Aswathanarayana Ravishankar, whose ever encouraging and highly positive approach has influenced and benefited me a lot. He has laid a very strong foundation for my research career. I express my deep sense of gratitude to him.

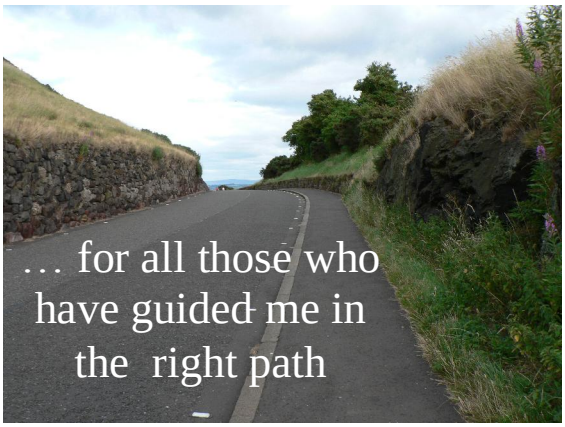
I am grateful to Dr.V.Prakash, Director, CFTRI, for providing me an opportunity to pursue my research work at CFTRI and the encouragement and support accorded during my work.

I place on record my sincere thanks to *Dr. Mallappa Mahadeva Swamy*, Retired scientist, Plant Cell Biotechnology Department for his invaluable support and encouragement during my tenure at PCBT, especially to deal with out-door cultivation in a comfortable fashion.
I am greatful to *Dr. Ravi Sarada* who gave me the cultures to start my research work and extended necessary help throughout my work.
My heartfelt thanks to *Dr. Bhagyalakshmi Neelwarne*, whose constant encouragement has benefited me immensely. I am ever grateful to *Dr. Mandayam Singara Narayan*, who was always more than willing to extend a helping hand during the hour of need. His suggestions have been very useful in this investigation.
I am thankful to the senior scientists of PCBT, *Dr. Thammanna Rajasekharan* and *Dr. Parvatam Giridhar,* for their kind support during this investigation. I also extend my sincere thanks to, *Kenchiah Shivanna*, *Yella Srinivasa Rao* and *Palaksha* who have been helpful in all ways during my tenure in the department.
I would like to express my special thanks to *Dr.Ravendra Pratap Singh*, Scientist, HRD, for introducing me to such a wonderful team of research. It is not an exaggeration if I said that he has been the guiding force behind my research career. I am extremely grateful for his constant support and encouragement throughout my stay at CFTRI.
I am grateful to all the scientists of CFTRI who have directly or indirectly helped me at all times.
I am thankful to all my friends, seniors and colleagues in the PCBT and other departments for their kind co-operation and support. I place on record my heartfelt thanks to all these people who made my stay at CFTRI a memorable one.
I gratefully acknowledge the co-operation from the staff of CIFS, HRD, FOSTIS, Pilot plant, Health center, Animal house and all the administrative departments at CFTRI during this investigation.
I would always be grateful to my uncle Sri.*K.S.Naganna* and my aunt Smt. *Anitha Naganna*, for being my guardians throughout my education. I will not be completing my duties if I am not extending my deep sense of gratitude to my parents, grand parents, brother, sister and all my relatives especially to my cousins Mr.*Guruswamy* and Mr.*Shiva kumar* without the encouragement and support of whom I would not have been what I am today.
Last but not the least, award of senior research fellowship by Council of Scientific and Industrial Research (CSIR), New Delhi is gratefully acknowledged.

(K.N.Chidambara Murthy)

LIST OF ABBREVIATIONS

µg	Microgram
µM	Micromolar
^{0}C	Degree centigrade
BHA	Butylated hydroxy anisole
Chl	Chlorophyll
D. bardawil	*Dunaliella bardawil*
D. salina	*Dunaliella salina*
DW	Dry weight
FW	Fresh weight
GC-MS	Gas Chromatography - Mass Spectroscopy
GLA	Gamma linoleic acid
HP	Horse power
HPLC	High Performance Liquid Chromatography
hrs	Hours
Kw	Kilowatts
Klux	Kilolux
L	Litre
LC-MS	Liquid Chromatography- Mass Spectroscopy
LDL	Low density lipoproteins
M	Molar
MALDI	Matrix Assisted Laser Desorption Ionization.
mg	Milligram
min	Minute(s)
mL	Milliliter
mM	Millimolar
MS	Mass Spectroscopy
OD	Optical Density
PCV	Packed cell volume
R_f/Rf	Retardation factor
Rt	Retardation time
rpm	Revolution per minutes
SOD	Super oxide desmutase
SD	Standard deviation
TLC	Thin Layer Chromatography
UV	Ultraviolet
v/v	Volume per volume
w/v	Weight per volume
w/w	Weight per weight


… for all those who
have guided me in
the right path

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1.0 General Introduction to algae.

1.1 History of algae.

History of algae is as old as that of plants. The first reference on algae was found in early Chinese literature and mentioned as 'Tsa'. It also appeared in Greek and Roman literature as *'phycos'* and *'Fucus '* respectively. While in the Latin language seaweed were designated by the term 'Algae'. Algae were used as food for long period, which was known by name ***'Limu'***. In the absence of microscope, no progress could be made to enhance the scientific knowledge on algae during the early centuries (Karagupta and Siddiqui 1995).

Table1. Eukaryotic algal division.

Division	**Organism**	**Major group of Alage**
Chlorophyta	*Chlorella vulgaris*	Green algae
	Chlamydomonas reinhardtii	
	Pcynacoccus provasoll	Parasinophytes
	Pseudossourfieldia marina	(Microbial green seaweeds)
Euglenophyta	*Ochromonas danica*	Euglenoids
	Astasia longa	
Chrysophyta	*Ochromonas danica*	Golden brown micro algae
	Fragillaria pinnata	Diatoms
	Prymensium parvam	Prymnesiophytes
	Yellow green micro algae	Eustigmatophytes
Pyrrophyta	*Prorocentrum micans*	Dinoflagellates
	Dinophysis	
Crytoptophyta	*Cryptomonas theta*	Cryptophytes
	Rhodomonas salina	
Rhodophyta	*Porphyridium umblicalis*	Red micro algae
Phaeophyta	*Ectocarpus, Fucus* sp	Seaweeds

(Adopted from Metting and Pyne 1986; Bhattacharya and Medlin 1998)

Apart from plant and synthetic sources, ocean is also one of the promising and potential sources of bioactive and pharmaceutically significant compounds. The reason for these marine organisms to produce these compounds is that they need to exist and survive in

fierce competitive environment. Some of the classical examples include antibiotics, antifungals and antimicrobial compounds.

The major pharmaceutical ingredients like, agar-agar, carrageenan, some of the antibiotics are also obtained from marine organisms. *Spirulina* is one such fresh water blue green algae known for pharmaceuticals, nutraceuticals, cosmetics and other applications. Various applications of microalgal forms are as shown in Figure-1.

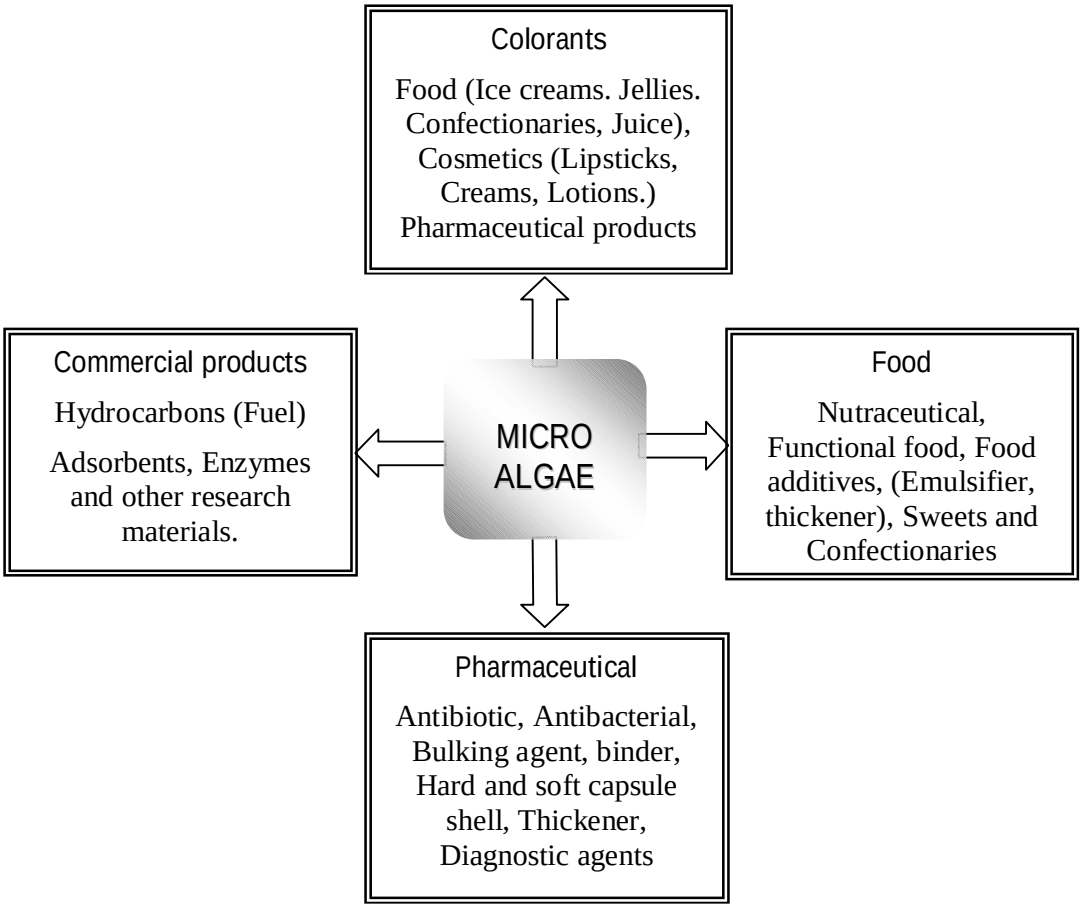


Figure 1. Micro algal forms as applicable in various fields.

1.2 Algae as source of nutrition and nutraceuticals.

Apart from their nutritional value, algal bioactive chemical compounds are known to have health promoting, disease preventing or medicinal properties. They are also referred to as functional foods, designer foods or pharmafood by different professionals. Total world market for nutraceutical in 2000 was US$ 38.9 million, 51.3 million and the same is expected to reach US$ 89.6 million in 2005 (Nutrition Business Journal Industry Overview 2001). This exponential growth indicates the demand and need for

nutraceuticals. The nutraceuticals that can be listed in A-Z fashion are as shown in Table-2.

Table 2. A-Z class of nutraceutical components.

Antioxidants and algae based products
Beverages and Bee based products
Carotenoids and Calcium rich products
Dietary fibers and Dairy products
Enzymes and Egg based products
Fiber diets and Fungi based products
Gelatin and Gum like products
Herb based products
Isoflavanoids and Iron rich products
Jellies and Juice (mineral and trace elements rich)
Kelp and other marine products
Lactoferrin and Lecithins
Meat proteins and Magnesium based products
Nut based products and Natural immune components
Oils - designer
Polyphenols and Pre and Probiotics
Q_{10} enzyme stumilating products
Resistant starches
Seaweed based products and Sugar alcohols
Tea and Tertiary butylhydroquinone
Unsaturated fatty acids
Vitamin based products
Wine and Whey proteins
Xanthophylls
Yogurt and its products
Zinc and its products

Increase in the consumer's health consciousness is leading to high demand in the market of nutraceuticals, hence there is a need for the search of molecules from known or newer

sources. In this connection algae particularly of marine origin have better scope in terms of both quality and quantity products,as sea represents more than 66 % of the earth. The major industries concerned with the nutraceuticals are, food, herbal and dietary and pharmaceutical industries.

Algal forms can serve as good source of nutraceuticals due to following reasons.

- θ Algae have short life cycle and can be multiplied fast to meet demand.
- θ Algae are easy to manipulate for desired components compared to plants.
- θ They are rich sources of all the vitamins and minerals.
- θ Available in natural habitat and also easy to cultivate.
- θ They have simple physiology and can be acclimatized to different environments.

Some of the commonly found nutraceutical components from algal forms include proteins of high quality, essential amino acids, antioxidants, vitamins, minerals, essential fatty acids and others.

Proteins: Most of the algal forms are rich sources of protein. Protein content in these algal forms range from (15-65 % w/w). They 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 ones. Protein content of well known micro and macro algal forms are as shown in Table-3.

Table 3. Protein content of different algal forms used as food ingredient.

Algal form	**% Protein content**	**Reference**
Microalgae		
Spirulina	55.0 - 66.0	Darcas 2004
Scenedesmus	45.0 – 65.0	Fabric 1970
Chlorella	54.0 - 63.0	Laguna et al., 1993
Nostoc	40.0 –42.0	Knubel et al., 1990
Haematococcus	17.30-27.16	Lorenz and Cysewski 2000
Dunaliella	16.0 –25.0	Liu et al., 2000
Macroalgae		
Porphyra	25.0 -35.0	Flurence 1999
Enteromorpha	29.2-35.6	Darcy-Vrillion 1993; Siva Kumar 2000
Kappaphycus	14.0 -21.0	Flurence 1999

Essential amino acids: The single alga can serve as a source of several essential amino acids. This is mainly because they produce all the amino acids required for the system. Amino acid composition of some of the edible algae are shown in Table-4.

Table 4. Amino acid composition of edible micro algal forms.

Amino acid	Amino acids content in g/100g (Values are mean of triplicates)				
	Spirulina	*Scenedesmus*	*Haematococcus*	*Porphyra*	*Gracilaria*
Isoleucine	6.7	3.6	0.79	4.4	4.1
Leucine	9.8	7.3	1.67	8.6	8.8
Valine	7.1	6.0	1.36	6.8	5.6
Phenylalanine	5.3	4.8	0.90	5.0	5.7
Tyrosine	5.3	3.2	0.52	2.5	2.3
Lysine	4.8	5.6	1.13	6.7	5.7
Metheonine	2.5	1.5	0.40	1.2	0.7
Cystine	0.9	0.6	0.19	-	-
Tryptophan	0.3	0.3	0.31	-	-
Threonine	6.2	5.1	1.04	6.2	6.1
Alanine	9.5	9.0	1.73	9.4	8.1
Arginine	7.3	7.1	1.07	5.1	4.7
Aspartic acid	11.8	8.4	1.89	13.3	12.2
Glutamic acid	10.3	10.7	2.19	13.7	12.6
Glycine	5.7	7.1	1.17	7.5	6.6
Histidine	2.2	2.1	0.61	3.2	2.9
Proline	4.2	3.9	0.89	4.9	5.1
Serine	5.1	3.8	0.94	5.7	5.3

(Adopted from Mayer and Gustafson, 2004; Guerin et al., 2003; Ichihara et al., 1999).

Antioxidants: Most of the edible algal forms are rich sources of one or the other antioxidant form. They accumulate high amount of antioxidant principles, since they have to survive in high stress conditions compared to higher plants. Some of the commonly found antioxidants are ascorbic acid, β-carotene, α- tocopherol, phycobiliproteins and other specific components. *Dunaliella* is an excellent source of

carotenoids and antioxidant compounds (Ichihara et al., 1999), *Enteromorpha* and *Kappaphycus alvarezzi* are good sources of ascorbic acid. Astaxanthin produced by *Haematococcus* is 5 folds more efficient as an antioxidant compared to β- carotene. *Scytosiphon lomentaria, Papenfussiella kuromo, Nemacystus decipiens* and *Porphyra* sp., used in Japan in various food products have shown to exhibit antioxidant properties measured in terms of superoxide desmutase (SOD), radical scavenging and hemoglobin induced linolenic acid model system.

Two famous algal species, *Caulerpa taxifolia* and *Caulerpa racemosa,* are good sources of antioxidant enzymes like, catalase, peroxidase, SOD and antilipid peroxidase (Kuda et al., 2005). Some of the algal forms well known for their antioxidant property are *Spirulina, Enteromorpha* and *Kappaphycus alvarezzi* (Fayaz et al., 2005).

Vitamins: Algae are also good sources of vitamins, they are known to contain both water soluble and fat-soluble vitamins. *Spirulina* is the only vegetarian source of cyanocobalamine (B_{12}). *Dunaliella* contains nature's highest carotenoid content, which is vitamin A precursor.

Enteromorpha and Porphyra are good source of ascorbic acid and *Kappaphycus alvarezzi* for vitamin C and E.

Minerals: Algae are the potential sources of minerals, they contain some of the biologically significant minerals like, calcium, zinc, selenium, cobalt, iodine, iron and potassium. They can also be fortified for essential minerals as they have tendency to take up these and are bioavailable for physiological benefits (Babu 1995).

Essential fatty acids: Most of the algal forms are rich sources of essential fatty acids and some contain high content of PUFA and ω-3 fatty acids. Algae of Rhodophyceae family contains 5.7 –24.6 mg g^{-1} of total lipids and 16:0, 18:1, 20:5, and n-3 and C_{20} PUFA are the major components of the lipid (Xiancui Li et al., 2002).

Phaeophyceae members have lipid content in the range of 2.5 –23.7 mg g^{-1} apart from the previously mentioned fatty acids they contain high percentage of 18:2 (n-6), 18:4 (n-3), 20:5 (n-3) and C_{18}, n-6 PUFA. Chlorophyceae members contain 7.2-12.0 mg g^{-1} lipids and they contain high percentage of 16:4 (n-3), 18:2 (n-6), 18:3 (n-3) and (n-3) PUFA (Levent Cavas et al., 2005). Green algae, *Tetrandrun minimum, Scenedesmus communis* and *Pediastrum boryanum* contains ω -hydroxy fatty acids (Xiancui Li et al., 2002). Six algae belonging to *Codium* species have shown the presence of 16:0, 18:1 ω

9, 18:3 ω3, 16:3 ω 3, 18:2 ω 6 and 20:4 ω 6 as major fatty acids. Total polyunsaturated fatty acid contents is reported to range from 17.2 to 54.4% (Blokker et al., 1998).

Another important fatty acid present in algae is gamma linoleic acid (GLA), which is of great significance in animal as well as human physiology. *Spirulina* is one of the richest sources of GLA among the microalgal forms. Nutritional benefits of some of the micro and macro algae are as shown in Table - 5.

Table 5. Nutritional benefits of some of commonly used micro and macro algal forms.

Algal forms	**Nutritional attributes**	**Reference**
Spirulina	High protein, essential amino acids, vitamin B complex and E, gamma linoleic acid, β-carotene	Xin-Qing Xu et al., 1998
Dunaliella	β - carotene, protein and glycerol	Johnson and Shubert 1986
Chlorella	Protein and minerals	Venkataraman et al., 1980
Scenedesmus	Protein and essential amino acids	
Kappaphycus	Protein, iron, α -tocopherol, ascorbic acid and β - carotene	Moore et al., 1988
Enteromorpha	Protein, ascorbic acid and iron	Moore et al., 1988
Porphyra	Protein, essential amino acids and vitamins	Moore et al., 1988

1.3 Algae as source of pharmaceuticals

The use of algae for therapeutic purpose has a long history (Glombitza et al., 1989). However systematic approach for the same was started in 1950's during which only *in vitro* studies were conducted. In 1970's the same was taken up for *in vivo* studies. Initial studies were focused on the macrophytes and only in 1980's the studies were taken on microalgae. These microalgae have certain advantages of cultivation due to shorter lifecycle, which can make them easy for utilization.

Some of the breakthrough researches in algae for medicine are,

« Heparins like sulphated polysaccharides from red algae, which 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.*

« Antiviral nucleotides from algae include idozuvidine, trifluridine, vivabaine and romovinyl dozyuridine (Damonte et al., 2004).

Structures of some of bioactive compounds are as shown in Figure - 2 and some of the bioactive compounds of algal origin are as shown in Table-6.

Table 6. Biologically active compounds from algae.

Algae	**Application**	**Reference**
Scenedesmus obligus	Antibacterial	Cohenz 1986
Asterionella sps	Anti viral and Anti fungal	Borowitzka, 1995
Chaetocerus and	Antifungal activity	Vilchenz et al., 1997
Fragilaria sps		
Sticocomus microbilis	Antiviral	Vilchenz et al., 1997
Chlorella sps	Antitumor	Pratt et al., 1944
Lyngbya majuscule	In Leukemia	Myndersa et al., 1977
Digenea simplex	Antibiotic	Richmond 1986
Plocamium cartilagenium	Antibiotic	Cueto et al., 1998

Major class of promising bioactive compounds from algal source include, antimicrobial, antiviral, anticancerous and other include anti-inflammatory agents, drugs acting on cardio vascular system and antihelmenthetics.

Antimicrobials: A number of micro algae have been screened for various antimicrobial activity, including antibacterial, antifungal and antiprotozoal (Pasado, 1990). Some of the compounds identified for this activity include fatty acids, glycolipids, acrylic acid, bromophenols, terpenoids and glycosides (Borowitzka 1995) . Antibiotics are the major class of the compounds obtained from algae.

Antiviral*:* Cyanobacterial extracts have shown activity against *Herpes simplex* virus type-II and also against respiratory syncytial virus at higher concentrations (Lau et al., 1993). A random screening of transcriptase of avian myloblastosis virus and human immuno- deficient virus type-I, 2.0 % have shown positive results, which mainly contain sulpholipids indicating that this is the possible molecule responsible for the activity.

Studies have shown that over 900 micro algal extracts screened for virus inhibition properties (Gustafson 1989).

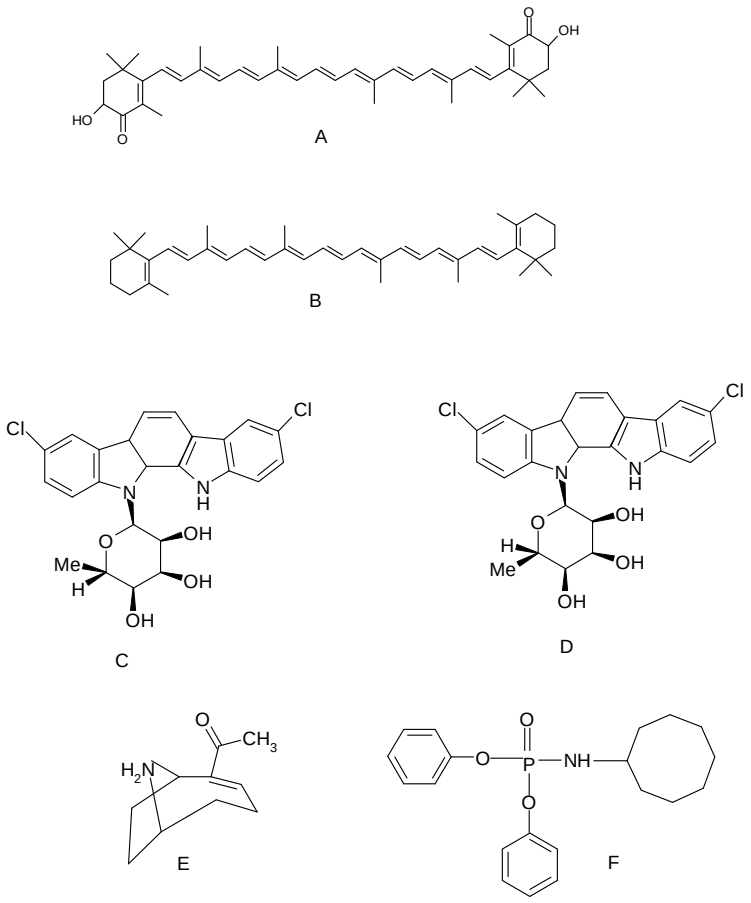
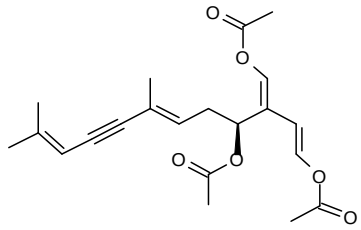


Figure 2. Bioactive Compounds of micro algal origin. A: Astaxanthin (*Haematococcus pluvialis)*, B: β-Carotene (*Dunaliella* sp.), C: Tijpanazole A1, D: Tijpanazole A2 (*Tolypothrix tjipanensis*), E: Anatoxin a (*Anabaena flos-aquae*) and F: PB-1 (*Ptychodiscus brevis*) (Borowitzka 1995).

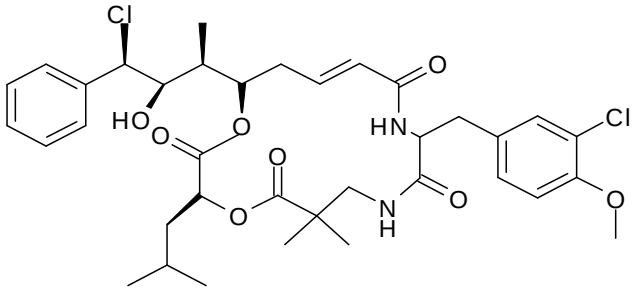
Anticancer*:* Cyanobacteria also produces a number of cytotoxic compounds namely, tubericidin and toyocamycin from *Streptomyces* (Patterson et al., 1991). Scytophycin B from *Scytonema pseudohoffmanni* has shown cytotoxic effect against KB (a human nasopharyngeal carcinoma) cell line at the concentration 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 Lewis lung carcinoma (Brachi et al.,

1984). *Spirulina* 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 anti cancer property exhibited by β- carotene and phycocyanin (Sude et al., 1986; Koto et al., 1984; Gerwick et al., 1994). Group of compounds from cyanobacteria known as cryptophytes and chrosophytes have shown promising anticancer activity (Helms et al., 1988).

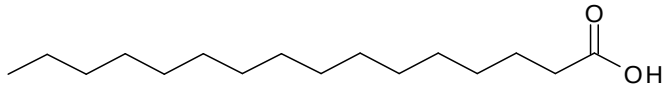
Around 250 marine compounds have been reported till date for anti tumor activity and more than 50 % of them are proven in pre-clinical trials. In 2000-01 itself around 30 compounds have been discovered from marine source. Among these caulerpenyne, cryptophycine, palmitic acid and mailiohydrin are of algal origin (Venkataraman et al., 1980) Structure of some of these are shown in Figure-3. Caulerpenyne is a sesquiterpene isolated form marine alga *Caulerpa taxifolia* and has shown antiproliferative activity in human neuroblastoma cell lines, as well as cytotoxic effect in several cell lines (Helms et al., 1988). 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). *Nostoc* a cyanobacterium is known to produce cryptophycine which is a depsipeptide, which can bind to microtubule end at vinca binding site to exhibit antimitotic activity (Morton and Bomber 1994).



Caulerpenyne



Cryptophycin-55



Palmitic acid

Figure 3. Structure of bioactive compounds of algal origin with antitumor and cytotoxic property. (Mayer and Gustafson 2004).

Other pharmacological activities: Some of the hydrophilic extracts of cyanobacteria have shown cardiotonic activity in isolated mouse atria (Borowitzka, 1995). Puwainaphycin C (*Anabaena* sp) and a cyclic peptide scytonemin A (*Scytonema* sp) have shown strong calcium agonist activity (Norton and Wells, 1982). Diatom *Nitzschia pungens* var. *multiseries* is a potential glutamate agonist (Laguna et al., 1993).

Brominated bi-indoles isolated from cyanobacteria *Rivularia firma* has shown anti-inflammatory activity and antihistamine activity tested in rat and mice models (Villar et al., 1992). Several species of cyanobacteria, chlorophyceae, dinophyceae and raphidophyceae have shown the angiotensin converting enzyme inhibitory activity (Benemann, 1990). *Dunaliella tertiolecta* was reported to exhibit antihypertensive, bronchodilator, antiseretonin, analgesic and anti oedema activity (Fox 1985), *Chlorella* has shown anti-dopaminergic activity (Ceferri 1983).

Some of the key factors for enhancing the active principles are bioactive compounds or secondary metabolites is by subjecting the cells to various physiological stresses like, nutrient, environmental and osmotic stress. Under such conditions algae will accumulate the secondary metabolites in stationary phase or in slow growing phase or vegetative phase. E.g. β- carotene in *Dunaliella* is produced under high salinity, high light and nutrient limitation. Thus when the cell growth is best the carotene content will be minimum. Nutrient limitations or stress is necessary for the production of bioactive principles. In some cases production of these compounds will also depends on growth phase and culture conditions Ex: In case of *Anabaena flos-aquae* (Utkilen and Golme 1992), environmental factors also play a vital role in metabolic production. Moreover lipid production and long chain polyunsaturated fatty acids production in diatoms is enhanced under low temperature. Microcystin production in *Microcystis oeruginosa* is enhanced under red or green light (Clament et al., 1967; Hauman 1981).

1.4 Algae as source of pigments.

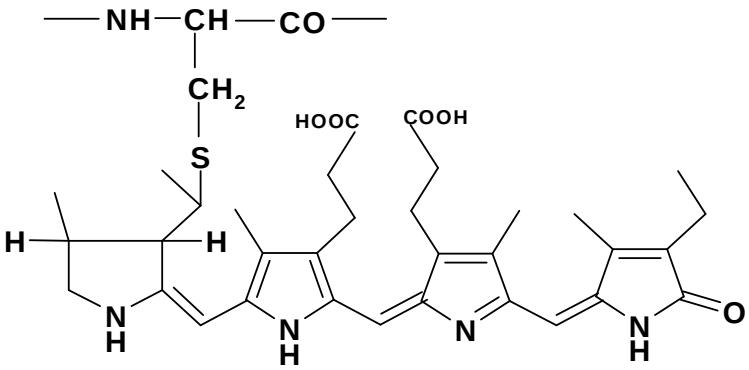
There is increasing demand for natural colors, which are of use in food, pharmaceuticals, cosmetics, textiles and printing dyes. However their utility is limited to few since the natural dyes have low tinctorial values and persistence. Due to the toxic effect of several synthetic dyes there is increasing preference to use natural colors for various end users. Only major problem of natural colors is the suststainability. Algae culture being ecofriendly and renewable, there is increasing affinity to use them as a source of natural

colors. Some of the sources of algal pigments are as shown in Table-7 and formulations available in market based on algal pigments are as shown in Table-8.

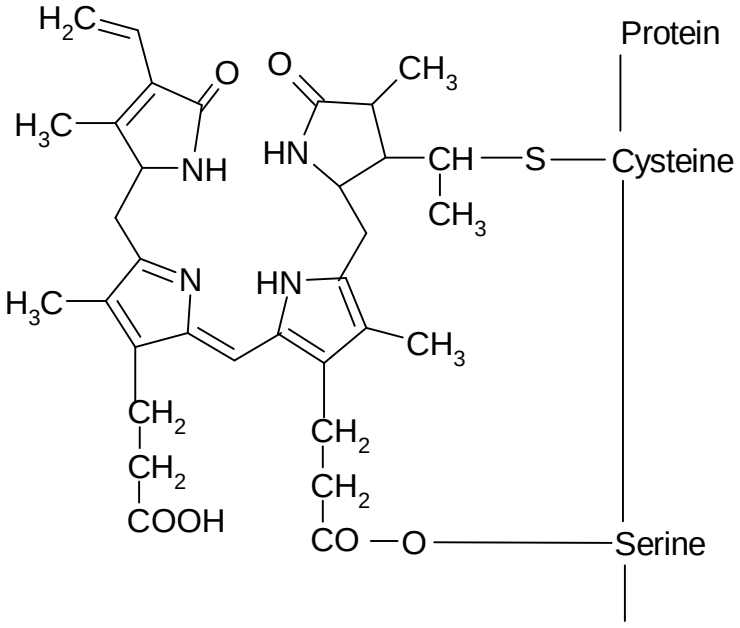
Major colors produced from algae, which are of commercial value, are phycocyanin (*Spirulina*), phycoerythrins (present in most of the seaweeds, Structure is given below Figure-4), astaxanthin (*Haematococcus*). β- carotene is also used as colorant in nutraceutical formulations.

Table 7. Pigments produced by algae and their application.

Pigment	**Algae**	**Applications**
Chlorophyll	*Chlorella, Spirulina*	Cosmetics, Antioxidant
Carotenoids	*Dunaliella* sp.	Pro vitamin-A, Food colorant, Bioactive compound.
Lutein	Mutants of *Dunaliella*	Food colorant, Antioxidant
Astaxanthin	*Haematococcus* sp.	Cosmetics, Coloring of fish Food colorant.
Phycocyanin	*Spirulina* sp.	Cosmetics, Diagnostic agent, Bioactive compound (anti cancer)
Phycoerythrin	*Porphyridium purpureum*	Cosmetics, Food colorant.



PHYCOCYANIN



R-PHYCOERYTHRIN

Figure 4. Structural formula of Phycocyanin and R-Phycoerythrin most used pigments of algal origin.

Table 8. Some of the market formulations containing micro algal pigments as one of the major component.

Formulation	Company	Intended use
Blue Manna Powder	Natural Zing, USA	Greater mental energy, attention, memory and focus. Emotional and mental balance Healthy joints and tissues
D and S control tablets	Max health Ltd., Hsi Chih, Taipei Hsien	Antiobesity
Glutamax	Max health Ltd., Hsi Chih, Taipei Hsien	Immunity booster
Alphastat (Astaxanthin)	Nature's Plus Rx-Formulations, USA	Food for healthy prostate
Marine Beta Carotene	Jarrow Formulations USA	Vitamin A deficiency, Immunity enhancer and antioxidant
Beta carotene soft gelatin capsules	Parry's pharmaceuticals, India	Antioxidant and vitamin supplement

1.5 Algal growth requirements.

Most of the research reports are available on the physiology and growth conditions for producing the compounds of interest from micro algal forms (Boussiba and Vonshak 1991, Lu et al., 1995). Major factors of significance in cultivation of algae are, nutrients, light, temperature and stress of various nature (Becker and Venkataraman 1982).

Nutrients: Nutrients required for growth of algae have been reported to be from organic and inorganic sources. In order to achieve optimum growth nutrients should be adequate in quantity (Kaplan et al., 1986; Borowitzka, 1988). Apart from carbon, hydrogen and oxygen, algae require additional compounds to grow, like nitrates, phosphates and sulphates, which are important for vital functions in algae. Carbon, nitrogen and oxygen

are provided either by inorganic salts (salts of carbonates and bicarbonates) or bubbling in the form of gas as in case of bioreactors. Some of the reported organic sources of nitrogen for algal cultivation are glycine, alanine, asparagine, aspartic acid, glutamine and succinamide (Sivasankar and Oaks 1996). Arnow et al., (1953) reported that citrulline, urea, arginine and ornithine can serve as source of nitrogen in *Chlorella*. Nutrient limitation will decrease the growth rate (Richardson et al., 1969; Parslow et al., 1984). Limitation of nitrogen leads to increase in the lipid content, and that of phosphorous leads to increased protein content in *Phaeodactylum tricornutum* (Youngmanitchai and Ward 1991). Nitrogen deficiency has been reported to cause chloroplast damage and size reduction in *Euglena gracilis* cells (Regnault et al., 1990). *Dunaliella* has shown to accumulate higher concentrations of carotenoids under nitrogen limitation (Ben Amotz 1983).

Glucose or acetate has been used as a source of organic carbon (Snoog 1980). Micro alga *Chamydomonas reinhardtii* was grown heterotrophically using acetate as carbon source by Chen and Johns (1994). Certain species of *Chlorella* could grow both autotrophically and heterotrophically in dark using organic substrates such as acetate, ethanol and glucose (Martinez and Drus 1991). *Spirulina* has been reported to assimilate glucose under mixotrophic conditions under varied intensity of light (Marquetz et al., 1995). Sodium bi carbonate is used as carbon source for *Spirulina* (Ceferri 1983) and *Scenedesmus obliquus* (Thielmann et al., 1990). Yield of biomass and growth are reported to be enhanced upon addition of CO_2 in case of *Tetraselmis suecica* (Fabregas et al., 1984), *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii* (Amoroso et al., 1998). Chen and Johns (1991) reported the effect of carbon/nitrogen (C/N) ratio on fatty acid composition of heterotrophically grown *Chlorella* sp. Enhanced C/N ratio has shown to enhance the accumulation of astaxanthin in *Haematococcus pluvialis* (Cifuentes et al., 2003).

Many algal forms require vitamins like, thiamin, cobalmine and biotin for their growth (Droop 1954; Pringshiem 1966). The actions of plant growth hormones like, auxins and others on growth and production of metabolites has not been studied, however an inhibitory action of 2,4-D on photosynthesis of *Chlorella* has been reported (Wedding et al., 1954). Bajguz and Czerpak (1998) reported the effect of brassinosteroids on the growth and carotenoid production in *Chlorella vulgaris.*

Light: Induces photosynthesis capacity of algae. Increases photosynthetic capacity of algae, Intensity, photoperiod and quality of light is of paramount significance to algae. Although most of the species grows well in continuous light, only few species are sensitive to continuous light (Brand and Guillard, 1981). Since light is the basic requirement for algal photosynthesis, enhancement of surface to volume ratio is necessary in order to maintain high growth rate, which is usually followed in tubular photobioreactors (Prit et al., 1979; Lee and Bazin 1990) in case of *Porphyridium creuntum* (Chaumount et al., 1988) and *Spirulina platensis* (Trozillo et al., 1986). High light intensity, leading to photoinhibition has been reported by many researchers in *Spirulina* (Chanawongse et al., 1994), *Anacystis nidulans* (Samuelson et al., 1985) and some green algae (Kyle and Ohad, 1986). Terry (1986) demonstrated enhancement of photosynthesis efficiency in various combinations of light and dark periods and this is supported by similar type of studies by Stitt (1986) and Laws et al., (1988).

Light plays a vital role in growth of *Dunaliella* as well as in carotenogenesis. Massive accumulation of β- carotene under high light intensity has been reported by Ben Amotz et al., (1989). Several kinetics as well as empherical models predicting the algal productivity as a function of light irradiance has been reported by Grobbelaar et al., (1984).

Temperature: Effect of extreme temperature has been studied in various algae (Volkman et al., 1991) Influence of temperature on growth is associated with various metabolic process such as photosynthesis, respiration, membrane permeability and uptake of ions (Soeder and Stengel 1974).

1.6 Algae cultivation methods.

Based on the nature of the organism and culture media composition, namely pH, salt content and structure of the algae they are cultivated using different systems. Two major class of cultivation are open-air cultivation and closed system cultivation. Most of the algae for commercial utility are grown by open-air method. Some algal forms can be easily cultivated using open-air system as the media composition and pH will not allow any contamination due to salt concentrations, like in case of *Spirulina*. However some algal forms cannot survive in open air conditions as pH and media composition may lead to contamination with other microbes ex: *Haematococcus*. In general, 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 enhancement of pH upon growth will make it less susceptible for contamination and hence it is possible to cultivate the same in open-air conditions. Some of the commonly used cultivation systems employed for algae are given below in Table 9.

Table 9. Utilization of cultivation techniques for different algal systems worldwide.

Type of tank	**Algae cultivated**	**Major Location**
Tanks	Most of the species	World wide
Extensive open ponds	*Dunaliella*	Australia
Circular ponds with rotating arm	*Chlorella*	Taiwan, Japan
Race way ponds	*Dunaliella, Spirulina,*	Japan, Taiwan, USA, Thailand,
	Chlorella	China, India, Vietnam, Chile, USA, Israel
Cascade system with baffels	*Chlorella*	Czech Republic, Bulgaria
Large Bags	Many species	World wide
Fermenters (Heterotropic)	*Chlorella* sp	Japan, Taiwan, Indonesia,
	Crypthecodinium cohnii	USA
Two stage systems	*Haematococcus*	USA

(Adopted from Borowitzka 1995)

1.7 Carotenoids

1.7.1 History of carotenoids.

Wackenroder first isolated carotene from carrots in 1831, seven year later Berzelius (1837) named the yellow pigments from autumn leaves xanthophylls. This opened the beginning of research on carotenoids. Since that time research on carotenoids is constantly in progress in the direction of chemistry, biology and pharmacology. However, recent research is focused more on the genetic aspect of these carotenoids for their best possible utilization as nutraceuticals and pharmaceuticals.

Carotenoids are lipid soluble plant pigments found in photosynthetic plants, algae and animal tissues. They also occur in photosynthetic bacteria, yeasts and molds. In nature about 700 carotenoids have been isolated and characterized. Among these more than 10 % are metabolized in the body to form vitamin A in different animals (Yeum and Russel 2002). Carotenoids provide wide range of colors to environment (Olson and Krinsky 1995). The well-known function of the carotenoids in plant system are giving characteristic color, protecting the plant against photosensitization and acting as protector against various free radical mediated damages.

1.7.2 Distribution of carotenoids.

The total carotenoids production in nature has been estimated to be approximately 100 million tons per annum by all the living organisms. They are distributed widely in a variety of living species like, fruits (pineapple, tomatoes, citrus, mango), flowers (narcissus, eschscholtzia), birds (flamingo, ibis, canary) insects (ladybird) and marine animals (salmanoids, crustaceans) and in most of the higher plants especially in leaf in association with chlorophyll (Gouveia and Emphis 2003). Carotenoids are found in the photosynthetic tissues of plant and animals in which many structural variations are found. It is considered as the only naturally occurring tetraterpenoids and very widely distributed throughout the living world. Carotenoids are synthesized *de novo* only by higher plants, mosses, liverworts, algae, photosynthetic and non-photosynthetic bacteria and fungi. All photosynthetic organisms contain carotenoids but are most often masked by chlorophyll present in chloroplast. The two pigments coexist because the carotenoids prevent the photodynamic sensitization of chlorophylls, which in their absence leads to destruction of the chloroplast (Goodwin 1979).

All the carotenoids in photosynthetic tissues are located in the grana of the chloroplast and consist of the same major group of pigments whatever the source. Major ones are β - carotene, lutein, violaxanthin and neoxanthin and smaller amount of α- carotene, β- cryptoxanthin, zeaxanthin and antheraxanthin. Xanthophylls are occur unesterified and upon senescence, when chloroplasts disintegrate, the xanthophylls released into the cytoplasm are esterified before they are oxidatively destroyed (Ladygin 2000).

Reproductive tissue: The first carotenoids isolated from anther was antheraxanthin and later it was found that it is present in trace quantity in all the green tissues. In flowers carotenoids are accumulated in chromoplasts, which are derived from chloroplasts. Flowers producing carotenoids are of three types viz., those containing highly oxidized pigments like furanoid oxide auroxanthin, hydrocarbons such as lycopene or β- carotene and highly species-specific carotenoids such as the retrocarotenoids-eschscholtzxanthin from *Eschscholtzia californica*. In some petals carotenoids concentration can be very high e.g. β- carotene represents 16.5 % of dry weight in case of *Narcissus poeticusrecurvis* (Goodwin 1979).

Fruits: In case of fruits they are divided into many groups based on the content viz., only traces of carotenoids, carotenoids similar to that of chloroplasts, lycopene and derivatives, large amount of β -carotene and its derivatives, very large amount of epoxides, pigments which are species specific, considerable amount of pro carotenes, secocarotenoids and apocarotenoids (Goodwin 1979).

Roots: The most important carotenogenic root is carrot containing β- carotene as major pigment along with xanthophylls constituting about 5.0 % of total pigments. Normal commercial strain contains about 60-120 μg of β -carotene per gram of fresh weight. Some sweet potatoes also contains significant amount of β- carotene. Roots of *Escobedia scabrifolia* (Family: Scrophulariaceae, grown in Northern America) is known to contain azafrin, an apocarotenoid (Goodwin 1979).

Bacteria: The photosynthetic bacteria differ from other photosynthetic tissue in accumulating acyclic pigments characterized by methoxy group at position C_2 and additional double bond at $C_{3,4}$ and keto groups conjugated to the conjugated double bond system. All these features are present in spheroidenone synthesized by *Rhodopseudomonas sphaeroides.* Chlorobactene is a aromatic ring containing carotene

produced from *Chlorobium* sp. Spirialloxanthin a carotene is present in non photosynthetic organism *Corynebacterium poinsette* (Jansen et al., 1965).

Fungi: Fungi are also known to contain carotenoids. Some of the carotenoids producing species are *Lycogala epidendron, Aleuria aurantia, Scutellinia scutellata, Neurospora crassa, Neurospora sitophila*. *Lycogala* is known to contain β- carotene, γ- carotene, lycopene, torulene. *Aleuria* contains same carotenoids except torulene. *Scutellinia* contains γ - carotene torulene and xanthophylls. Both species of *Neurospora* contains phytofluene, ζ- carotene and neurosporene apart from carotenoids mentioned above (Jansen et al., 1965). Another fungus *Epicoccum nigrum* is known to produce β-carotene (32%), γ- carotene (22%), rhodoxanthin (40%), torularhodin (6%) (Sassu and Foppen 1967). Lycopene cyclase which is a key enzyme in biosynthesis of β -carotene was isolated and characterized from fungus *Erwinia uredovora* and it is reported to be of 43 kD molecular mass (Schnurr et al., 1996).

Algae: Almost all classes of algae contain one or the other type of carotenoids, major being β-carotene and xanthophylls. Algal carotenoids are present in chloroplast as a complex mixture that is the characteristic of each class of algae. The red alga Rhodophyta has α and β -carotene and their hydroxylated derivatives (South and Whittick 1987). Chloromonadophyta contains diadinoxanthin, heteroxanthin and vaucheriaxanthin. Chlorophyta are characterized by acetylenic carotenoids namely, alloxanthin, monadoxanthin and crocoxanthin (Cheng et al. 1974). *Spirulina* is known for accumulation of β -carotene of upto 0.8-1.0 % w/w. *Dunaliella* is the highest carotenoid producing organism among the algae and other organisms. Commercially cultivated species are *Dunaliella salina* and *D. bardawil*. Other carotenoids containing algal species include *Haematococcus and Chlorella.*

1.7.3 Chemistry of carotenoids.

Carotenoids are hydrocarbons containing C_{40} polyene chain backbones. The linear chain is cyclized at terminals, the molecule with hydrocarbon terminal are known as carotene and that of oxygenated terminals are known as xanthophylls (Goodwin 1980b).

Those carotenes with at least half of the β- carotene molecules i.e. an unsaturated ionone ring having 11 carbon polyene side chain are classified as metabolic precursors of vitamin A. Each carotene can occur in 282 different geometrical isomers. Most of the carotenoids can be described by the general formula $C_{40}H_{56}O_n$, where n is 0-6.

Hydrocarbons (n= 0) are termed as carotenes. According to International Union of Pure and Applied Chemistry's (IUPAC) recommendation semi-synthetic names should have symbols like, β,ε - and β,β - carotene.

Beta-carotene is a $C_{40}H_{56}$ molecule containing hydrocarbon cycle at terminal. It consists of eight isoprenoid units joined in such a manner that the joining of isoprenoid units at the middle is reversed at the center of molecule so that 2 central methyl groups are at $C_{1,6}$ position and remaining non-terminal methyl groups are at $C_{1,5}$ position (Goodwin 1980a). This long chain is formed by hydrogenation, dehydrogenation, cyclization and oxidation.

The rule for naming carotenoids was published by IUPAC in 1975. Zechmecster (1962) first reported stereochemistry of carotenoids. It was also reported that mainly there are two different forms namely *cis-trans* or E-Z isomers. These have different activities, based on the number of double bond isomers exists.

The first total synthesis of β-carotene was also in 1950. However, the industrial large-scale synthesis was done in 1954.

There are two basic methods in synthesis of C_{40} slice, by joining two smaller molecules. symmetric, $C_{16} + C_8 + C_{16} = C_{40}$ and asymmetric / unsymmetrical, $C_{25} + C_{15} = C_{40}$

The most popular industrial methods are given below:

i). Badische Aniline and Soda – Fabric (BASF) synthesis

Here β-retinyltriphenyl phosphonium chloride and retinal were made to react using KOH and methanol to form β-carotene (Figure-5).

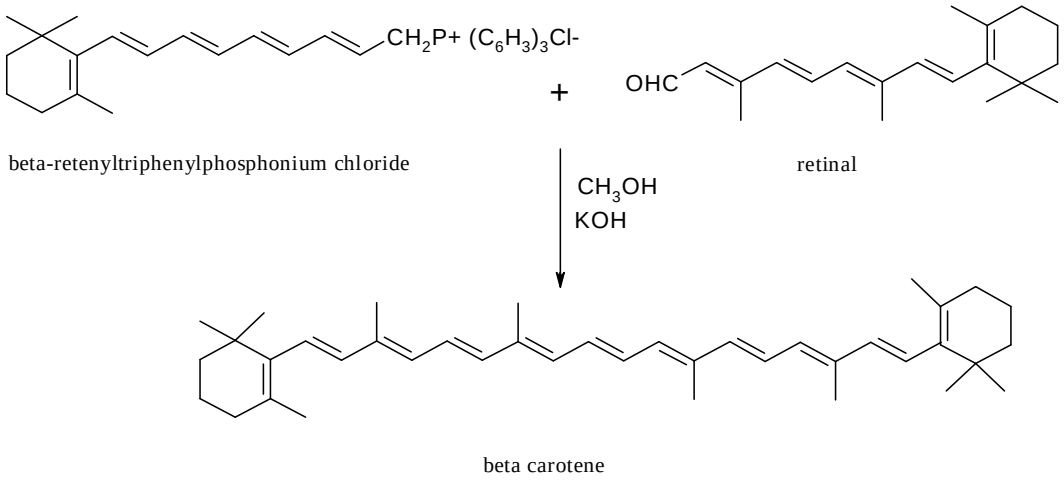


Figure 5. Badische Aniline and Soda – Fabric (BASF) synthesis of β - carotene.

ii). Roche synthesis

This is based on Grignard reaction. In this method two molecules of β -C_{19} aldehyde are made to react using acetylene dimagnesium bromide to form C_{40} diol, which further converts to 15-15′ didehydro-beta-carotene, they will be converting to 15′ β -carotene and all-*trans* β-carotene which takes very few seconds (Figure-6).

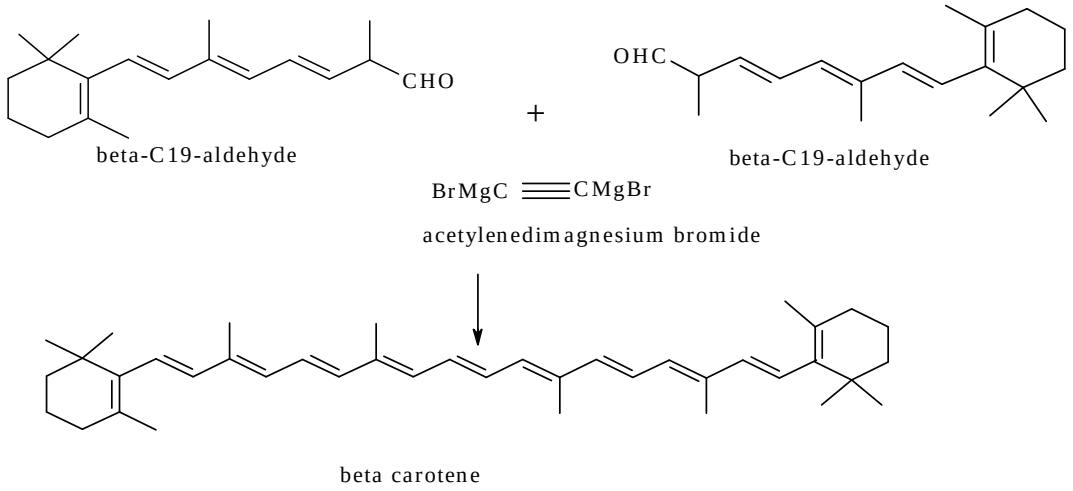


Figure 6: Roche synthesis of β - carotene by Grignard reaction.

Biologically *cis* isomers are highly efficient in converting to vitamin A due to high polarity and the activity is good in contrast to *trans* (Bertone et al., 2001). Therefore there are many ways by which *tans* isomers can be converted to *cis* form including cooking of vegetables. The important ones that occur in natural process or can be induced by external influence are heat induced and light induced process.

1.7.3.1 Stereochemistry of carotenoids.

Beta-carotene occurs naturally as all-*trans* beta-carotene and 9-*cis* beta-carotene, and to a lesser extent of 13-*cis* beta-carotene. Synthetic beta-carotene consists mainly of all-*trans* beta-carotene with smaller amounts of 13-*cis* beta-carotene and even smaller amounts of 9-*cis* beta-carotene. Dietary intake of beta-carotene in the American diet ranges from 1.3 to 2.9 milligrams daily. The consumption of five or more servings of fruits and vegetables per day, which is recommended by a number of federal agencies and other organizations, including the NCI (National Cancer Institute) would provide three to six milligrams of beta-carotene daily.

Vitamin A and more than 600 carotenoids have been crystallized and fully characterized by a variety of chemical and physical methods. Furthermore, vitamin A and many of its

analogs, as well as selected carotenoids, have been synthesized chemically from simple and readily available precursors. Because of the structure of conjugated double bonds that are characteristic of both vitamin A and carotenoids, these substances are sensitive to oxidation. Vitamin A is now considered, chemically, as a subgroup of the retinoids, which are defined as a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner and customarily containing five conjugated double bonds (Olson 1993).

Carotenoids are rich in conjugated double bonds, each of which can theoretically undergo isomerization to produce an array of mono or poly-*cis* isomers. Introversion is thought to occur with the absorption of light, exposure to thermo energy or by participation in specific chemical reaction. In most foods carotenoids occur in *trans* configuration, which is the most thermodynamically stable form (Clinton 1998).

The structure of the carotenoids is what determines the physical properties, chemical reactivity and biological properties or pharmacological behavior of compounds. Upon biosynthesis by plants and microorganisms, each carotenoid is incorporated into very precise orientations within sub cellular structures of the photosynthetic apparatus (Demming-Adems et al., 1996). Most of the time, chemical and physical properties of the carotenoids are strongly influenced by other molecules within the microenvironment, particularly associated with proteins and membrane lipids. In turn carotenoids can also influence the properties of the sub cellular structures. The chemical features of each carotenoids like, size, shape, hydrophobicity and polarity, determine its ability to interact and function in plant, bacteria or fungus. Hence behavior of each carotenoid in biological system is governed by these properties (Stahl et al., 1992).

1.7.4 Carotenoids as nutrient.

A number of nutritional benefits have been reported from β - carotene. Most of them are as source of Vitamin A and they are correlated with biological properties. Most common and well-known properties include pigment synthesis required for the vision, maintenance of skin properties by protecting against UV- light and so on. Other role of Vitamin A include protein synthesis, RNA synthesis, cell division, cell membrane stability, mucous production, sexual and reproductive process, sperm production, egg development, function of adrenal and thyroid glands, which in turn maintains metabolic rates, energy level, body temperature and growth rates, skin integrity, functions of inner lining of digestive tract, bone development, remodeling and liver functions.

1.7.5 Biological activity of carotenoids in plants.

Carotenoids are known for their biological activity due to their capacity to transfer energy in photosynthesis and photoprotection (Krinsky 1994b). Carotenoids play vital role in photosynthesis in association with chlorophyll. The major functions of carotenoids include accessory pigments in light harvesting system and as photoprotective agents against oxidative damages. They absorb the visual light and act as photo protector by forming complexes with chlorophyll and proteins known as photosystems (PS I and PS II). β- carotene involves in PS I and Lutein in PS II (Armstrong and Hearst 1996; Cogdell and Gardiner 1992). These utilize the energy absorbed by direct sunlight for various biochemical conversions and inturn protect the plant forms. Similarly when there are free radicals like, singlet oxygen, hydroxyl radical, it will be utilized for inter conversion of carotenoids. Some of the examples are, increase in astaxanthin content as a protective mechanism when *Phaffia rhodozyama* is exposed to singlet oxygen (Schroeder et al., 1996). When leaves are exposed to high light, conversion of various xanthophylls takes place. Violoxanthin, upon losing epoxy xanthophylls forms antheraxanthin followed by zeaxanthin. Therefore number of carotenoid molecules will be more in light exposed plant when compared to those grown in dark (Olson and Krinsky 1995).

1.7.6 Pharmacology of carotenoids.

The efficiency of absorption of beta-carotene is highly variable. The efficiency of absorption of beta-carotene from carrots and other beta-carotene containing raw foods is less than 5%. Carotenoids are absorbed from the intestine with the aid of dietary fat and incorporated into chylomicrons for transport in the serum (Rapola et al., 1998). On the other hand, the efficiency of absorption of beta-carotene from beta-carotene containing nutritional supplements can be as high as 70% or more. In foods, beta-carotene exists either as a solution in oil (e.g., red palm oil) or as part of a matrix within the vegetable or fruit. For example, in carrots, beta -carotene exists in a complex matrix, comprised of indigestible polysaccharides, digestible polysaccharides and protein.

Absorption: Absorption and metabolism varies to great extent in animal species. In case of humans, carotenoids can be absorbed intact by mucosal cells and subsequently appear unchanged in circulation and peripheral tissues (Bowen et al., 1993). In some of the rodents and animals β -carotene and other provitamin -A carotenoids are metabolized

into vitamin A in intestinal mucosal cells. If not it will not be absorbed in the body hence plasma concentration is very low and not comparable with that of human beings. Different proportion of carotenoids present in food and supplements are taken up by intestinal cells and are metabolized to vitamin A in the process of absorption in humans. This complicates the interpretation of plasma carotenoids (Parker 1996). Absorption of carotenoids takes place in the intestinal mucosa and uptake of these compounds by duodenal mucosal cells appears to be by passive diffusion, with a concentration difference between mixed micelle and that of cell membrane and this decides the rate of diffusion (Parker 1996). After passive diffusion into enterocytes, unmetabolized carotenoids are incorporated into chylomicron and secreted by the lymph, followed by uptake by liver and release into circulation in association with very low density lipoproteins (VLDL) and ultimately in association with low density lipoproteins (LDL) (Erdman et al., 1993). Generally two peaks are observed in plasma analysis of β-carotene first one for the chylomicron associated β- carotene and later for the β -carotene associated with LDL. Retinol formed in enterocytes is secreted in lymph chylomicra as retinyl ester, where as retinoic acid and other polar metabolites exit the intestinal tissue through the portal circulation. It is hypothesized that carotenoids may bind to cytosolic protein and this may play a role in the intracellular transport of these compounds in intestine or liver (Rock 1997). Factors which affect the absorption of carotenoids in general and β- carotene in particular are as shown in Table -10.

Only a small percentage of beta-carotene is released from the matrix during the passage of foods. The different structural features possessed by carotenoids account for selective distribution in organ tissue, biological activity and pro-vitamin A potency, or *in vivo* conversion to vitamin A. Due to the hydrophobic character, carotenoids are associated with lipid portions of human tissues, cells, and membranes. In general, 80-85% of carotenoids are distributed in adipose tissue, with smaller amounts found in the liver, muscle, adrenal glands and reproductive organs. Approximately 1% circulates in the serum on high and low density lipoproteins. The major serum carotenoids are β-carotene, α-carotene, lutein, zeaxanthin, lycopene and cryptoxanthin. Smaller amounts of polyenes such as phytoene and phytofluene are also present (Redlich 1999).

Table 10. Factors determining the absorption of carotenoids in human.

Dietary factors: Fat ↑, Soluble fiber ↑↓, Dosage of carotenoids administered ↓, Competitive interaction between carotenoids ↓.
Food forms: Location in plant tissue (Chromoplast are more available compared to chloroplast), Mild heat (cooking)↑, reduced particle size by process like, blending/ grinding↑.
Biochemical & metabolic: Isomeric forms ↑↓ , Large individual variability due to absorption and metabolic polymorphism.
Individual subject: Intestinal parasites↓, malabsorption syndrome↓, Vitamin A status ↑↓, Increase in gastric pH ↓.
↑- Increase, ↓ - Decrease, ↑↓ - Variable.

Distribution: Carotenoids in bloodstream are transported in association with the lipoproteins, with a distribution similar to cholesterol. Hence the plasma cholesterol concentrations are highly correlated with circulating carotenoids concentration using *in vivo* trials (Olson 1994). Approximately 75% of plasma carotenoids are associated with LDL and remaining will be distributed in VLDL and HDL. More polar carotenoids like lutein are associated with HDL and non-polar ones like, β- carotene, α- carotene and lycopene are associated with LDL. In case of human study, it has shown that circulating carotenoids concentration is lower in case of non-smokers compared to smoking individuals (Handelman et al., 1996). The inverse pattern of carotenoids concentration has been observed in case of alcohol consuming individuals (Brady et al., 1996).

Adipose tissue is the largest body pool for carotenoids. Serum concentrations are fairly constant and slow to change during periods of low intake. The estimated half-life is 11-14 days for lycopene, α-carotene, β-carotene, lutein and zeaxanthin (Santos et al., 1998; Santos et al., 1996) Evidence for the existence of more than one body pool has been published (Tavani and La Vecchia 1999).

Serum levels of carotenoids reflect lifestyle choices and dietary habits within and between cultures. Variations can be attributed to different intake, unequal abilities to absorb certain carotenoids, and different rates of metabolism and tissue uptake. Decreased serum levels occur with alcohol consumption, the use of oral contraceptives, smoking (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994; van Poppel 1993) and prolonged exposure to UV light (Hankinson et al., 1992).

Beta-carotene, from supplements, oils or foods is either solubilized in the lipid core of micelles (formed from bile salts and dietary fat) in the lumen of the small intestine or forms clathrate complexes with conjugated bile salts. Micelles and clathrate complexes deliver beta-carotene to the enterocytes. All of the beta-carotene isomers viz., all-*trans* beta-carotene, 9-*cis* beta-carotene and 13-*cis* beta-carotene are absorbed from the lumen of the small intestine into the enterocytes. Within the enterocytes, a fraction of all-*trans* beta-carotene is oxidized to retinal and then reduced to retinol. Retinol is then esterified to form retinyl esters. It appears that 9-*cis* beta-carotene is isomerized to the all-trans form before being released into the lymphatics. The principal enzyme involved in the oxidation of beta-carotene is called beta-carotene 15, 15' dioxygenase.

Beta-carotene and retinyl esters are released from the enterocytes into the lymphatics in the form of chylomicrons. Beta-carotene is transported by the lymphatics to the general circulation via the thoracic duct. In the circulation, lipoprotein lipase hydrolyzes much of the triglycerides in the chylomicrons, resulting in the formation of chylomicron remnants. Chylomicron remnants retain apolipoproteins E and B48 on their surfaces and are mainly taken up by hepatocytes and to smaller extent by other tissues. Within hepatocytes, beta-carotene is incorporated into lipoproteins. Beta-carotene is released into the blood from the hepatocytes in the form of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). In the plasma, VLDLs are converted by lipoprotein lipase to LDLs. Beta-carotene is transported in the plasma predominantly in the form of LDLs.

Storage: Adipose tissue and liver are the major tissue storage depots for the carotenoids, although carotenoid traces have been found in lungs, kidney, cervix, prostate and other tissues (Shimitz et al., 1991). High concentration of carotenoids are found in tissues rich in LDL receptors, like corpus luteum, adrenal tissue, testas probably resulting from nonspecific intake of lipoproteins. Some of the carotenoids are specific in absorption, like macula of eye is rich in lutein and zeaxanthin, but not in other carotenoids (Handleman et al., 1992). Some of the concentration is found in pineal gland but not in case of brain stem (Olson 1994). Difference in the distribution is also seen with respect to isomeric forms. In general, amount of *trans* exceeds that of *cis* in case of circulation in plasma, where as increase in the proportion of cis β- carotene is observed in peripheral tissue (Stahl and Sies 1993).

In general carotenoids tissue content is directly proportional to dietary intake. Hence carotenoids are used as biomarkers for assessing intake of vegetables and fruits. The

concentration of carotenoids will also increase in peripheral tissue on high intake apart from plasma. Deposition of carotenoids in stratum corneum of epidermis occurs with repeated high dose of carotenoids leading to yellowing of skin and the condition is referred as '*carotenodermia'*. Average resident time for β -carotene is 51 days as revealed from human isotope tracer study (Novotny et al., 1995).

Metabolism: Only those carotenoids with at least one unsubstitued β- ionone ring attached to an intact conjugated polyene structure from C-7 to C-15 can be metabolized into retinal, which is then reduced to retinol (Goodwin 1986). Because of this structural requirement β -carotene is the most important provitamin-A carotenoid. The basic central cleavage takes place by cytosolic enzyme 15, 15′ dioxygenase. Hence β -carotene will ideally be metabolized to retinal (Goodwin 1986). Excentric cleavage, which produces apocarotenol intermediates, is also demonstrated in human intestinal tissue (Krinsky et al., 1993). Research has shown that carotenoids are also converted into compounds other than vitamin A and these retinoid like metabolites may affect growth regulation and other cellular activities (Krinsky 1994). Metabolism also takes place in peripheral tissues, such as adipose tissue in human, lung and kidney of primate, bovine corpus luteum apart from intestinal mucosa and hepatocytes (Wang et al., 1991). Provitamin -A carotenoids are not converted into retinol unless there is need for Vitamin-A. The rate is proportional to the demand for vitamin A. β -carotene is known to have $1/6^{th}$ value of vitamin A, considering 33.0 % average bioconversion and absorption ability of carotenoids in comparison with vitamin A, whereas others are known to have $1/12^{th}$ the vitamin A value of retinol (Solomons and Bulux 1993).

Exact nature of excreting metabolites of carotenoids is not known, however it is assumed that they are similar to that of retinol and vitamin A. Liver cytochrome- 450 has shown to take part in the metabolism and it converts retinol and retinoic acid into polar metabolites (Ranet et al., 1996). Due to inefficient absorption of the compounds most of the carotenoids that are ingested will exit the human body in the feces (Rock et al., 1996).

Safety and toxicity: United State Food and Drug Administration has declared β- carotene as generally recognized as safe (GRAS) for the consumption in 1979, as food colorant and dietary supplement (Diplock, 1995). This was supported by various studies like, mutagenicity, embryotoxicity, teratogenecity of carotenoids (Bendich 1988). Except in case of ATBC (Alpha tocopherol and beta carotene study) and CARET (carotene and

retinol efficiency trail) where they found that beta-carotene is associated with increased risk of lung cancer in heavy smokers, it was considered to be completely safe in humans. Exact mechanism of increasing the risk of carcinogenesis associated with smoking and alcohol consumption is not known (Erdman et al., 1996). Excess consumption by β -carotene or other provitamin A carotenoids will not result in hypervitaminosis A as it is converted on demand of the body. But yellowing of skin or hyper carotenemia occurs with high dose of β- carotene (30 mg day^{-1}) for long period of time, and this will spontaneously reduce upon decrease in the carotenoids dose. Apart from these reports no serious side effect has been observed in any normal human subjects.

1.7.7 Reported pharmacological activities.

Among the pharmacological activities almost all are antioxidant mediated. Carotenoids are an integral part of membranes but xanthophylls are variable in their position. Therefore carotenoids are effective antioxidants if radicals are generated inside the membrane. Apart from this, carotenoids have a remarkable effect in the immune response and intracellular communication (Britton 1995; Charluex 1996; Honh and Sporn 1997). Many of the carotenoids including β-carotene show an efficient induction of Gap junction communication (these are water-filled pores, connecting the cytosol of two neighboring cells, allowing the exchange of low-molecular mass compounds). Induction and free radical scavenging are two different mechanisms, which act independently to prevent cancer (Stahl et al., 1997). Carotenoids are associated with LDL, where as xanthophylls are distributed equally between high and low-density lipoproteins. Carotenoids upon biotransformation convert to retinol, retinoic acid and other products.

Pharmacological activity in human can be classified as ,

- Vitamin A (Retinol)
- Antioxidant
- Immunomodulatory
- Anticancer
- Skin protection (sunburns and other mediated injuries)
- Heart disease prevention and prevention of cataracts and macular degeneration.
- Biological signaling.

***Retinol (Vitamin A)*:** Majority of biological activity of the β-carotene are retinal and other vitamin A analogs mediated. β -carotene is found to convert into vitamin A analogs

in intestine of human and rodents, which is a cleavage process mediated by β,β-carotene-15,15'-oxygenase (BCO) soluble in alkaline medium. It is the major component in the vision mechanism, where it synthesizes the pigment required for vision. It is responsible for maintenance of epithelial cells, control of mucous secretion and reproduction (Olson 1993: Radlwimmer and Yokoyama 1997; Taylor and Mayne 1996). Some of conditions in which vitamin A is essential for the body are, cystofibrosis, diahorrea, long term injury, liver disease, malabsorption, pancreatic disorder

***Antioxidant*:** Beta-carotene has been demonstrated to quench singlet oxygen (O_2^-), scavenge peroxyl radicals and inhibit lipid peroxidation. The mechanism of beta-carotene's antioxidant activity is not clearly understood. Some, but not all, studies have shown a difference in the *in vitro* activities of the beta-carotene isomers. One study showed that 9-*cis* beta-carotene, which is a naturally occurring form of beta-carotene, protected methyl linoleate from oxidation more efficiently than all-*trans* beta-carotene (Liu et al., 2000). Results from some human studies have shown improvement of measures of antioxidant activity (decreased copper-induced LDL oxidation), decreased DNA strand breaks and oxidized pyrimidine bases in lymphocytes, decreased serum lipid peroxide levels, decreased serum malondialdehyde (MDA) and increased erythrocytes copper/zinc-superoxide desmutase activity in those receiving relatively high intakes of beta-carotene. It is possible that *in vivo* antioxidant activity of beta-carotene is unlikely to be a consequence of its conversion to retinal (Keys et al., 1999).

Immunomodulatory activity: Beta-carotene has demonstrated immunomodulatory effects. In healthy male nonsmokers, beta-carotene supplementation (15 mg day^{-1}) was found to significantly increase the percentage of monocytes expressing the major histocompatibility complex class II molecule HLA-DR, to increase the expression of intercellular adhesion molecule-1 and leukocyte function-associated antigen-3 and to increase *ex-vivo* secretion of tumor necrosis factor (TNF)-alpha by blood monocytes. Beta-carotene supplementation has also been found to enhance natural killer cell activity in elderly men, to increase lymphocyte response to mitogens in healthy male cigarette smokers and to increase the CD_4 lymphocyte count in some subjects with AIDS. The mechanism of the possible immunomodulatory activity of beta-carotene is not known, however it is thought that the possible immunomodulatory activity may be independent of beta-carotene's role as a precursor of retinol (Lyn Patrick 1999).

Anti cancer: Beta-carotene has been found to inhibit the growth of some malignant cells, including human prostate cancer cells, *in vitro*. The mechanism of this activity is not well understood. The ability of beta-carotene to modulate the carcinogenic process, at least *in vitro*, may be due, in part, to its conversion to retinoids (Yeh 2003). Beta-carotene may act as a prooxidant when present in high concentrations in an oxidative environment such as the lungs of smokers in the advanced promotional stage of the neoplastic process. Supplemental beta-carotene is known to inhibit the absorption of the carotenoid lutein which itself may have chemopreventive activity (Bertram 2004). Beta-carotene is known to reprogramme the oncogene through antioxidant property by changes in phosphorylation and ultimately transcription.

Beta-carotene has shown anticarcinogenic activity in the case of prostate cancer in human studies. The mechanism of this possible anticarcinogenic effect is unclear (Redlich et al., 1999). A review of the postulated mechanisms of possible anticarcinogenic activity in certain circumstances explains that beta-carotene which is metabolically converted to retinoids will modulate gene expression of factors linked to differentiation and cell proliferation via retinoic acid, which in turn helps in cancer.

Beta-carotene may also modulate the activity of enzymes that metabolize xenobiotics. It is possible that antioxidant activity may result in prevention of oxidative damage to DNA and inhibition of lipid peroxidation as well as regulation of the expression of genes sensitive to the intracellular redox state that may be involved in carcinogenesis. Beta-carotene may modulate the gene expression of connexin- 43 (first gene expressed independently by carotenoids) resulting in the induction of gap junctions with a consequent inhibition of neoplastic transformations (Jian et al., 2005).

***Cardiovascular protection*:** Epidemiological studies and some, but not all, intervention studies suggest an inverse association between coronary artery disease and beta-carotene intake. The possible antiatherogenic activity of beta-carotene may be accounted for, in part, by its possible antioxidant activity. Humans supplemented with beta-carotene, but not lycopene, were found to have low-density lipoproteins that were less oxidized than controls using endothelial cell-initiated autoxidation (Olson and Krinsky 1995).

Biological signaling: Carotenoids of natural origin are known to influence the intracellular communication (Stahl et al., 2002). Lycopene and tocopherol have shown inhibitory action on the growth of human prostate carcinoma cell lines (Pastori et al., 1998). Lycopene is known to lower the cyclin D levels which in turn leads to decreases

of phosphorylation of the retinoblastoma protein leading to growth suppression (Nahum et al., 2001). β - carotene is known to facilitate gap junction communication. GJC (gap junction communication) will help in the enhancement of the regulation of cell growth (Trosko et al., 2000). Carotenoids are known for induction of phase I and phase II metabolic enzymes, which play a role in the detoxification of carcinogens (Stahl et al., 2002).

Other activities: Include hepatoprotection, antiageing, and protection against some forms of cancer in some populations. It may also play a role in protecting against heart disease in some. Beta-carotene has demonstrated positive effects in the immune system. Diminished beta-carotene status has been observed in subjects with non insulin-dependent diabetes, but supplementation with beta-carotene has so far produced no notable benefits in diabetic patients. The carotenoids have been found to increase the levels of phase II detoxifying enzymes such as glutathione S-transferase m (GST-m) and glutathione peroxidase. Benefits sometimes attributed to beta-carotene in the prevention of cataracts and age-related macular degeneration may actually be due to other carotenoids, notably lutein and zeaxanthin (Bone et al., 2003).

***Some of the findings from clinical studies*:** Six major chemoprevention trials have been reported in different parts of world including, intervention studies in China in cancer subjects (Bolt et al., 1971), skin cancer prevention studies (Greenburg et al., 1990), cardiovascular diseases and malignant neoplasm (Hennekens et al., 1996) and lung cancer (Omenn et al., 1996) and some of the findings of these are given in the table 11.

Table 11. Some of the clinical research findings of carotenoids in various disorders.

Activity	**Reference**
In smokers synthetic β-carotene is known to increase the risk of lung cancer	Santos et al., 1998 Albanes et al., 1996
All *trans* carotenoids can convert to Vitamin A (94 %)	Omenn et al., 1996
Before reaching blood stream most of *cis* carotenoids will be converted to *trans* form	You et al., 1996
Natural carotenoids possess beneficial activities compared to synthetic ones	Tamai et al., 1995 ; Bitterman et al., 1994
β - carotene is helpful in asthma induced by exercise	Ben-Amotz, et al., 1996
A clinical trial involving smokers has shown carotenoid with α - tocopherol will decrease the incidence of lung cancer	Neuman et al., 2000
Dietary β- carotene can scavenge nitrogen dioxide, thiol and sulfonyl free radicals	Albanes 1999
β-carotene along with a ascorbic acid and α -tocopherol is beneficial in prevention of cardiovascular disease	Everett et al., 1996
Risk of coronary artery disease (CAD) is associated with low serum levels of β-carotene in urban patients	Singh et al., 1995
β-carotene is known to reprogramme the oncogene	Schwartz et al., 1993

1.8 *Dunaliella*

Table 12. Taxonomical classification of *Dunaliella*.

Super kingdom	Eukaryota
Kingdom	Viridiplantse
Subkingdom	Phycobionta
Phylum	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	Dunaliellaceae / Chlorophyceae
Genus	*Dunaliella*

The genus *Dunaliella* consists of 23 Sub species. Some of the important and extensively found species are, *Dunaliella bardawil, D.salina, D.bioculata, D.teritolecta, D.parva, D. spec, D. granulata* and *D. primolecta*. Commercially cultivated strains for the production of natural carotenoids are *Dunaliella bardawil* and *Dunaliella salina.*

1.8.1 Organism and life cycle.

Dunaliella is an unicellular biflagellate alga with two equal and long flagella, contains one large cup shaped chloroplast, which occupies half of the cell volume. *Dunaliella* are generally ovoid in shape 4-10 μm wide and 6-15 μm long (Ben-Amotz, 1983). 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* cell responsible for its rapid change in shape and response to osmotic changes (Ben-Amotz and Avron 1981).

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) upto 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. Initially cells can grow in low salt concentration and

upon growth they can adapt to the 2-3 fold hyper tonic and hypotonic change in salt concentrations (Ben-Amotz and Avron 1983).

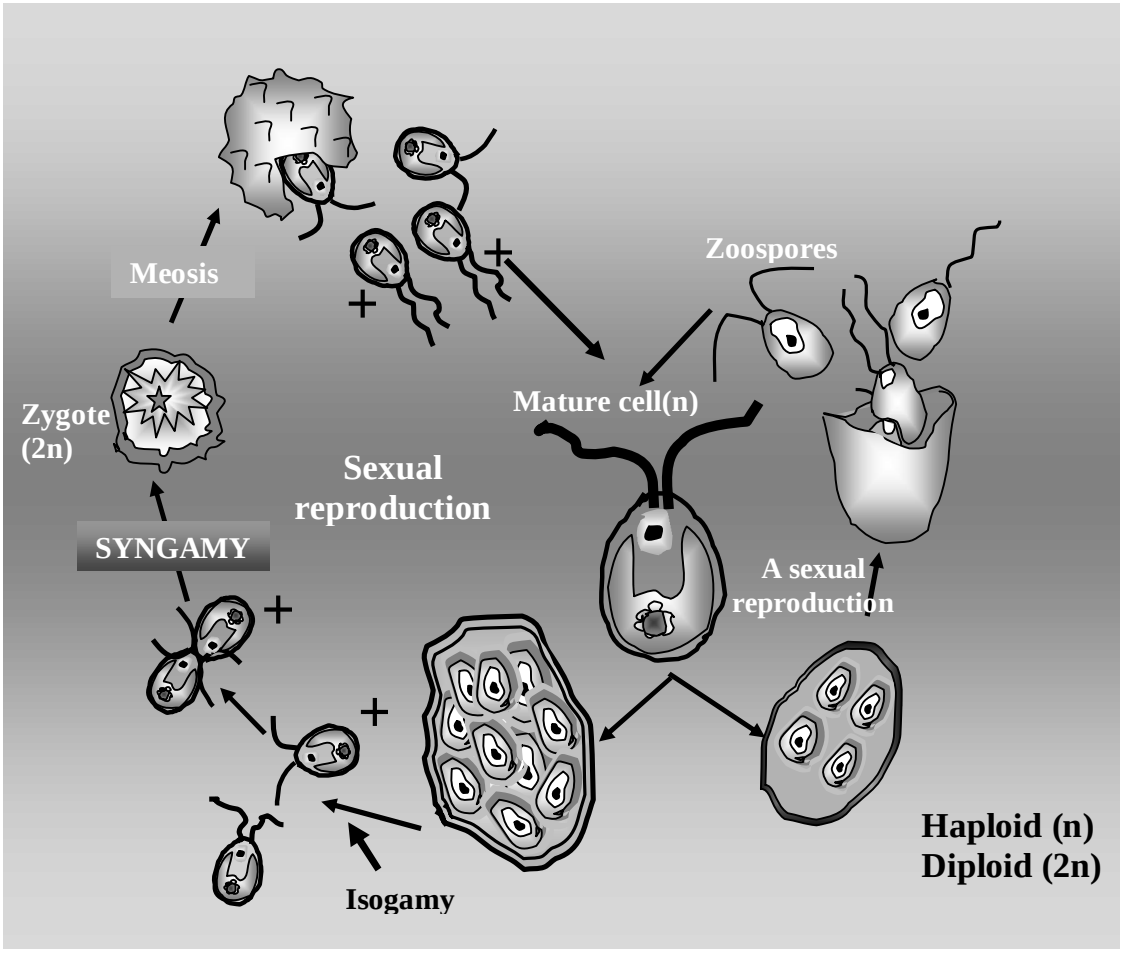


Figure 7. Cartoon showing different stages of reproduction in *Dunaliella*

1.8.2 Reproduction in *Dunaliella*.

Lack a rigid cell wall, and reproduce by longitudinal division of the motile cell or by fusion of two motile cells to form a zygote. This will undergo complex life cycle that includes, in addition to divisions of motile vegetative cells, the possibility of sexual reproduction. 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). She reported that the formation of zygote in *Dunaliella* can be induced by reduction in salt concentration from 10 to 3.0 % .

In the process of 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 upto 32 haploid daughter cells through a tear in the cell envelope. Lerche performed a series of experiment in which carotenoids rich red cells were crossed with green cells, enableling them to form zygote (Figure-7). Possibility of formation of asexual resting cysts by *D.salina* was explained by Hamburger (1905) and later Loeblich (1961) reported the formation of such cysts in media of reduced salinity. Some of the *Dunaliella* species can also develop a vegetative palmelliod stage consisting of round non-motile cells. Larche (1937) has reported this phenomenon under low salinity and Brock (1975) observed the same in benthic algal mats of Great salt lake, Utah.

1.9 *Dunaliella* cultivation, utilization and biological activity.

Number of research reports are available on the algae *Dunaliella*, most of which focus on carotenoids production and their biological activity for various health benefits both therapeutic and prophylactic. Some of those highlighting cultivations systems, carotenoids accumulation and biological activity are reviewed here.

Dunaliella bardawil has been reported to have higher content of β- carotene under high light of 1650 microeinsteins per square meter and the same was compared with white light of 27 microeinsteins. High light resulted in accumulation of β- carotene upto 10, upon illumination for 24 hours. Algae could accumulate upto 85 pg $cell^{-1}$ from 2.5 pg $cell^{-1}$ on exposure for 200 hours (Lears et al., 1990).

A study by Ben Amotz et al., (1988) reveals that exposing *Dunaliella bardawil* cells to high intensity white light during division cycle induces phytoene production up to 8% with a *cis* to *trans* β- carotene ratio of 1.0.

Large scale cultivation of two stage as described by Ben Amotz et al., (1995) consists of stage one for growth of cells for maximum production of biomass containing low β-carotene to chlorophyll ratio, followed by stage two in which culture is diluted to three volumes in order to induce carotenogenesis, which has increased production of β-

carotene to 450 mg m^{-2} d^{-1} compared to 200 mg m^{-2} d^{-1} in conventional single phase method.

Ben Amotz and Avron (1983) have reported various factors influencing accumulation of β- carotene in *D. bardawil*. Some of the factors are stress related like, high salt (upto 4.0 M), nutrient deficiency (sulphate and nitrate), light (continuous and diurnal cycle). Optimum carotenoids production was observed at 1.5 mM of nitrate, 2.0-2.5 mM sulphate and β- carotene to chlorophyll ratio of 13.1 was achieved at light intensity of 550 Kergs.Cm^{-2} Sec^{-1} intensity.

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 the condition. Light intensity of 20-50 μmol m^{-2} s^{-1} has promoted a high ratio of 9-*cis* to all *trans* β- carotene, the same was decreased when exposed to high intensity of light (Orset and Young 2000).

Rabbani et al., (1998) have shown that the synthesis of β- carotene is dependent on the triacylglycerol deposition in *D.bardawil.* Upon blocking triacylglycerol production it was observed that over production of β- carotene was blocked indicating the inter dependency. This also indicates that under normal growth conditions carotenoids pathway is not maximally active and the same can be appreciably stimulated in the presence of sequestering structures, creating a plastid-localized sink for the end product of the carotenoid biosynthetic pathway.

Jahnke (1999) has reported that ultraviolet A (320-400 nm) radiation can induce massive accumulation of carotenoids in *D.bardawil*. Supplementing various levels of UV-A radiation (38 μmol $m^{-2}s^{-1}$) during growth has shown to enhance the carotenoids: chlorophyll ratio by 80-310 %. The increase was observed in all the salinities ranging from 0.5 to 3.0 M and it was also confirmed that the effect was not due to UV-B (290-320 nm). Similar type of results were reported in case of *D.salina* under low and high light, effect of UV-A and UV-C was eliminated using potassium chromate solution (White and Jahnke 2002 ; Masi and Melis 1997).

For the first time Mokady et al., (1984) reported the safety of *D.bardawil* tested using rats for two generations,. A feed formulation containing 5.0 and 10.0 % of *Dunaliella* was fed to rats and observations were made up to three generations (12 months). Algae had no significant effect on the body weight of the animals in both the sexes. Feed

conversion efficiency of 2.7 was observed in these rats (ratio of food consumption to weight gain) against 2.54 of control diet (F_0). These rats have shown to have slightly enhanced level of serum biochemical markers (alkaline phosphatase and acid phosphatase) responsible for hepatoprotection compared to ones fed with normal diet.

Recently sub chronic toxicity of carotenoids of *Dunaliella* in F344 rats has been reported by feeding pigments for 90 days (Kuroiwa, et al., 2006). *Dunaliella* carotene were fed at the rate of 352, 696, 1420 and 2750 mg $kg^{-1}day^{-1}$ respectively, for males, and 370, 748, 1444 and 2879 mg $kg^{-1}day^{-1}$ for females using 0.63%, 1.25%, 2.5% and 5.0% in algal powdered basal diet. No symptoms of morbidity or any kind of abnormality were not seen during and after treatment. Increase in the platelet content was observed at higher dose i.e., 2.5 and 5.0 % based diets. Significant enhancement in total serum cholesterol and calcium content were observed in both the sexes. Based on the reports no-observed-adverse-effect-levels (NOAELs) was estimated to be 1.25% (696 mg $kg^{-1}day^{-1}$) for males and 5% (2879 mg $kg^{-1}day^{-1}$) for females. Results of these study indicate that the treatment of algae shows slight variation in some of the biochemical compositions, like calcium and cholesterol content. Safe concentration without any change is 2.5 % for male and 5.0 % in case of female (Kuroiwa et al., 2006).

Finney et al., (1984) reported the possible utilization of *Dunaliella* as a protein supplement in bread. They have used whole algae, carotenoids extracted algae, water-soluble fraction and water insoluble fractions for the purpose of bread making. One gram of protein and water-soluble fraction of *Dunaliella* have contributed to loaf volume equal to 10% of wheat flour. Green color was imparted in the final product in most of the case which can be removed if the same is not acceptable using suitable techniques employed for leaf extracts.

A study carried out by Giordano et al., (2000) revealed that sulfur depletion will influence the imbalance between carbon and nitrogen. This has shown abundance in alanine and there was variation in the regulation of enzymes responsible for the metabolism of carbon, sulfur and nitrogen. Adenosine 5' triphosphate sulfurylase activity increased by 4-folds, while the activity of nitrate reductase and phosphoenolpyruvate (PEP) carboxylase activities decreased by 4 - folds. Ben Amotz and Avron (1983) reported that the osmoregulation of algae is due to the metabolism of intracellular

glycerol in response to salt concentration of both intracellular and extra cellular, no leak of glycerol from cell was observed up to sodium chloride concentration of (0.6M).

Salt tolerance in *Dunaliella tertiolecta* is found to be via osmoregulation by producing glycerol. There was also enhancement of glycerol production upon increasing pH of the medium and glycerol dehydrogenase is the key enzyme for the process (Borowitzka et al., 1977).

Regulation of Lhcb gene responsible for initiation of carotenoids production as a response to the irradiance to high intensity light is reported (Webb and Melis 1995).

The carbohydrates were the only fractions to accumulate maximum under nutrient stress ranging from 57-10% of the organic fraction, however content of lipid increased with increase in the nutrient availability. Results of the study reveal that the *D. tertiolecta* has high capacity of changing its biomass productivity and biochemical composition (as protein) in semi continuous cultures, on the basis of nutrient concentration and rate of renewal (Fabregas et al., 1995).

Genetic recombination of algae *Porphyridium cruenturm* and *Dunaliella salina* by interphylum protoplast fusion was reported by Lee and Tan (1988). The same was confirmed by testing resistance to penicillin and erythromycin.

Reactivity of various carotenoid isomers of *Dunaliella* to oxygen radicals in different light intensity was reported by Jimenez and Pick (1993). It was found that all the oxidants used methyl viologen, H_2O_2, rose Bengal and 2,2'-azobis (2-amidinopropane) HCl (AAPH) could induce light mediated degradation of β - carotene and chlorophyll. It was also found that degradation of 9- cis β- carotene was faster compared to all trans β - carotene under oxidants, both in intact cells and extracted biomass. It was also shown that the ratio of degradation of chlorophyll and β- carotene remains constant.

Two-phase aqueous organic system has provided an effective methodology for enhancement of productivity of poorly water-soluble compound like carotenoids. Various solvents used were pentane to hexadecane in the order of carbon content, among these decane is found to be suitable for maintenance of viability of cells as well as production of carotenoids (Leon et al., 2003).

Ergosterol and 7-dehydroporiferasterol are reported to be the major sterols of *Dunaliella,* It contain phospholipids, glycolopids and neutral lipids as major class of lipids. Major fatty acids of algae are palmitic (31%), oleic (13%), linoleic (20%), and γ- linoleic (17%)

acid. It is also known to contain sterol peroxides (Sheffer et al., 1986). There was increase in the degree of fatty acids saturation which, reduces the fluidity and permeability of the microalgal membranes (Xin-Qing Xu and Beardall 1997).

Dunaliella has been employed as a medium for biotransformation of aromatic aldehydes viz., benzaldehyde, salicylaldehydes, methoxybenzaldehyde and mono and didichloro benzaldehyde into respective alcohols. *Dunaliella parva* has shown significant biotransformations compared to other five marine species used for the study (Hook et al., 1999).

Olmos Soto et al., (2002) have reported technique for molecular identification of β-carotene hyper producing strains of *Dunaliella* using species-specific oligonucleotides. This technique with the help of single-cell can be used as taxonomical markers at species levels and for phylogenetic characterization of natural population.

Role of Ca^{2+} on glycerol production was reported by Tsukahara et al., (1999). Results suggest that the calcium influx from extra cellular space via the stretch- activated $Ca2^{+}$ channels localized in the plasma membrane is essential for the transduction of osmotic signals in *Dunaliella* for the glycerol production.

Protein of 38-kD was identified and purified from β- carotene globules and the same was designated as carotene globule protein (Cgp). Induction of Cgp occurs parallel to accumulation of β- carotene in *D.bardawil* grown under different inductive conditions (Katz et al., 1995).

β-carotene of *Dunaliella* has shown beneficial effect on irradiance of whole body in experimental rats. Rats whole body was exposed to 4 Gy of irradiance followed by treating animals with *Dunaliella* rich in carotenoids equivalent to 50.0 mg Kg^{-1} b. w of carotene for one week. Both liver and blood was analyzed for content of β- carotene, retinal and metabolic products like, retinal ester, oxy carotenoids. Exposure to irradiance has decreased the level of β-carotene and retinal in liver compared to control animals indicating the protection effect of carotenoids by preventing oxidation at cellular level (Ben Amotz et al., 1996).

9-*cis* and *all-trans* stereoisomers of phytoene and phytofluene producing strains of *Dunaliella* were selected by treating cells with the bleaching herbicide norflurazon. Ratio of 1:1 was maintained (9-*cis*-to-*all-trans*) in serum as in case of diet, and the same

was reduced to 1:3 in liver, spleen and kidney. This was attributed to the stronger antioxidative effect of 9-*cis* phytoene over the *all-trans* isomer (Werman et al., 2002).

1.10 Cultivation in various geographical locations.

Dunaliella, was first identified by Dunal a French scientist in 1838 and he described it as unicellular biflagellate alga living in concentrated brines (Dunl 1838). It was reported to be growing near Montpellier, the costs of Meditaranian costs of France. Later in 19^{th} century, algae similar to the one reported by Dunal was observed by other biologist in hyper salinity lakes near Crimea (Butschinsky 1897), Algeria (Blanchard 1891), Lorrine, France (Florentin 1899) and Romania (Bujor 1900).

Nutritional requirement of *Dunaliella* was first studied in depth by Gibor (1956) and Johnson et al., (1968). They have found that salt requirement of *Dunaliella* depends upon the strain and it is in the range of 5-12 %. It was found that actual salt concentration in the environment from which strain had been isolated was always much higher than the salt concentration found to be optimal in laboratory conditions. From this it was confirmed that *Dunaliella*, which grew in marine conditions not necessarily means that it is optimal for cultivation. This lead to idea of cultivation of algae in simulated environmental conditions where it can perform still better and it need not struggle with competitors for existence. First quantitative occurrence of the algae was reported in lakes of Dead Sea, cell density of 4×10^4 cells ml^{-1} was observed on surface water (Kaplan and Friedmann 1970). It was also observed that high content of magnesium and calcium ions known for inhibition of *Dunaliella* (Bass-Becking 1931). It was also observed that temperature and pH play a major role in growth of the algae, small green motile cells known as 'chlorospores' use to turn to red motile cells known as 'erythrospore' in winter after dilution with rain and this formation was directly proportional to salt concentration (Nicolai and Bass Becking 1935). *Dunaliella* is one of the most environmentally tolerant eukaryotic organism known and can cope with a salinity range from seawater (= 3% NaCl) to NaCl saturation (= 31% NaCl), and a temperature range from <0 °C to >38 °C (Ginzburg 1987; Borowitzka and Borowitzka 1989).

Based on the above scientific observation and environmental utilization *Dunaliella* is mainly cultivated for commercial utility in Israel, Australia, USA and also in part of India and China, where the commercial production have begun very recently. In Australia algae is mostly exported and used in nutritional supplements and food processing.

Australia is the world leader in *Dunaliella* sales and technology, with two companies recording sales of $10 million per annum. Two major companies producing algae are Western Biotechnology Ltd. Perth, Western Australia (5 ha each production pond and total area of production is 50 ha) and Betatene Ltd. Melbourne, Victoria (production is much bigger than other company). They grow the algae in very large and shallow (approx. 20 cm deep) ponds constructed either on the bed of hypersaline coastal lagoon, or formed by artificially expanding a lagoon (Curtain et al., 1987; Borowitzka and Borowitzka, 1989). Production rate and nutrient requirement depends upon the environmental condition and varies throughout the year. Paddlewheel-driven raceway ponds are used in Israel and USA for the cultivation of *Dunaliella*. In California, aquaculturalists "rotate" farming *Dunaliella* with brine shrimp. The cultivation is highly preferred in the area where environmental conditions favors the growth for more than 8-9 months to make it economically viable (Oren 2005).

Alternate method is closed culture systems at which seems to be too expensive at this time. The advantages of closed systems such as the tubular photobioreactors are that contamination by other species of *Dunaliella* and protozoa can be virtually eliminated (Chaumont 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. This also reduces harvesting costs. On the other hand, closed systems require pumping of the culture for circulation, and *Dunaliella* species are very sensitive to shear damage. As well as this, there is also the high capital cost of these systems and a higher operating cost. Therefore open system is invariably preferred and is adopted in conditions favorable for *Dunaliella* as a source for carotenoids production.

1.11 Need for β- carotene for developing world.

Research indicates that Natural β- carotene possesses numerous benefits for the human body. Since it cannot be synthesized in human body, should be consumed from food and other ingredients. Human body converts β - carotene to Vitamin A via body tissues as opposed to the liver, hence avoiding a build up of toxins in the liver. Vitamin A is essential for the human body in that it assists the body's immune system and helps battle eye diseases, such as cataracts and night blindness, various skin ailments such as acne, signs of aging, and various forms of cancer (Agarwal and Rao 2000).

Food deficient of β- carotene, leads to deficiency of vitamin A as there are limited direct dietary sources of vitamin A. It plays an important role in the early embryonic development of all mammals, and in proper functioning of the immune system, the rod cells in the retina of the eye and mucous membranes throughout the body

Requirement of β- carotene plays a vital role in children. Some of the facts about vitamin A deficiency are,

- Roughly 400 million people in the world are at risk of Vitamin A deficiency.
- 100-200 million children were affected by Vitamin A deficiency in 2000 and recently it has reached 124 as reported by UNICEF.
- 1.0 to 2.5 million deaths per year of preschool children -upto 30% of total deaths in that age group - could potentially be averted by bringing Vitamin A deficiency under control worldwide (http://www.who.int/nut/vad.html).

In early 1980s, researchers studying Vitamin A deficiency in Indonesia observed that young children diagnosed with mild night blindness were at a significantly higher risk of dying from other diseases in the next three to four months. The greatest vitamin A deficiency occurs in South and Southeast Asia, where 70% of the children under five are affected (Gary 2000).

Recommended dietary allowance of vitamin A is around 800 μg for normal adult and 1300 μg for lactating woman. In case of children it range from 375-700 μg. Since 6 μg of β-carotene is considered as one retinal equivalent, the requirement in terms of β -carotene is 4.8 mg for adults, 7.8 mg for lactating mother and in the range of 2.25-4.2 mg for children. The same is not provided in the daily food in case of children of south and southern Asia and some of African countries due to poverty and related problems. In order to overcome these problems there is a need for renewable and sustainable source of β-carotene.

In this direction various scientific approaches have been attempted to produce β-carotene rich food ingredient of plant and microbial origin. Production of rice rich in β-carotene is one such successful biotechnological approach, which resulted in ‘golden rice’. Genetic engineering of some of vegetables, fruits as well as large-scale production of micro algae via biotechnology are some of success stories in this direction.

1.12 Biotechnological efforts to meet β- carotene requirements.

Carotenoids are intracellular components of plant cells and can be extracted into medium by fermentation process. In order to adopt this technology selection of biomass, amount of total carotenoids and in that quantity of interested carotenoids are factors which limit the process and make it economically not viable.

Other biotechnological approaches includes, increasing the biomass production containing β- carotene. This can be done in selected host but the amount of biomass enhanced remains only as academic interest as it will not be economically viable for commercial utility (Ausich 1997). Like all secondary metabolites biosynthesis of carotenoids in cells is governed by various enzymes. Use of recombinant technology can help in altering the levels and activity of enzymes, which in turn help to increase the productivity of carotenoids in plant cells. Utilization of this technique has some requirements like, availability of the gene responsible for the synthesis of carotenoids and availability of the host system, which have those genes incorporated and expressed in a stable manner through many generations of the cells (Ausich 1997).

Carotenoids are isoprenoids compounds, having seven biochemical conversion steps which are involved in conversion of precursor of isoprenoid unit 'acetyl CoA' into C_{15} molecule fernesyl diphosphate. All the genes involved in this have been isolated and characterized (Armstrong and Hearst 1996). The gene for conversion of fernesyl diphosphate to C_{20} compound geranylgeranyl diphosphate and then into first specific precursor of carotenoids phytoene have been isolated and characterized. A number of different carotenoids biosynthetic genes encoding enzymes that converts phytoene to lycopene, ζ- carotene, α-carotene, β- carotene and enzymes that further coverts β-carotene in to zeaxanthin, canthaxanthin, astaxanthin, capsorubin and capsanthin have been isolated and charecterized (Armstrong and Hearst 1996). Using these genes, various research have been undergone to develop protocols to clone and transfer to various hosts. *Phycomyces blacken* is a fungus which produces β- carotene, a protocol has been developed in which DNA from one strain could be introduced in a stable manner into different strains by micro injection (Clerda et al., 1993). Similar attempts have been successfully made to introduce DNA into the astaxanthin producing yeast *Phaffia rhodozyma* (van Ooyen and Van Ooije 1994). One which has seen maximum utility and enjoyed market among the genetic transformants of β- carotene is golden rice.

1.13 Golden rice and β - carotene.

The term "golden rice" was coined by a Thai businessman who is active in initiatives aimed at reducing the birth rate, a major cause of the food security problem (Potrykus 2001).

The first Golden Rice encodes phytoene synthase (PSY), which utilizes the endogenously synthesized geranygeranyl-diphosphate to form phytoene, a colorless carotene with a triene chromophore (Burkhardt et al., 1997; Ye et al., 2000). The second and more recent version all rely on the expression of two transgenes which encodes the bacterial CRT1, a carotene desaturase that introduces conjugation by adding four double bonds. This rice is known to contain upto 20-23 times more of β- carotene (Figure-8). The genes used for the creation of golden rice were, ***psy*** (photoene synthase), ***lyc*** (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).

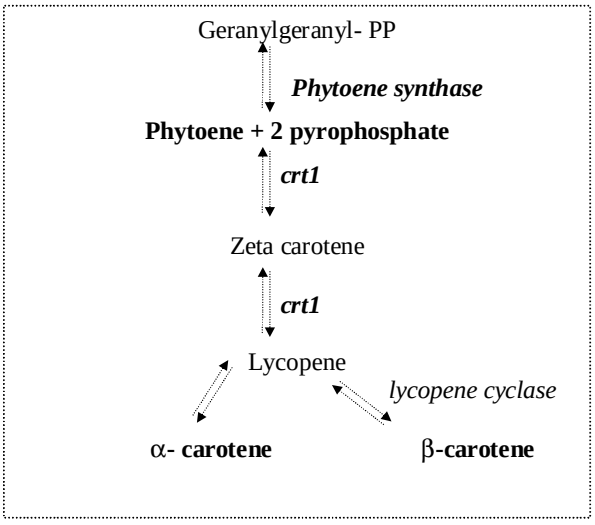


Figure 8. A simplified overview of the carotenoid biosynthesis pathway showing the enzymes expressed in the endosperm of golden rice.

Eventhough it is undergoing number of Intellectual Property Rights issue around the world, with an expectation to help vitamin A deficiency, agricultural aspects of cultivation of golden rice are under consideration for its effective utility.

Another biotechnological approach for effective production of β- carotene is through micro algae *Dunaliella,* which can produce highest amount of β- carotene along with other carotenoids under manipulated condition. Details of the cultivation and utility of algal is the aim of present research work.

In view of requirement in demand for β- carotene and possibility of utilization of algal source for large-scale production. This study was undertaken with the following objectives. The aspects of safety and efficacy of the product both biomass and isolated carotenoids is also borne in mind to add value to production and utilization of algal biomass in an eco friendly manner.

1.14 Objectives of the study.

- « Collection of germplasm of *Dunaliella* sp. from various sources.
- « Optimization of media, culture conditions, nutrient and environmental factors for the growth and production of β- carotene.
- « Identification of different carotenoids and isomers of β- carotene.
- « Downstream processing for the production of carotenoids and separation in various vehicles including edible oils.
- « Stability studies of various carotenoid fractions.
- « Antioxidant properties and bio-efficacy of various isomers of β- carotene using *in vitro* and *in vivo* models.

1.0 General Introduction to algae.

1.1 History of algae.

History of algae is as old as that of plants. The first reference on algae was found in early Chinese literature and mentioned as 'Tsa'. It also appeared in Greek and Roman literature as *'phycos'* and *'Fucus '* respectively. While in the Latin language seaweed were designated by the term 'Algae'. Algae were used as food for long period, which was known by name ***'Limu'***. In the absence of microscope, no progress could be made to enhance the scientific knowledge on algae during the early centuries (Karagupta and Siddiqui 1995).

Table1. Eukaryotic algal division.

Division	**Organism**	**Major group of Alage**
Chlorophyta	*Chlorella vulgaris*	Green algae
	Chlamydomonas reinhardtii	
	Pcynacoccus provasoll	Parasinophytes
	Pseudossourfieldia marina	(Microbial green seaweeds)
Euglenophyta	*Ochromonas danica*	Euglenoids
	Astasia longa	
Chrysophyta	*Ochromonas danica*	Golden brown micro algae
	Fragillaria pinnata	Diatoms
	Prymensium parvam	Prymnesiophytes
	Yellow green micro algae	Eustigmatophytes
Pyrrophyta	*Prorocentrum micans*	Dinoflagellates
	Dinophysis	
Crytoptophyta	*Cryptomonas theta*	Cryptophytes
	Rhodomonas salina	
Rhodophyta	*Porphyridium umblicalis*	Red micro algae
Phaeophyta	*Ectocarpus, Fucus* sp	Seaweeds

(Adopted from Metting and Pyne 1986; Bhattacharya and Medlin 1998)

Apart from plant and synthetic sources, ocean is also one of the promising and potential sources of bioactive and pharmaceutically significant compounds. The reason for these marine organisms to produce these compounds is that they need to exist and survive in

fierce competitive environment. Some of the classical examples include antibiotics, antifungals and antimicrobial compounds.

The major pharmaceutical ingredients like, agar-agar, carrageenan, some of the antibiotics are also obtained from marine organisms. *Spirulina* is one such fresh water blue green algae known for pharmaceuticals, nutraceuticals, cosmetics and other applications. Various applications of microalgal forms are as shown in Figure-1.

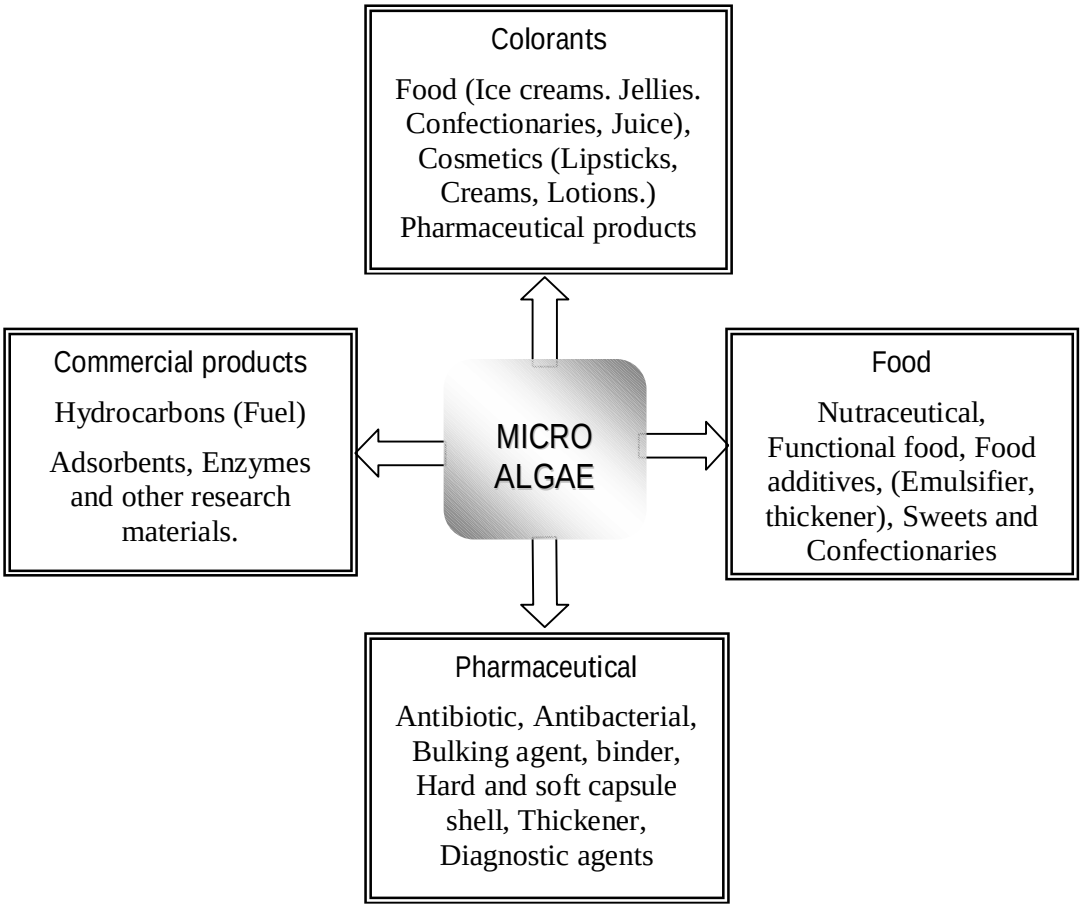


Figure 1. Micro algal forms as applicable in various fields.

1.2 Algae as source of nutrition and nutraceuticals.

Apart from their nutritional value, algal bioactive chemical compounds are known to have health promoting, disease preventing or medicinal properties. They are also referred to as functional foods, designer foods or pharmafood by different professionals. Total world market for nutraceutical in 2000 was US$ 38.9 million, 51.3 million and the same is expected to reach US$ 89.6 million in 2005 (Nutrition Business Journal Industry Overview 2001). This exponential growth indicates the demand and need for

nutraceuticals. The nutraceuticals that can be listed in A-Z fashion are as shown in Table-2.

Table 2. A-Z class of nutraceutical components.

Antioxidants and algae based products
Beverages and Bee based products
Carotenoids and Calcium rich products
Dietary fibers and Dairy products
Enzymes and Egg based products
Fiber diets and Fungi based products
Gelatin and Gum like products
Herb based products
Isoflavanoids and Iron rich products
Jellies and Juice (mineral and trace elements rich)
Kelp and other marine products
Lactoferrin and Lecithins
Meat proteins and Magnesium based products
Nut based products and Natural immune components
Oils - designer
Polyphenols and Pre and Probiotics
Q_{10} enzyme stumilating products
Resistant starches
Seaweed based products and Sugar alcohols
Tea and Tertiary butylhydroquinone
Unsaturated fatty acids
Vitamin based products
Wine and Whey proteins
Xanthophylls
Yogurt and its products
Zinc and its products

Increase in the consumer's health consciousness is leading to high demand in the market of nutraceuticals, hence there is a need for the search of molecules from known or newer

sources. In this connection algae particularly of marine origin have better scope in terms of both quality and quantity products,as sea represents more than 66 % of the earth. The major industries concerned with the nutraceuticals are, food, herbal and dietary and pharmaceutical industries.

Algal forms can serve as good source of nutraceuticals due to following reasons.

- θ Algae have short life cycle and can be multiplied fast to meet demand.
- θ Algae are easy to manipulate for desired components compared to plants.
- θ They are rich sources of all the vitamins and minerals.
- θ Available in natural habitat and also easy to cultivate.
- θ They have simple physiology and can be acclimatized to different environments.

Some of the commonly found nutraceutical components from algal forms include proteins of high quality, essential amino acids, antioxidants, vitamins, minerals, essential fatty acids and others.

Proteins: Most of the algal forms are rich sources of protein. Protein content in these algal forms range from (15-65 % w/w). They 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 ones. Protein content of well known micro and macro algal forms are as shown in Table-3.

Table 3. Protein content of different algal forms used as food ingredient.

Algal form	**% Protein content**	**Reference**
Microalgae		
Spirulina	55.0 - 66.0	Darcas 2004
Scenedesmus	45.0 – 65.0	Fabric 1970
Chlorella	54.0 - 63.0	Laguna et al., 1993
Nostoc	40.0 –42.0	Knubel et al., 1990
Haematococcus	17.30-27.16	Lorenz and Cysewski 2000
Dunaliella	16.0 –25.0	Liu et al., 2000
Macroalgae		
Porphyra	25.0 -35.0	Flurence 1999
Enteromorpha	29.2-35.6	Darcy-Vrillion 1993; Siva Kumar 2000
Kappaphycus	14.0 -21.0	Flurence 1999

Essential amino acids: The single alga can serve as a source of several essential amino acids. This is mainly because they produce all the amino acids required for the system. Amino acid composition of some of the edible algae are shown in Table-4.

Table 4. Amino acid composition of edible micro algal forms.

Amino acid	Amino acids content in g/100g (Values are mean of triplicates)				
	Spirulina	*Scenedesmus*	*Haematococcus*	*Porphyra*	*Gracilaria*
Isoleucine	6.7	3.6	0.79	4.4	4.1
Leucine	9.8	7.3	1.67	8.6	8.8
Valine	7.1	6.0	1.36	6.8	5.6
Phenylalanine	5.3	4.8	0.90	5.0	5.7
Tyrosine	5.3	3.2	0.52	2.5	2.3
Lysine	4.8	5.6	1.13	6.7	5.7
Metheonine	2.5	1.5	0.40	1.2	0.7
Cystine	0.9	0.6	0.19	-	-
Tryptophan	0.3	0.3	0.31	-	-
Threonine	6.2	5.1	1.04	6.2	6.1
Alanine	9.5	9.0	1.73	9.4	8.1
Arginine	7.3	7.1	1.07	5.1	4.7
Aspartic acid	11.8	8.4	1.89	13.3	12.2
Glutamic acid	10.3	10.7	2.19	13.7	12.6
Glycine	5.7	7.1	1.17	7.5	6.6
Histidine	2.2	2.1	0.61	3.2	2.9
Proline	4.2	3.9	0.89	4.9	5.1
Serine	5.1	3.8	0.94	5.7	5.3

(Adopted from Mayer and Gustafson, 2004; Guerin et al., 2003; Ichihara et al., 1999).

Antioxidants: Most of the edible algal forms are rich sources of one or the other antioxidant form. They accumulate high amount of antioxidant principles, since they have to survive in high stress conditions compared to higher plants. Some of the commonly found antioxidants are ascorbic acid, β-carotene, α- tocopherol, phycobiliproteins and other specific components. *Dunaliella* is an excellent source of

carotenoids and antioxidant compounds (Ichihara et al., 1999), *Enteromorpha* and *Kappaphycus alvarezzi* are good sources of ascorbic acid. Astaxanthin produced by *Haematococcus* is 5 folds more efficient as an antioxidant compared to β- carotene. *Scytosiphon lomentaria, Papenfussiella kuromo, Nemacystus decipiens* and *Porphyra* sp., used in Japan in various food products have shown to exhibit antioxidant properties measured in terms of superoxide desmutase (SOD), radical scavenging and hemoglobin induced linolenic acid model system.

Two famous algal species, *Caulerpa taxifolia* and *Caulerpa racemosa,* are good sources of antioxidant enzymes like, catalase, peroxidase, SOD and antilipid peroxidase (Kuda et al., 2005). Some of the algal forms well known for their antioxidant property are *Spirulina, Enteromorpha* and *Kappaphycus alvarezzi* (Fayaz et al., 2005).

Vitamins: Algae are also good sources of vitamins, they are known to contain both water soluble and fat-soluble vitamins. *Spirulina* is the only vegetarian source of cyanocobalamine (B_{12}). *Dunaliella* contains nature's highest carotenoid content, which is vitamin A precursor.

Enteromorpha and Porphyra are good source of ascorbic acid and *Kappaphycus alvarezzi* for vitamin C and E.

Minerals: Algae are the potential sources of minerals, they contain some of the biologically significant minerals like, calcium, zinc, selenium, cobalt, iodine, iron and potassium. They can also be fortified for essential minerals as they have tendency to take up these and are bioavailable for physiological benefits (Babu 1995).

Essential fatty acids: Most of the algal forms are rich sources of essential fatty acids and some contain high content of PUFA and ω-3 fatty acids. Algae of Rhodophyceae family contains 5.7 –24.6 mg g^{-1} of total lipids and 16:0, 18:1, 20:5, and n-3 and C_{20} PUFA are the major components of the lipid (Xiancui Li et al., 2002).

Phaeophyceae members have lipid content in the range of 2.5 –23.7 mg g^{-1} apart from the previously mentioned fatty acids they contain high percentage of 18:2 (n-6), 18:4 (n-3), 20:5 (n-3) and C_{18}, n-6 PUFA. Chlorophyceae members contain 7.2-12.0 mg g^{-1} lipids and they contain high percentage of 16:4 (n-3), 18:2 (n-6), 18:3 (n-3) and (n-3) PUFA (Levent Cavas et al., 2005). Green algae, *Tetrandrun minimum, Scenedesmus communis* and *Pediastrum boryanum* contains ω -hydroxy fatty acids (Xiancui Li et al., 2002). Six algae belonging to *Codium* species have shown the presence of 16:0, 18:1 ω

9, 18:3 ω3, 16:3 ω 3, 18:2 ω 6 and 20:4 ω 6 as major fatty acids. Total polyunsaturated fatty acid contents is reported to range from 17.2 to 54.4% (Blokker et al., 1998).

Another important fatty acid present in algae is gamma linoleic acid (GLA), which is of great significance in animal as well as human physiology. *Spirulina* is one of the richest sources of GLA among the microalgal forms. Nutritional benefits of some of the micro and macro algae are as shown in Table - 5.

Table 5. Nutritional benefits of some of commonly used micro and macro algal forms.

Algal forms	**Nutritional attributes**	**Reference**
Spirulina	High protein, essential amino acids, vitamin B complex and E, gamma linoleic acid, β-carotene	Xin-Qing Xu et al., 1998
Dunaliella	β - carotene, protein and glycerol	Johnson and Shubert 1986
Chlorella	Protein and minerals	Venkataraman et al., 1980
Scenedesmus	Protein and essential amino acids	
Kappaphycus	Protein, iron, α -tocopherol, ascorbic acid and β - carotene	Moore et al., 1988
Enteromorpha	Protein, ascorbic acid and iron	Moore et al., 1988
Porphyra	Protein, essential amino acids and vitamins	Moore et al., 1988

1.3 Algae as source of pharmaceuticals

The use of algae for therapeutic purpose has a long history (Glombitza et al., 1989). However systematic approach for the same was started in 1950's during which only *in vitro* studies were conducted. In 1970's the same was taken up for *in vivo* studies. Initial studies were focused on the macrophytes and only in 1980's the studies were taken on microalgae. These microalgae have certain advantages of cultivation due to shorter lifecycle, which can make them easy for utilization.

Some of the breakthrough researches in algae for medicine are,

« Heparins like sulphated polysaccharides from red algae, which 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.*

« Antiviral nucleotides from algae include idozuvidine, trifluridine, vivabaine and romovinyl dozyuridine (Damonte et al., 2004).

Structures of some of bioactive compounds are as shown in Figure - 2 and some of the bioactive compounds of algal origin are as shown in Table-6.

Table 6. Biologically active compounds from algae.

Algae	**Application**	**Reference**
Scenedesmus obligus	Antibacterial	Cohenz 1986
Asterionella sps	Anti viral and Anti fungal	Borowitzka, 1995
Chaetocerus and	Antifungal activity	Vilchenz et al., 1997
Fragilaria sps		
Sticocomus microbilis	Antiviral	Vilchenz et al., 1997
Chlorella sps	Antitumor	Pratt et al., 1944
Lyngbya majuscule	In Leukemia	Myndersa et al., 1977
Digenea simplex	Antibiotic	Richmond 1986
Plocamium cartilagenium	Antibiotic	Cueto et al., 1998

Major class of promising bioactive compounds from algal source include, antimicrobial, antiviral, anticancerous and other include anti-inflammatory agents, drugs acting on cardio vascular system and antihelmenthetics.

Antimicrobials: A number of micro algae have been screened for various antimicrobial activity, including antibacterial, antifungal and antiprotozoal (Pasado, 1990). Some of the compounds identified for this activity include fatty acids, glycolipids, acrylic acid, bromophenols, terpenoids and glycosides (Borowitzka 1995) . Antibiotics are the major class of the compounds obtained from algae.

Antiviral*:* Cyanobacterial extracts have shown activity against *Herpes simplex* virus type-II and also against respiratory syncytial virus at higher concentrations (Lau et al., 1993). A random screening of transcriptase of avian myloblastosis virus and human immuno- deficient virus type-I, 2.0 % have shown positive results, which mainly contain sulpholipids indicating that this is the possible molecule responsible for the activity.

Studies have shown that over 900 micro algal extracts screened for virus inhibition properties (Gustafson 1989).

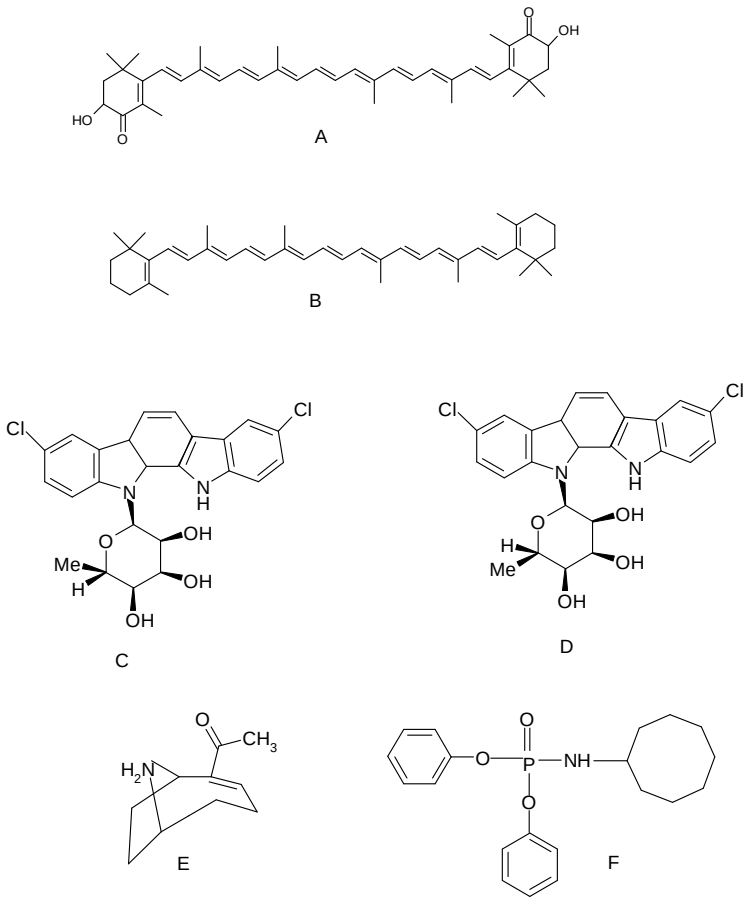
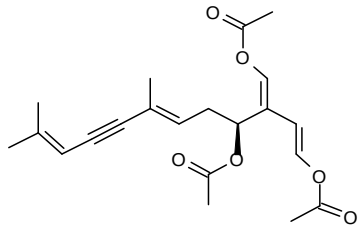


Figure 2. Bioactive Compounds of micro algal origin. A: Astaxanthin (*Haematococcus pluvialis)*, B: β-Carotene (*Dunaliella* sp.), C: Tijpanazole A1, D: Tijpanazole A2 (*Tolypothrix tjipanensis*), E: Anatoxin a (*Anabaena flos-aquae*) and F: PB-1 (*Ptychodiscus brevis*) (Borowitzka 1995).

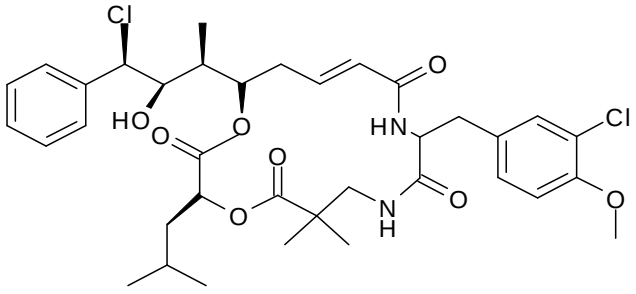
Anticancer*:* Cyanobacteria also produces a number of cytotoxic compounds namely, tubericidin and toyocamycin from *Streptomyces* (Patterson et al., 1991). Scytophycin B from *Scytonema pseudohoffmanni* has shown cytotoxic effect against KB (a human nasopharyngeal carcinoma) cell line at the concentration 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 Lewis lung carcinoma (Brachi et al.,

1984). *Spirulina* 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 anti cancer property exhibited by β- carotene and phycocyanin (Sude et al., 1986; Koto et al., 1984; Gerwick et al., 1994). Group of compounds from cyanobacteria known as cryptophytes and chrosophytes have shown promising anticancer activity (Helms et al., 1988).

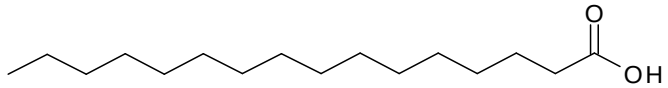
Around 250 marine compounds have been reported till date for anti tumor activity and more than 50 % of them are proven in pre-clinical trials. In 2000-01 itself around 30 compounds have been discovered from marine source. Among these caulerpenyne, cryptophycine, palmitic acid and mailiohydrin are of algal origin (Venkataraman et al., 1980) Structure of some of these are shown in Figure-3. Caulerpenyne is a sesquiterpene isolated form marine alga *Caulerpa taxifolia* and has shown antiproliferative activity in human neuroblastoma cell lines, as well as cytotoxic effect in several cell lines (Helms et al., 1988). 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). *Nostoc* a cyanobacterium is known to produce cryptophycine which is a depsipeptide, which can bind to microtubule end at vinca binding site to exhibit antimitotic activity (Morton and Bomber 1994).



Caulerpenyne



Cryptophycin-55



Palmitic acid

Figure 3. Structure of bioactive compounds of algal origin with antitumor and cytotoxic property. (Mayer and Gustafson 2004).

Other pharmacological activities: Some of the hydrophilic extracts of cyanobacteria have shown cardiotonic activity in isolated mouse atria (Borowitzka, 1995). Puwainaphycin C (*Anabaena* sp) and a cyclic peptide scytonemin A (*Scytonema* sp) have shown strong calcium agonist activity (Norton and Wells, 1982). Diatom *Nitzschia pungens* var. *multiseries* is a potential glutamate agonist (Laguna et al., 1993).

Brominated bi-indoles isolated from cyanobacteria *Rivularia firma* has shown anti-inflammatory activity and antihistamine activity tested in rat and mice models (Villar et al., 1992). Several species of cyanobacteria, chlorophyceae, dinophyceae and raphidophyceae have shown the angiotensin converting enzyme inhibitory activity (Benemann, 1990). *Dunaliella tertiolecta* was reported to exhibit antihypertensive, bronchodilator, antiseretonin, analgesic and anti oedema activity (Fox 1985), *Chlorella* has shown anti-dopaminergic activity (Ceferri 1983).

Some of the key factors for enhancing the active principles are bioactive compounds or secondary metabolites is by subjecting the cells to various physiological stresses like, nutrient, environmental and osmotic stress. Under such conditions algae will accumulate the secondary metabolites in stationary phase or in slow growing phase or vegetative phase. E.g. β- carotene in *Dunaliella* is produced under high salinity, high light and nutrient limitation. Thus when the cell growth is best the carotene content will be minimum. Nutrient limitations or stress is necessary for the production of bioactive principles. In some cases production of these compounds will also depends on growth phase and culture conditions Ex: In case of *Anabaena flos-aquae* (Utkilen and Golme 1992), environmental factors also play a vital role in metabolic production. Moreover lipid production and long chain polyunsaturated fatty acids production in diatoms is enhanced under low temperature. Microcystin production in *Microcystis oeruginosa* is enhanced under red or green light (Clament et al., 1967; Hauman 1981).

1.4 Algae as source of pigments.

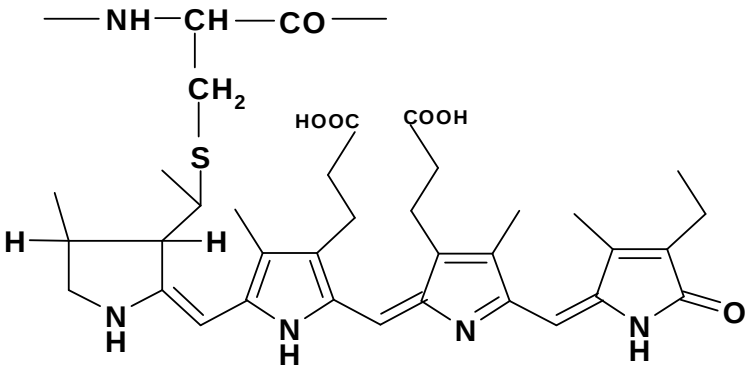
There is increasing demand for natural colors, which are of use in food, pharmaceuticals, cosmetics, textiles and printing dyes. However their utility is limited to few since the natural dyes have low tinctorial values and persistence. Due to the toxic effect of several synthetic dyes there is increasing preference to use natural colors for various end users. Only major problem of natural colors is the suststainability. Algae culture being ecofriendly and renewable, there is increasing affinity to use them as a source of natural

colors. Some of the sources of algal pigments are as shown in Table-7 and formulations available in market based on algal pigments are as shown in Table-8.

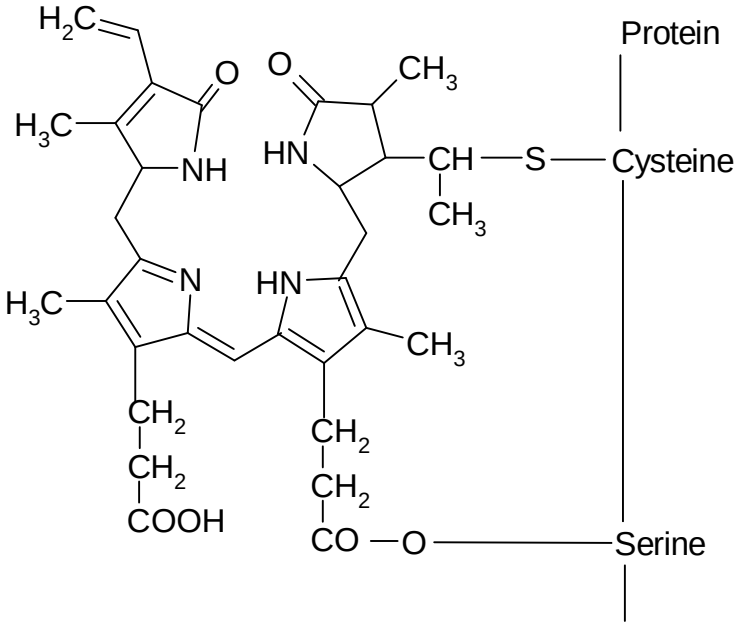
Major colors produced from algae, which are of commercial value, are phycocyanin (*Spirulina*), phycoerythrins (present in most of the seaweeds, Structure is given below Figure-4), astaxanthin (*Haematococcus*). β- carotene is also used as colorant in nutraceutical formulations.

Table 7. Pigments produced by algae and their application.

Pigment	**Algae**	**Applications**
Chlorophyll	*Chlorella, Spirulina*	Cosmetics, Antioxidant
Carotenoids	*Dunaliella* sp.	Pro vitamin-A, Food colorant, Bioactive compound.
Lutein	Mutants of *Dunaliella*	Food colorant, Antioxidant
Astaxanthin	*Haematococcus* sp.	Cosmetics, Coloring of fish Food colorant.
Phycocyanin	*Spirulina* sp.	Cosmetics, Diagnostic agent, Bioactive compound (anti cancer)
Phycoerythrin	*Porphyridium purpureum*	Cosmetics, Food colorant.



PHYCOCYANIN



R-PHYCOERYTHRIN

Figure 4. Structural formula of Phycocyanin and R-Phycoerythrin most used pigments of algal origin.

Table 8. Some of the market formulations containing micro algal pigments as one of the major component.

Formulation	Company	Intended use
Blue Manna Powder	Natural Zing, USA	Greater mental energy, attention, memory and focus. Emotional and mental balance Healthy joints and tissues
D and S control tablets	Max health Ltd., Hsi Chih, Taipei Hsien	Antiobesity
Glutamax	Max health Ltd., Hsi Chih, Taipei Hsien	Immunity booster
Alphastat (Astaxanthin)	Nature's Plus Rx-Formulations, USA	Food for healthy prostate
Marine Beta Carotene	Jarrow Formulations USA	Vitamin A deficiency, Immunity enhancer and antioxidant
Beta carotene soft gelatin capsules	Parry's pharmaceuticals, India	Antioxidant and vitamin supplement

1.5 Algal growth requirements.

Most of the research reports are available on the physiology and growth conditions for producing the compounds of interest from micro algal forms (Boussiba and Vonshak 1991, Lu et al., 1995). Major factors of significance in cultivation of algae are, nutrients, light, temperature and stress of various nature (Becker and Venkataraman 1982).

Nutrients: Nutrients required for growth of algae have been reported to be from organic and inorganic sources. In order to achieve optimum growth nutrients should be adequate in quantity (Kaplan et al., 1986; Borowitzka, 1988). Apart from carbon, hydrogen and oxygen, algae require additional compounds to grow, like nitrates, phosphates and sulphates, which are important for vital functions in algae. Carbon, nitrogen and oxygen

are provided either by inorganic salts (salts of carbonates and bicarbonates) or bubbling in the form of gas as in case of bioreactors. Some of the reported organic sources of nitrogen for algal cultivation are glycine, alanine, asparagine, aspartic acid, glutamine and succinamide (Sivasankar and Oaks 1996). Arnow et al., (1953) reported that citrulline, urea, arginine and ornithine can serve as source of nitrogen in *Chlorella*. Nutrient limitation will decrease the growth rate (Richardson et al., 1969; Parslow et al., 1984). Limitation of nitrogen leads to increase in the lipid content, and that of phosphorous leads to increased protein content in *Phaeodactylum tricornutum* (Youngmanitchai and Ward 1991). Nitrogen deficiency has been reported to cause chloroplast damage and size reduction in *Euglena gracilis* cells (Regnault et al., 1990). *Dunaliella* has shown to accumulate higher concentrations of carotenoids under nitrogen limitation (Ben Amotz 1983).

Glucose or acetate has been used as a source of organic carbon (Snoog 1980). Micro alga *Chamydomonas reinhardtii* was grown heterotrophically using acetate as carbon source by Chen and Johns (1994). Certain species of *Chlorella* could grow both autotrophically and heterotrophically in dark using organic substrates such as acetate, ethanol and glucose (Martinez and Drus 1991). *Spirulina* has been reported to assimilate glucose under mixotrophic conditions under varied intensity of light (Marquetz et al., 1995). Sodium bi carbonate is used as carbon source for *Spirulina* (Ceferri 1983) and *Scenedesmus obliquus* (Thielmann et al., 1990). Yield of biomass and growth are reported to be enhanced upon addition of CO_2 in case of *Tetraselmis suecica* (Fabregas et al., 1984), *Dunaliella tertiolecta* and *Chlamydomonas reinhardtii* (Amoroso et al., 1998). Chen and Johns (1991) reported the effect of carbon/nitrogen (C/N) ratio on fatty acid composition of heterotrophically grown *Chlorella* sp. Enhanced C/N ratio has shown to enhance the accumulation of astaxanthin in *Haematococcus pluvialis* (Cifuentes et al., 2003).

Many algal forms require vitamins like, thiamin, cobalmine and biotin for their growth (Droop 1954; Pringshiem 1966). The actions of plant growth hormones like, auxins and others on growth and production of metabolites has not been studied, however an inhibitory action of 2,4-D on photosynthesis of *Chlorella* has been reported (Wedding et al., 1954). Bajguz and Czerpak (1998) reported the effect of brassinosteroids on the growth and carotenoid production in *Chlorella vulgaris.*

Light: Induces photosynthesis capacity of algae. Increases photosynthetic capacity of algae, Intensity, photoperiod and quality of light is of paramount significance to algae. Although most of the species grows well in continuous light, only few species are sensitive to continuous light (Brand and Guillard, 1981). Since light is the basic requirement for algal photosynthesis, enhancement of surface to volume ratio is necessary in order to maintain high growth rate, which is usually followed in tubular photobioreactors (Prit et al., 1979; Lee and Bazin 1990) in case of *Porphyridium creuntum* (Chaumount et al., 1988) and *Spirulina platensis* (Trozillo et al., 1986). High light intensity, leading to photoinhibition has been reported by many researchers in *Spirulina* (Chanawongse et al., 1994), *Anacystis nidulans* (Samuelson et al., 1985) and some green algae (Kyle and Ohad, 1986). Terry (1986) demonstrated enhancement of photosynthesis efficiency in various combinations of light and dark periods and this is supported by similar type of studies by Stitt (1986) and Laws et al., (1988).

Light plays a vital role in growth of *Dunaliella* as well as in carotenogenesis. Massive accumulation of β- carotene under high light intensity has been reported by Ben Amotz et al., (1989). Several kinetics as well as empherical models predicting the algal productivity as a function of light irradiance has been reported by Grobbelaar et al., (1984).

Temperature: Effect of extreme temperature has been studied in various algae (Volkman et al., 1991) Influence of temperature on growth is associated with various metabolic process such as photosynthesis, respiration, membrane permeability and uptake of ions (Soeder and Stengel 1974).

1.6 Algae cultivation methods.

Based on the nature of the organism and culture media composition, namely pH, salt content and structure of the algae they are cultivated using different systems. Two major class of cultivation are open-air cultivation and closed system cultivation. Most of the algae for commercial utility are grown by open-air method. Some algal forms can be easily cultivated using open-air system as the media composition and pH will not allow any contamination due to salt concentrations, like in case of *Spirulina*. However some algal forms cannot survive in open air conditions as pH and media composition may lead to contamination with other microbes ex: *Haematococcus*. In general, 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 enhancement of pH upon growth will make it less susceptible for contamination and hence it is possible to cultivate the same in open-air conditions. Some of the commonly used cultivation systems employed for algae are given below in Table 9.

Table 9. Utilization of cultivation techniques for different algal systems worldwide.

Type of tank	**Algae cultivated**	**Major Location**
Tanks	Most of the species	World wide
Extensive open ponds	*Dunaliella*	Australia
Circular ponds with rotating arm	*Chlorella*	Taiwan, Japan
Race way ponds	*Dunaliella, Spirulina,*	Japan, Taiwan, USA, Thailand,
	Chlorella	China, India, Vietnam, Chile, USA, Israel
Cascade system with baffels	*Chlorella*	Czech Republic, Bulgaria
Large Bags	Many species	World wide
Fermenters (Heterotropic)	*Chlorella* sp	Japan, Taiwan, Indonesia,
	Crypthecodinium cohnii	USA
Two stage systems	*Haematococcus*	USA

(Adopted from Borowitzka 1995)

1.7 Carotenoids

1.7.1 History of carotenoids.

Wackenroder first isolated carotene from carrots in 1831, seven year later Berzelius (1837) named the yellow pigments from autumn leaves xanthophylls. This opened the beginning of research on carotenoids. Since that time research on carotenoids is constantly in progress in the direction of chemistry, biology and pharmacology. However, recent research is focused more on the genetic aspect of these carotenoids for their best possible utilization as nutraceuticals and pharmaceuticals.

Carotenoids are lipid soluble plant pigments found in photosynthetic plants, algae and animal tissues. They also occur in photosynthetic bacteria, yeasts and molds. In nature about 700 carotenoids have been isolated and characterized. Among these more than 10 % are metabolized in the body to form vitamin A in different animals (Yeum and Russel 2002). Carotenoids provide wide range of colors to environment (Olson and Krinsky 1995). The well-known function of the carotenoids in plant system are giving characteristic color, protecting the plant against photosensitization and acting as protector against various free radical mediated damages.

1.7.2 Distribution of carotenoids.

The total carotenoids production in nature has been estimated to be approximately 100 million tons per annum by all the living organisms. They are distributed widely in a variety of living species like, fruits (pineapple, tomatoes, citrus, mango), flowers (narcissus, eschscholtzia), birds (flamingo, ibis, canary) insects (ladybird) and marine animals (salmanoids, crustaceans) and in most of the higher plants especially in leaf in association with chlorophyll (Gouveia and Emphis 2003). Carotenoids are found in the photosynthetic tissues of plant and animals in which many structural variations are found. It is considered as the only naturally occurring tetraterpenoids and very widely distributed throughout the living world. Carotenoids are synthesized *de novo* only by higher plants, mosses, liverworts, algae, photosynthetic and non-photosynthetic bacteria and fungi. All photosynthetic organisms contain carotenoids but are most often masked by chlorophyll present in chloroplast. The two pigments coexist because the carotenoids prevent the photodynamic sensitization of chlorophylls, which in their absence leads to destruction of the chloroplast (Goodwin 1979).

All the carotenoids in photosynthetic tissues are located in the grana of the chloroplast and consist of the same major group of pigments whatever the source. Major ones are β - carotene, lutein, violaxanthin and neoxanthin and smaller amount of α- carotene, β- cryptoxanthin, zeaxanthin and antheraxanthin. Xanthophylls are occur unesterified and upon senescence, when chloroplasts disintegrate, the xanthophylls released into the cytoplasm are esterified before they are oxidatively destroyed (Ladygin 2000).

Reproductive tissue: The first carotenoids isolated from anther was antheraxanthin and later it was found that it is present in trace quantity in all the green tissues. In flowers carotenoids are accumulated in chromoplasts, which are derived from chloroplasts. Flowers producing carotenoids are of three types viz., those containing highly oxidized pigments like furanoid oxide auroxanthin, hydrocarbons such as lycopene or β- carotene and highly species-specific carotenoids such as the retrocarotenoids-eschscholtzxanthin from *Eschscholtzia californica*. In some petals carotenoids concentration can be very high e.g. β- carotene represents 16.5 % of dry weight in case of *Narcissus poeticusrecurvis* (Goodwin 1979).

Fruits: In case of fruits they are divided into many groups based on the content viz., only traces of carotenoids, carotenoids similar to that of chloroplasts, lycopene and derivatives, large amount of β -carotene and its derivatives, very large amount of epoxides, pigments which are species specific, considerable amount of pro carotenes, secocarotenoids and apocarotenoids (Goodwin 1979).

Roots: The most important carotenogenic root is carrot containing β- carotene as major pigment along with xanthophylls constituting about 5.0 % of total pigments. Normal commercial strain contains about 60-120 μg of β -carotene per gram of fresh weight. Some sweet potatoes also contains significant amount of β- carotene. Roots of *Escobedia scabrifolia* (Family: Scrophulariaceae, grown in Northern America) is known to contain azafrin, an apocarotenoid (Goodwin 1979).

Bacteria: The photosynthetic bacteria differ from other photosynthetic tissue in accumulating acyclic pigments characterized by methoxy group at position C_2 and additional double bond at $C_{3,4}$ and keto groups conjugated to the conjugated double bond system. All these features are present in spheroidenone synthesized by *Rhodopseudomonas sphaeroides.* Chlorobactene is a aromatic ring containing carotene

produced from *Chlorobium* sp. Spirialloxanthin a carotene is present in non photosynthetic organism *Corynebacterium poinsette* (Jansen et al., 1965).

Fungi: Fungi are also known to contain carotenoids. Some of the carotenoids producing species are *Lycogala epidendron, Aleuria aurantia, Scutellinia scutellata, Neurospora crassa, Neurospora sitophila*. *Lycogala* is known to contain β- carotene, γ- carotene, lycopene, torulene. *Aleuria* contains same carotenoids except torulene. *Scutellinia* contains γ - carotene torulene and xanthophylls. Both species of *Neurospora* contains phytofluene, ζ- carotene and neurosporene apart from carotenoids mentioned above (Jansen et al., 1965). Another fungus *Epicoccum nigrum* is known to produce β-carotene (32%), γ- carotene (22%), rhodoxanthin (40%), torularhodin (6%) (Sassu and Foppen 1967). Lycopene cyclase which is a key enzyme in biosynthesis of β -carotene was isolated and characterized from fungus *Erwinia uredovora* and it is reported to be of 43 kD molecular mass (Schnurr et al., 1996).

Algae: Almost all classes of algae contain one or the other type of carotenoids, major being β-carotene and xanthophylls. Algal carotenoids are present in chloroplast as a complex mixture that is the characteristic of each class of algae. The red alga Rhodophyta has α and β -carotene and their hydroxylated derivatives (South and Whittick 1987). Chloromonadophyta contains diadinoxanthin, heteroxanthin and vaucheriaxanthin. Chlorophyta are characterized by acetylenic carotenoids namely, alloxanthin, monadoxanthin and crocoxanthin (Cheng et al. 1974). *Spirulina* is known for accumulation of β -carotene of upto 0.8-1.0 % w/w. *Dunaliella* is the highest carotenoid producing organism among the algae and other organisms. Commercially cultivated species are *Dunaliella salina* and *D. bardawil*. Other carotenoids containing algal species include *Haematococcus and Chlorella.*

1.7.3 Chemistry of carotenoids.

Carotenoids are hydrocarbons containing C_{40} polyene chain backbones. The linear chain is cyclized at terminals, the molecule with hydrocarbon terminal are known as carotene and that of oxygenated terminals are known as xanthophylls (Goodwin 1980b).

Those carotenes with at least half of the β- carotene molecules i.e. an unsaturated ionone ring having 11 carbon polyene side chain are classified as metabolic precursors of vitamin A. Each carotene can occur in 282 different geometrical isomers. Most of the carotenoids can be described by the general formula $C_{40}H_{56}O_n$, where n is 0-6.

Hydrocarbons (n= 0) are termed as carotenes. According to International Union of Pure and Applied Chemistry's (IUPAC) recommendation semi-synthetic names should have symbols like, β,ε - and β,β - carotene.

Beta-carotene is a $C_{40}H_{56}$ molecule containing hydrocarbon cycle at terminal. It consists of eight isoprenoid units joined in such a manner that the joining of isoprenoid units at the middle is reversed at the center of molecule so that 2 central methyl groups are at $C_{1,6}$ position and remaining non-terminal methyl groups are at $C_{1,5}$ position (Goodwin 1980a). This long chain is formed by hydrogenation, dehydrogenation, cyclization and oxidation.

The rule for naming carotenoids was published by IUPAC in 1975. Zechmecster (1962) first reported stereochemistry of carotenoids. It was also reported that mainly there are two different forms namely *cis-trans* or E-Z isomers. These have different activities, based on the number of double bond isomers exists.

The first total synthesis of β-carotene was also in 1950. However, the industrial large-scale synthesis was done in 1954.

There are two basic methods in synthesis of C_{40} slice, by joining two smaller molecules. symmetric, $C_{16} + C_8 + C_{16} = C_{40}$ and asymmetric / unsymmetrical, $C_{25} + C_{15} = C_{40}$

The most popular industrial methods are given below:

i). Badische Aniline and Soda – Fabric (BASF) synthesis

Here β-retinyltriphenyl phosphonium chloride and retinal were made to react using KOH and methanol to form β-carotene (Figure-5).

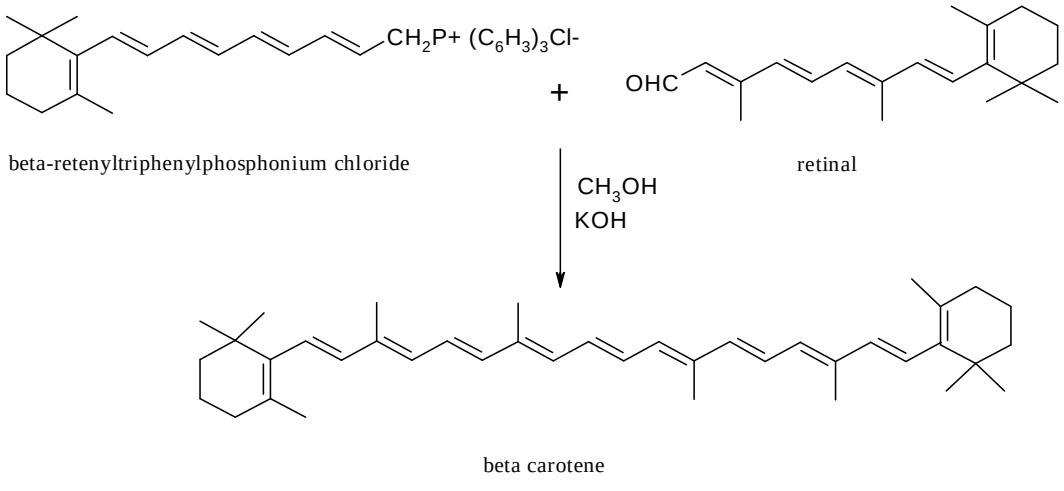


Figure 5. Badische Aniline and Soda – Fabric (BASF) synthesis of β - carotene.

ii). Roche synthesis

This is based on Grignard reaction. In this method two molecules of β -C_{19} aldehyde are made to react using acetylene dimagnesium bromide to form C_{40} diol, which further converts to 15-15′ didehydro-beta-carotene, they will be converting to 15′ β -carotene and all-*trans* β-carotene which takes very few seconds (Figure-6).

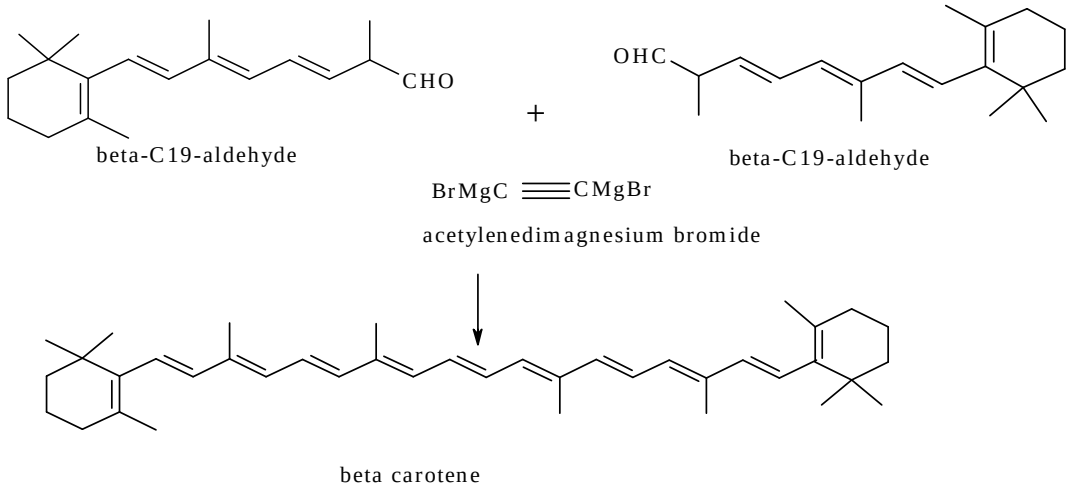


Figure 6: Roche synthesis of β - carotene by Grignard reaction.

Biologically *cis* isomers are highly efficient in converting to vitamin A due to high polarity and the activity is good in contrast to *trans* (Bertone et al., 2001). Therefore there are many ways by which *tans* isomers can be converted to *cis* form including cooking of vegetables. The important ones that occur in natural process or can be induced by external influence are heat induced and light induced process.

1.7.3.1 Stereochemistry of carotenoids.

Beta-carotene occurs naturally as all-*trans* beta-carotene and 9-*cis* beta-carotene, and to a lesser extent of 13-*cis* beta-carotene. Synthetic beta-carotene consists mainly of all-*trans* beta-carotene with smaller amounts of 13-*cis* beta-carotene and even smaller amounts of 9-*cis* beta-carotene. Dietary intake of beta-carotene in the American diet ranges from 1.3 to 2.9 milligrams daily. The consumption of five or more servings of fruits and vegetables per day, which is recommended by a number of federal agencies and other organizations, including the NCI (National Cancer Institute) would provide three to six milligrams of beta-carotene daily.

Vitamin A and more than 600 carotenoids have been crystallized and fully characterized by a variety of chemical and physical methods. Furthermore, vitamin A and many of its

analogs, as well as selected carotenoids, have been synthesized chemically from simple and readily available precursors. Because of the structure of conjugated double bonds that are characteristic of both vitamin A and carotenoids, these substances are sensitive to oxidation. Vitamin A is now considered, chemically, as a subgroup of the retinoids, which are defined as a class of compounds consisting of four isoprenoid units joined in a head-to-tail manner and customarily containing five conjugated double bonds (Olson 1993).

Carotenoids are rich in conjugated double bonds, each of which can theoretically undergo isomerization to produce an array of mono or poly-*cis* isomers. Introversion is thought to occur with the absorption of light, exposure to thermo energy or by participation in specific chemical reaction. In most foods carotenoids occur in *trans* configuration, which is the most thermodynamically stable form (Clinton 1998).

The structure of the carotenoids is what determines the physical properties, chemical reactivity and biological properties or pharmacological behavior of compounds. Upon biosynthesis by plants and microorganisms, each carotenoid is incorporated into very precise orientations within sub cellular structures of the photosynthetic apparatus (Demming-Adems et al., 1996). Most of the time, chemical and physical properties of the carotenoids are strongly influenced by other molecules within the microenvironment, particularly associated with proteins and membrane lipids. In turn carotenoids can also influence the properties of the sub cellular structures. The chemical features of each carotenoids like, size, shape, hydrophobicity and polarity, determine its ability to interact and function in plant, bacteria or fungus. Hence behavior of each carotenoid in biological system is governed by these properties (Stahl et al., 1992).

1.7.4 Carotenoids as nutrient.

A number of nutritional benefits have been reported from β - carotene. Most of them are as source of Vitamin A and they are correlated with biological properties. Most common and well-known properties include pigment synthesis required for the vision, maintenance of skin properties by protecting against UV- light and so on. Other role of Vitamin A include protein synthesis, RNA synthesis, cell division, cell membrane stability, mucous production, sexual and reproductive process, sperm production, egg development, function of adrenal and thyroid glands, which in turn maintains metabolic rates, energy level, body temperature and growth rates, skin integrity, functions of inner lining of digestive tract, bone development, remodeling and liver functions.

1.7.5 Biological activity of carotenoids in plants.

Carotenoids are known for their biological activity due to their capacity to transfer energy in photosynthesis and photoprotection (Krinsky 1994b). Carotenoids play vital role in photosynthesis in association with chlorophyll. The major functions of carotenoids include accessory pigments in light harvesting system and as photoprotective agents against oxidative damages. They absorb the visual light and act as photo protector by forming complexes with chlorophyll and proteins known as photosystems (PS I and PS II). β- carotene involves in PS I and Lutein in PS II (Armstrong and Hearst 1996; Cogdell and Gardiner 1992). These utilize the energy absorbed by direct sunlight for various biochemical conversions and inturn protect the plant forms. Similarly when there are free radicals like, singlet oxygen, hydroxyl radical, it will be utilized for inter conversion of carotenoids. Some of the examples are, increase in astaxanthin content as a protective mechanism when *Phaffia rhodozyama* is exposed to singlet oxygen (Schroeder et al., 1996). When leaves are exposed to high light, conversion of various xanthophylls takes place. Violoxanthin, upon losing epoxy xanthophylls forms antheraxanthin followed by zeaxanthin. Therefore number of carotenoid molecules will be more in light exposed plant when compared to those grown in dark (Olson and Krinsky 1995).

1.7.6 Pharmacology of carotenoids.

The efficiency of absorption of beta-carotene is highly variable. The efficiency of absorption of beta-carotene from carrots and other beta-carotene containing raw foods is less than 5%. Carotenoids are absorbed from the intestine with the aid of dietary fat and incorporated into chylomicrons for transport in the serum (Rapola et al., 1998). On the other hand, the efficiency of absorption of beta-carotene from beta-carotene containing nutritional supplements can be as high as 70% or more. In foods, beta-carotene exists either as a solution in oil (e.g., red palm oil) or as part of a matrix within the vegetable or fruit. For example, in carrots, beta -carotene exists in a complex matrix, comprised of indigestible polysaccharides, digestible polysaccharides and protein.

Absorption: Absorption and metabolism varies to great extent in animal species. In case of humans, carotenoids can be absorbed intact by mucosal cells and subsequently appear unchanged in circulation and peripheral tissues (Bowen et al., 1993). In some of the rodents and animals β -carotene and other provitamin -A carotenoids are metabolized

into vitamin A in intestinal mucosal cells. If not it will not be absorbed in the body hence plasma concentration is very low and not comparable with that of human beings. Different proportion of carotenoids present in food and supplements are taken up by intestinal cells and are metabolized to vitamin A in the process of absorption in humans. This complicates the interpretation of plasma carotenoids (Parker 1996). Absorption of carotenoids takes place in the intestinal mucosa and uptake of these compounds by duodenal mucosal cells appears to be by passive diffusion, with a concentration difference between mixed micelle and that of cell membrane and this decides the rate of diffusion (Parker 1996). After passive diffusion into enterocytes, unmetabolized carotenoids are incorporated into chylomicron and secreted by the lymph, followed by uptake by liver and release into circulation in association with very low density lipoproteins (VLDL) and ultimately in association with low density lipoproteins (LDL) (Erdman et al., 1993). Generally two peaks are observed in plasma analysis of β-carotene first one for the chylomicron associated β- carotene and later for the β -carotene associated with LDL. Retinol formed in enterocytes is secreted in lymph chylomicra as retinyl ester, where as retinoic acid and other polar metabolites exit the intestinal tissue through the portal circulation. It is hypothesized that carotenoids may bind to cytosolic protein and this may play a role in the intracellular transport of these compounds in intestine or liver (Rock 1997). Factors which affect the absorption of carotenoids in general and β- carotene in particular are as shown in Table -10.

Only a small percentage of beta-carotene is released from the matrix during the passage of foods. The different structural features possessed by carotenoids account for selective distribution in organ tissue, biological activity and pro-vitamin A potency, or *in vivo* conversion to vitamin A. Due to the hydrophobic character, carotenoids are associated with lipid portions of human tissues, cells, and membranes. In general, 80-85% of carotenoids are distributed in adipose tissue, with smaller amounts found in the liver, muscle, adrenal glands and reproductive organs. Approximately 1% circulates in the serum on high and low density lipoproteins. The major serum carotenoids are β-carotene, α-carotene, lutein, zeaxanthin, lycopene and cryptoxanthin. Smaller amounts of polyenes such as phytoene and phytofluene are also present (Redlich 1999).

Table 10. Factors determining the absorption of carotenoids in human.

Dietary factors: Fat ↑, Soluble fiber ↑↓, Dosage of carotenoids administered ↓, Competitive interaction between carotenoids ↓.
Food forms: Location in plant tissue (Chromoplast are more available compared to chloroplast), Mild heat (cooking)↑, reduced particle size by process like, blending/ grinding↑.
Biochemical & metabolic: Isomeric forms ↑↓ , Large individual variability due to absorption and metabolic polymorphism.
Individual subject: Intestinal parasites↓, malabsorption syndrome↓, Vitamin A status ↑↓, Increase in gastric pH ↓.
↑- Increase, ↓ - Decrease, ↑↓ - Variable.

Distribution: Carotenoids in bloodstream are transported in association with the lipoproteins, with a distribution similar to cholesterol. Hence the plasma cholesterol concentrations are highly correlated with circulating carotenoids concentration using *in vivo* trials (Olson 1994). Approximately 75% of plasma carotenoids are associated with LDL and remaining will be distributed in VLDL and HDL. More polar carotenoids like lutein are associated with HDL and non-polar ones like, β- carotene, α- carotene and lycopene are associated with LDL. In case of human study, it has shown that circulating carotenoids concentration is lower in case of non-smokers compared to smoking individuals (Handelman et al., 1996). The inverse pattern of carotenoids concentration has been observed in case of alcohol consuming individuals (Brady et al., 1996).

Adipose tissue is the largest body pool for carotenoids. Serum concentrations are fairly constant and slow to change during periods of low intake. The estimated half-life is 11-14 days for lycopene, α-carotene, β-carotene, lutein and zeaxanthin (Santos et al., 1998; Santos et al., 1996) Evidence for the existence of more than one body pool has been published (Tavani and La Vecchia 1999).

Serum levels of carotenoids reflect lifestyle choices and dietary habits within and between cultures. Variations can be attributed to different intake, unequal abilities to absorb certain carotenoids, and different rates of metabolism and tissue uptake. Decreased serum levels occur with alcohol consumption, the use of oral contraceptives, smoking (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994; van Poppel 1993) and prolonged exposure to UV light (Hankinson et al., 1992).

Beta-carotene, from supplements, oils or foods is either solubilized in the lipid core of micelles (formed from bile salts and dietary fat) in the lumen of the small intestine or forms clathrate complexes with conjugated bile salts. Micelles and clathrate complexes deliver beta-carotene to the enterocytes. All of the beta-carotene isomers viz., all-*trans* beta-carotene, 9-*cis* beta-carotene and 13-*cis* beta-carotene are absorbed from the lumen of the small intestine into the enterocytes. Within the enterocytes, a fraction of all-*trans* beta-carotene is oxidized to retinal and then reduced to retinol. Retinol is then esterified to form retinyl esters. It appears that 9-*cis* beta-carotene is isomerized to the all-trans form before being released into the lymphatics. The principal enzyme involved in the oxidation of beta-carotene is called beta-carotene 15, 15' dioxygenase.

Beta-carotene and retinyl esters are released from the enterocytes into the lymphatics in the form of chylomicrons. Beta-carotene is transported by the lymphatics to the general circulation via the thoracic duct. In the circulation, lipoprotein lipase hydrolyzes much of the triglycerides in the chylomicrons, resulting in the formation of chylomicron remnants. Chylomicron remnants retain apolipoproteins E and B48 on their surfaces and are mainly taken up by hepatocytes and to smaller extent by other tissues. Within hepatocytes, beta-carotene is incorporated into lipoproteins. Beta-carotene is released into the blood from the hepatocytes in the form of very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL). In the plasma, VLDLs are converted by lipoprotein lipase to LDLs. Beta-carotene is transported in the plasma predominantly in the form of LDLs.

Storage: Adipose tissue and liver are the major tissue storage depots for the carotenoids, although carotenoid traces have been found in lungs, kidney, cervix, prostate and other tissues (Shimitz et al., 1991). High concentration of carotenoids are found in tissues rich in LDL receptors, like corpus luteum, adrenal tissue, testas probably resulting from nonspecific intake of lipoproteins. Some of the carotenoids are specific in absorption, like macula of eye is rich in lutein and zeaxanthin, but not in other carotenoids (Handleman et al., 1992). Some of the concentration is found in pineal gland but not in case of brain stem (Olson 1994). Difference in the distribution is also seen with respect to isomeric forms. In general, amount of *trans* exceeds that of *cis* in case of circulation in plasma, where as increase in the proportion of cis β- carotene is observed in peripheral tissue (Stahl and Sies 1993).

In general carotenoids tissue content is directly proportional to dietary intake. Hence carotenoids are used as biomarkers for assessing intake of vegetables and fruits. The

concentration of carotenoids will also increase in peripheral tissue on high intake apart from plasma. Deposition of carotenoids in stratum corneum of epidermis occurs with repeated high dose of carotenoids leading to yellowing of skin and the condition is referred as '*carotenodermia'*. Average resident time for β -carotene is 51 days as revealed from human isotope tracer study (Novotny et al., 1995).

Metabolism: Only those carotenoids with at least one unsubstitued β- ionone ring attached to an intact conjugated polyene structure from C-7 to C-15 can be metabolized into retinal, which is then reduced to retinol (Goodwin 1986). Because of this structural requirement β -carotene is the most important provitamin-A carotenoid. The basic central cleavage takes place by cytosolic enzyme 15, 15′ dioxygenase. Hence β -carotene will ideally be metabolized to retinal (Goodwin 1986). Excentric cleavage, which produces apocarotenol intermediates, is also demonstrated in human intestinal tissue (Krinsky et al., 1993). Research has shown that carotenoids are also converted into compounds other than vitamin A and these retinoid like metabolites may affect growth regulation and other cellular activities (Krinsky 1994). Metabolism also takes place in peripheral tissues, such as adipose tissue in human, lung and kidney of primate, bovine corpus luteum apart from intestinal mucosa and hepatocytes (Wang et al., 1991). Provitamin -A carotenoids are not converted into retinol unless there is need for Vitamin-A. The rate is proportional to the demand for vitamin A. β -carotene is known to have $1/6^{th}$ value of vitamin A, considering 33.0 % average bioconversion and absorption ability of carotenoids in comparison with vitamin A, whereas others are known to have $1/12^{th}$ the vitamin A value of retinol (Solomons and Bulux 1993).

Exact nature of excreting metabolites of carotenoids is not known, however it is assumed that they are similar to that of retinol and vitamin A. Liver cytochrome- 450 has shown to take part in the metabolism and it converts retinol and retinoic acid into polar metabolites (Ranet et al., 1996). Due to inefficient absorption of the compounds most of the carotenoids that are ingested will exit the human body in the feces (Rock et al., 1996).

Safety and toxicity: United State Food and Drug Administration has declared β- carotene as generally recognized as safe (GRAS) for the consumption in 1979, as food colorant and dietary supplement (Diplock, 1995). This was supported by various studies like, mutagenicity, embryotoxicity, teratogenecity of carotenoids (Bendich 1988). Except in case of ATBC (Alpha tocopherol and beta carotene study) and CARET (carotene and

retinol efficiency trail) where they found that beta-carotene is associated with increased risk of lung cancer in heavy smokers, it was considered to be completely safe in humans. Exact mechanism of increasing the risk of carcinogenesis associated with smoking and alcohol consumption is not known (Erdman et al., 1996). Excess consumption by β -carotene or other provitamin A carotenoids will not result in hypervitaminosis A as it is converted on demand of the body. But yellowing of skin or hyper carotenemia occurs with high dose of β- carotene (30 mg day^{-1}) for long period of time, and this will spontaneously reduce upon decrease in the carotenoids dose. Apart from these reports no serious side effect has been observed in any normal human subjects.

1.7.7 Reported pharmacological activities.

Among the pharmacological activities almost all are antioxidant mediated. Carotenoids are an integral part of membranes but xanthophylls are variable in their position. Therefore carotenoids are effective antioxidants if radicals are generated inside the membrane. Apart from this, carotenoids have a remarkable effect in the immune response and intracellular communication (Britton 1995; Charluex 1996; Honh and Sporn 1997). Many of the carotenoids including β-carotene show an efficient induction of Gap junction communication (these are water-filled pores, connecting the cytosol of two neighboring cells, allowing the exchange of low-molecular mass compounds). Induction and free radical scavenging are two different mechanisms, which act independently to prevent cancer (Stahl et al., 1997). Carotenoids are associated with LDL, where as xanthophylls are distributed equally between high and low-density lipoproteins. Carotenoids upon biotransformation convert to retinol, retinoic acid and other products.

Pharmacological activity in human can be classified as ,

- Vitamin A (Retinol)
- Antioxidant
- Immunomodulatory
- Anticancer
- Skin protection (sunburns and other mediated injuries)
- Heart disease prevention and prevention of cataracts and macular degeneration.
- Biological signaling.

***Retinol (Vitamin A)*:** Majority of biological activity of the β-carotene are retinal and other vitamin A analogs mediated. β -carotene is found to convert into vitamin A analogs

in intestine of human and rodents, which is a cleavage process mediated by β,β-carotene-15,15'-oxygenase (BCO) soluble in alkaline medium. It is the major component in the vision mechanism, where it synthesizes the pigment required for vision. It is responsible for maintenance of epithelial cells, control of mucous secretion and reproduction (Olson 1993: Radlwimmer and Yokoyama 1997; Taylor and Mayne 1996). Some of conditions in which vitamin A is essential for the body are, cystofibrosis, diahorrea, long term injury, liver disease, malabsorption, pancreatic disorder

***Antioxidant*:** Beta-carotene has been demonstrated to quench singlet oxygen (O_2^-), scavenge peroxyl radicals and inhibit lipid peroxidation. The mechanism of beta-carotene's antioxidant activity is not clearly understood. Some, but not all, studies have shown a difference in the *in vitro* activities of the beta-carotene isomers. One study showed that 9-*cis* beta-carotene, which is a naturally occurring form of beta-carotene, protected methyl linoleate from oxidation more efficiently than all-*trans* beta-carotene (Liu et al., 2000). Results from some human studies have shown improvement of measures of antioxidant activity (decreased copper-induced LDL oxidation), decreased DNA strand breaks and oxidized pyrimidine bases in lymphocytes, decreased serum lipid peroxide levels, decreased serum malondialdehyde (MDA) and increased erythrocytes copper/zinc-superoxide desmutase activity in those receiving relatively high intakes of beta-carotene. It is possible that *in vivo* antioxidant activity of beta-carotene is unlikely to be a consequence of its conversion to retinal (Keys et al., 1999).

Immunomodulatory activity: Beta-carotene has demonstrated immunomodulatory effects. In healthy male nonsmokers, beta-carotene supplementation (15 mg day^{-1}) was found to significantly increase the percentage of monocytes expressing the major histocompatibility complex class II molecule HLA-DR, to increase the expression of intercellular adhesion molecule-1 and leukocyte function-associated antigen-3 and to increase *ex-vivo* secretion of tumor necrosis factor (TNF)-alpha by blood monocytes. Beta-carotene supplementation has also been found to enhance natural killer cell activity in elderly men, to increase lymphocyte response to mitogens in healthy male cigarette smokers and to increase the CD_4 lymphocyte count in some subjects with AIDS. The mechanism of the possible immunomodulatory activity of beta-carotene is not known, however it is thought that the possible immunomodulatory activity may be independent of beta-carotene's role as a precursor of retinol (Lyn Patrick 1999).

Anti cancer: Beta-carotene has been found to inhibit the growth of some malignant cells, including human prostate cancer cells, *in vitro*. The mechanism of this activity is not well understood. The ability of beta-carotene to modulate the carcinogenic process, at least *in vitro*, may be due, in part, to its conversion to retinoids (Yeh 2003). Beta-carotene may act as a prooxidant when present in high concentrations in an oxidative environment such as the lungs of smokers in the advanced promotional stage of the neoplastic process. Supplemental beta-carotene is known to inhibit the absorption of the carotenoid lutein which itself may have chemopreventive activity (Bertram 2004). Beta-carotene is known to reprogramme the oncogene through antioxidant property by changes in phosphorylation and ultimately transcription.

Beta-carotene has shown anticarcinogenic activity in the case of prostate cancer in human studies. The mechanism of this possible anticarcinogenic effect is unclear (Redlich et al., 1999). A review of the postulated mechanisms of possible anticarcinogenic activity in certain circumstances explains that beta-carotene which is metabolically converted to retinoids will modulate gene expression of factors linked to differentiation and cell proliferation via retinoic acid, which in turn helps in cancer.

Beta-carotene may also modulate the activity of enzymes that metabolize xenobiotics. It is possible that antioxidant activity may result in prevention of oxidative damage to DNA and inhibition of lipid peroxidation as well as regulation of the expression of genes sensitive to the intracellular redox state that may be involved in carcinogenesis. Beta-carotene may modulate the gene expression of connexin- 43 (first gene expressed independently by carotenoids) resulting in the induction of gap junctions with a consequent inhibition of neoplastic transformations (Jian et al., 2005).

***Cardiovascular protection*:** Epidemiological studies and some, but not all, intervention studies suggest an inverse association between coronary artery disease and beta-carotene intake. The possible antiatherogenic activity of beta-carotene may be accounted for, in part, by its possible antioxidant activity. Humans supplemented with beta-carotene, but not lycopene, were found to have low-density lipoproteins that were less oxidized than controls using endothelial cell-initiated autoxidation (Olson and Krinsky 1995).

Biological signaling: Carotenoids of natural origin are known to influence the intracellular communication (Stahl et al., 2002). Lycopene and tocopherol have shown inhibitory action on the growth of human prostate carcinoma cell lines (Pastori et al., 1998). Lycopene is known to lower the cyclin D levels which in turn leads to decreases

of phosphorylation of the retinoblastoma protein leading to growth suppression (Nahum et al., 2001). β - carotene is known to facilitate gap junction communication. GJC (gap junction communication) will help in the enhancement of the regulation of cell growth (Trosko et al., 2000). Carotenoids are known for induction of phase I and phase II metabolic enzymes, which play a role in the detoxification of carcinogens (Stahl et al., 2002).

Other activities: Include hepatoprotection, antiageing, and protection against some forms of cancer in some populations. It may also play a role in protecting against heart disease in some. Beta-carotene has demonstrated positive effects in the immune system. Diminished beta-carotene status has been observed in subjects with non insulin-dependent diabetes, but supplementation with beta-carotene has so far produced no notable benefits in diabetic patients. The carotenoids have been found to increase the levels of phase II detoxifying enzymes such as glutathione S-transferase m (GST-m) and glutathione peroxidase. Benefits sometimes attributed to beta-carotene in the prevention of cataracts and age-related macular degeneration may actually be due to other carotenoids, notably lutein and zeaxanthin (Bone et al., 2003).

***Some of the findings from clinical studies*:** Six major chemoprevention trials have been reported in different parts of world including, intervention studies in China in cancer subjects (Bolt et al., 1971), skin cancer prevention studies (Greenburg et al., 1990), cardiovascular diseases and malignant neoplasm (Hennekens et al., 1996) and lung cancer (Omenn et al., 1996) and some of the findings of these are given in the table 11.

Table 11. Some of the clinical research findings of carotenoids in various disorders.

Activity	**Reference**
In smokers synthetic β-carotene is known to increase the risk of lung cancer	Santos et al., 1998 Albanes et al., 1996
All *trans* carotenoids can convert to Vitamin A (94 %)	Omenn et al., 1996
Before reaching blood stream most of *cis* carotenoids will be converted to *trans* form	You et al., 1996
Natural carotenoids possess beneficial activities compared to synthetic ones	Tamai et al., 1995 ; Bitterman et al., 1994
β - carotene is helpful in asthma induced by exercise	Ben-Amotz, et al., 1996
A clinical trial involving smokers has shown carotenoid with α - tocopherol will decrease the incidence of lung cancer	Neuman et al., 2000
Dietary β- carotene can scavenge nitrogen dioxide, thiol and sulfonyl free radicals	Albanes 1999
β-carotene along with a ascorbic acid and α -tocopherol is beneficial in prevention of cardiovascular disease	Everett et al., 1996
Risk of coronary artery disease (CAD) is associated with low serum levels of β-carotene in urban patients	Singh et al., 1995
β-carotene is known to reprogramme the oncogene	Schwartz et al., 1993

1.8 *Dunaliella*

Table 12. Taxonomical classification of *Dunaliella*.

Super kingdom	Eukaryota
Kingdom	Viridiplantse
Subkingdom	Phycobionta
Phylum	Chlorophyta
Class	Chlorophyceae
Order	Volvocales
Family	Dunaliellaceae / Chlorophyceae
Genus	*Dunaliella*

The genus *Dunaliella* consists of 23 Sub species. Some of the important and extensively found species are, *Dunaliella bardawil, D.salina, D.bioculata, D.teritolecta, D.parva, D. spec, D. granulata* and *D. primolecta*. Commercially cultivated strains for the production of natural carotenoids are *Dunaliella bardawil* and *Dunaliella salina.*

1.8.1 Organism and life cycle.

Dunaliella is an unicellular biflagellate alga with two equal and long flagella, contains one large cup shaped chloroplast, which occupies half of the cell volume. *Dunaliella* are generally ovoid in shape 4-10 μm wide and 6-15 μm long (Ben-Amotz, 1983). 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* cell responsible for its rapid change in shape and response to osmotic changes (Ben-Amotz and Avron 1981).

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) upto 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. Initially cells can grow in low salt concentration and

upon growth they can adapt to the 2-3 fold hyper tonic and hypotonic change in salt concentrations (Ben-Amotz and Avron 1983).

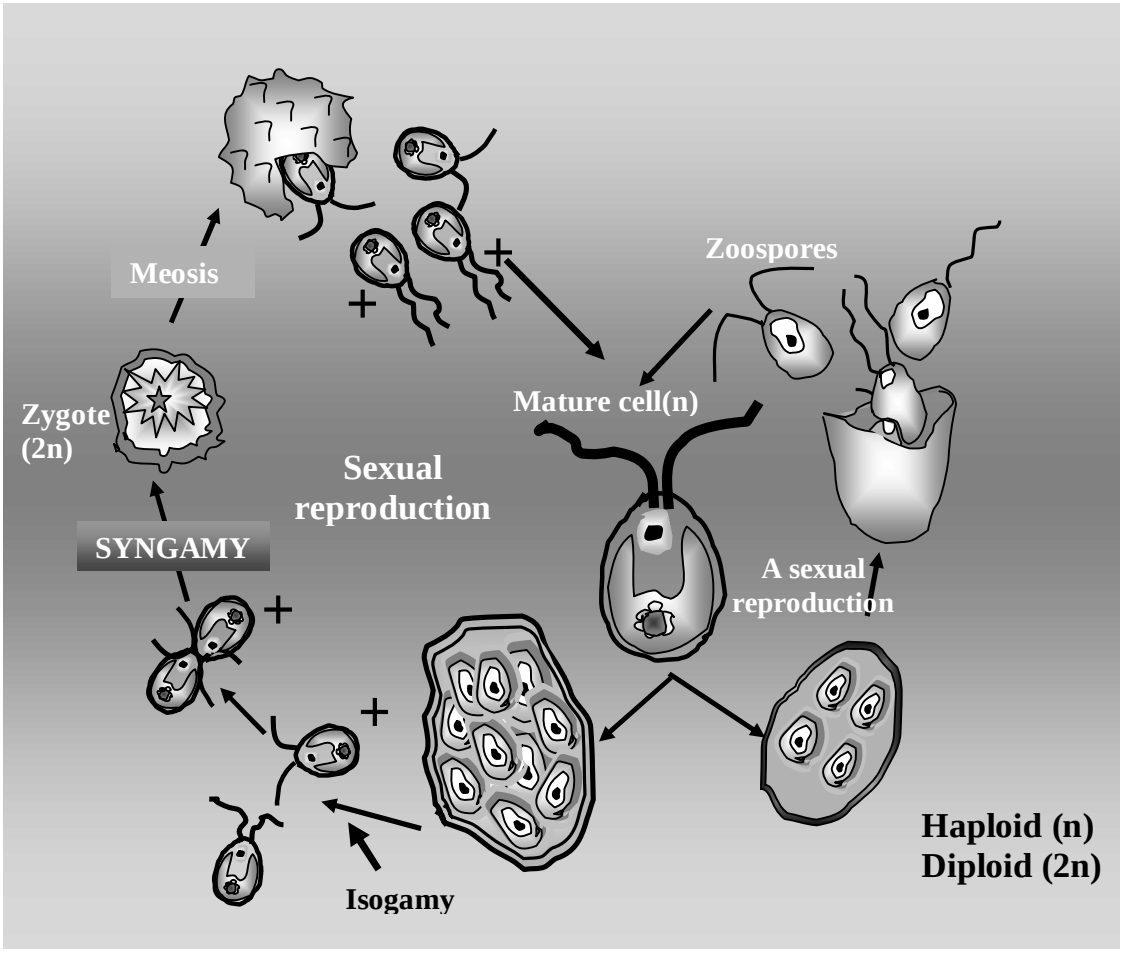


Figure 7. Cartoon showing different stages of reproduction in *Dunaliella*

1.8.2 Reproduction in *Dunaliella*.

Lack a rigid cell wall, and reproduce by longitudinal division of the motile cell or by fusion of two motile cells to form a zygote. This will undergo complex life cycle that includes, in addition to divisions of motile vegetative cells, the possibility of sexual reproduction. 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). She reported that the formation of zygote in *Dunaliella* can be induced by reduction in salt concentration from 10 to 3.0 % .

In the process of 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 upto 32 haploid daughter cells through a tear in the cell envelope. Lerche performed a series of experiment in which carotenoids rich red cells were crossed with green cells, enableling them to form zygote (Figure-7). Possibility of formation of asexual resting cysts by *D.salina* was explained by Hamburger (1905) and later Loeblich (1961) reported the formation of such cysts in media of reduced salinity. Some of the *Dunaliella* species can also develop a vegetative palmelliod stage consisting of round non-motile cells. Larche (1937) has reported this phenomenon under low salinity and Brock (1975) observed the same in benthic algal mats of Great salt lake, Utah.

1.9 *Dunaliella* cultivation, utilization and biological activity.

Number of research reports are available on the algae *Dunaliella*, most of which focus on carotenoids production and their biological activity for various health benefits both therapeutic and prophylactic. Some of those highlighting cultivations systems, carotenoids accumulation and biological activity are reviewed here.

Dunaliella bardawil has been reported to have higher content of β- carotene under high light of 1650 microeinsteins per square meter and the same was compared with white light of 27 microeinsteins. High light resulted in accumulation of β- carotene upto 10, upon illumination for 24 hours. Algae could accumulate upto 85 pg $cell^{-1}$ from 2.5 pg $cell^{-1}$ on exposure for 200 hours (Lears et al., 1990).

A study by Ben Amotz et al., (1988) reveals that exposing *Dunaliella bardawil* cells to high intensity white light during division cycle induces phytoene production up to 8% with a *cis* to *trans* β- carotene ratio of 1.0.

Large scale cultivation of two stage as described by Ben Amotz et al., (1995) consists of stage one for growth of cells for maximum production of biomass containing low β-carotene to chlorophyll ratio, followed by stage two in which culture is diluted to three volumes in order to induce carotenogenesis, which has increased production of β-

carotene to 450 mg m^{-2} d^{-1} compared to 200 mg m^{-2} d^{-1} in conventional single phase method.

Ben Amotz and Avron (1983) have reported various factors influencing accumulation of β- carotene in *D. bardawil*. Some of the factors are stress related like, high salt (upto 4.0 M), nutrient deficiency (sulphate and nitrate), light (continuous and diurnal cycle). Optimum carotenoids production was observed at 1.5 mM of nitrate, 2.0-2.5 mM sulphate and β- carotene to chlorophyll ratio of 13.1 was achieved at light intensity of 550 Kergs.Cm^{-2} Sec^{-1} intensity.

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 the condition. Light intensity of 20-50 μmol m^{-2} s^{-1} has promoted a high ratio of 9-*cis* to all *trans* β- carotene, the same was decreased when exposed to high intensity of light (Orset and Young 2000).

Rabbani et al., (1998) have shown that the synthesis of β- carotene is dependent on the triacylglycerol deposition in *D.bardawil.* Upon blocking triacylglycerol production it was observed that over production of β- carotene was blocked indicating the inter dependency. This also indicates that under normal growth conditions carotenoids pathway is not maximally active and the same can be appreciably stimulated in the presence of sequestering structures, creating a plastid-localized sink for the end product of the carotenoid biosynthetic pathway.

Jahnke (1999) has reported that ultraviolet A (320-400 nm) radiation can induce massive accumulation of carotenoids in *D.bardawil*. Supplementing various levels of UV-A radiation (38 μmol $m^{-2}s^{-1}$) during growth has shown to enhance the carotenoids: chlorophyll ratio by 80-310 %. The increase was observed in all the salinities ranging from 0.5 to 3.0 M and it was also confirmed that the effect was not due to UV-B (290-320 nm). Similar type of results were reported in case of *D.salina* under low and high light, effect of UV-A and UV-C was eliminated using potassium chromate solution (White and Jahnke 2002 ; Masi and Melis 1997).

For the first time Mokady et al., (1984) reported the safety of *D.bardawil* tested using rats for two generations,. A feed formulation containing 5.0 and 10.0 % of *Dunaliella* was fed to rats and observations were made up to three generations (12 months). Algae had no significant effect on the body weight of the animals in both the sexes. Feed

conversion efficiency of 2.7 was observed in these rats (ratio of food consumption to weight gain) against 2.54 of control diet (F_0). These rats have shown to have slightly enhanced level of serum biochemical markers (alkaline phosphatase and acid phosphatase) responsible for hepatoprotection compared to ones fed with normal diet.

Recently sub chronic toxicity of carotenoids of *Dunaliella* in F344 rats has been reported by feeding pigments for 90 days (Kuroiwa, et al., 2006). *Dunaliella* carotene were fed at the rate of 352, 696, 1420 and 2750 mg $kg^{-1}day^{-1}$ respectively, for males, and 370, 748, 1444 and 2879 mg $kg^{-1}day^{-1}$ for females using 0.63%, 1.25%, 2.5% and 5.0% in algal powdered basal diet. No symptoms of morbidity or any kind of abnormality were not seen during and after treatment. Increase in the platelet content was observed at higher dose i.e., 2.5 and 5.0 % based diets. Significant enhancement in total serum cholesterol and calcium content were observed in both the sexes. Based on the reports no-observed-adverse-effect-levels (NOAELs) was estimated to be 1.25% (696 mg $kg^{-1}day^{-1}$) for males and 5% (2879 mg $kg^{-1}day^{-1}$) for females. Results of these study indicate that the treatment of algae shows slight variation in some of the biochemical compositions, like calcium and cholesterol content. Safe concentration without any change is 2.5 % for male and 5.0 % in case of female (Kuroiwa et al., 2006).

Finney et al., (1984) reported the possible utilization of *Dunaliella* as a protein supplement in bread. They have used whole algae, carotenoids extracted algae, water-soluble fraction and water insoluble fractions for the purpose of bread making. One gram of protein and water-soluble fraction of *Dunaliella* have contributed to loaf volume equal to 10% of wheat flour. Green color was imparted in the final product in most of the case which can be removed if the same is not acceptable using suitable techniques employed for leaf extracts.

A study carried out by Giordano et al., (2000) revealed that sulfur depletion will influence the imbalance between carbon and nitrogen. This has shown abundance in alanine and there was variation in the regulation of enzymes responsible for the metabolism of carbon, sulfur and nitrogen. Adenosine 5' triphosphate sulfurylase activity increased by 4-folds, while the activity of nitrate reductase and phosphoenolpyruvate (PEP) carboxylase activities decreased by 4 - folds. Ben Amotz and Avron (1983) reported that the osmoregulation of algae is due to the metabolism of intracellular

glycerol in response to salt concentration of both intracellular and extra cellular, no leak of glycerol from cell was observed up to sodium chloride concentration of (0.6M).

Salt tolerance in *Dunaliella tertiolecta* is found to be via osmoregulation by producing glycerol. There was also enhancement of glycerol production upon increasing pH of the medium and glycerol dehydrogenase is the key enzyme for the process (Borowitzka et al., 1977).

Regulation of Lhcb gene responsible for initiation of carotenoids production as a response to the irradiance to high intensity light is reported (Webb and Melis 1995).

The carbohydrates were the only fractions to accumulate maximum under nutrient stress ranging from 57-10% of the organic fraction, however content of lipid increased with increase in the nutrient availability. Results of the study reveal that the *D. tertiolecta* has high capacity of changing its biomass productivity and biochemical composition (as protein) in semi continuous cultures, on the basis of nutrient concentration and rate of renewal (Fabregas et al., 1995).

Genetic recombination of algae *Porphyridium cruenturm* and *Dunaliella salina* by interphylum protoplast fusion was reported by Lee and Tan (1988). The same was confirmed by testing resistance to penicillin and erythromycin.

Reactivity of various carotenoid isomers of *Dunaliella* to oxygen radicals in different light intensity was reported by Jimenez and Pick (1993). It was found that all the oxidants used methyl viologen, H_2O_2, rose Bengal and 2,2'-azobis (2-amidinopropane) HCl (AAPH) could induce light mediated degradation of β - carotene and chlorophyll. It was also found that degradation of 9- cis β- carotene was faster compared to all trans β - carotene under oxidants, both in intact cells and extracted biomass. It was also shown that the ratio of degradation of chlorophyll and β- carotene remains constant.

Two-phase aqueous organic system has provided an effective methodology for enhancement of productivity of poorly water-soluble compound like carotenoids. Various solvents used were pentane to hexadecane in the order of carbon content, among these decane is found to be suitable for maintenance of viability of cells as well as production of carotenoids (Leon et al., 2003).

Ergosterol and 7-dehydroporiferasterol are reported to be the major sterols of *Dunaliella,* It contain phospholipids, glycolopids and neutral lipids as major class of lipids. Major fatty acids of algae are palmitic (31%), oleic (13%), linoleic (20%), and γ- linoleic (17%)

acid. It is also known to contain sterol peroxides (Sheffer et al., 1986). There was increase in the degree of fatty acids saturation which, reduces the fluidity and permeability of the microalgal membranes (Xin-Qing Xu and Beardall 1997).

Dunaliella has been employed as a medium for biotransformation of aromatic aldehydes viz., benzaldehyde, salicylaldehydes, methoxybenzaldehyde and mono and didichloro benzaldehyde into respective alcohols. *Dunaliella parva* has shown significant biotransformations compared to other five marine species used for the study (Hook et al., 1999).

Olmos Soto et al., (2002) have reported technique for molecular identification of β-carotene hyper producing strains of *Dunaliella* using species-specific oligonucleotides. This technique with the help of single-cell can be used as taxonomical markers at species levels and for phylogenetic characterization of natural population.

Role of Ca^{2+} on glycerol production was reported by Tsukahara et al., (1999). Results suggest that the calcium influx from extra cellular space via the stretch- activated $Ca2^{+}$ channels localized in the plasma membrane is essential for the transduction of osmotic signals in *Dunaliella* for the glycerol production.

Protein of 38-kD was identified and purified from β- carotene globules and the same was designated as carotene globule protein (Cgp). Induction of Cgp occurs parallel to accumulation of β- carotene in *D.bardawil* grown under different inductive conditions (Katz et al., 1995).

β-carotene of *Dunaliella* has shown beneficial effect on irradiance of whole body in experimental rats. Rats whole body was exposed to 4 Gy of irradiance followed by treating animals with *Dunaliella* rich in carotenoids equivalent to 50.0 mg Kg^{-1} b. w of carotene for one week. Both liver and blood was analyzed for content of β- carotene, retinal and metabolic products like, retinal ester, oxy carotenoids. Exposure to irradiance has decreased the level of β-carotene and retinal in liver compared to control animals indicating the protection effect of carotenoids by preventing oxidation at cellular level (Ben Amotz et al., 1996).

9-*cis* and *all-trans* stereoisomers of phytoene and phytofluene producing strains of *Dunaliella* were selected by treating cells with the bleaching herbicide norflurazon. Ratio of 1:1 was maintained (9-*cis*-to-*all-trans*) in serum as in case of diet, and the same

was reduced to 1:3 in liver, spleen and kidney. This was attributed to the stronger antioxidative effect of 9-*cis* phytoene over the *all-trans* isomer (Werman et al., 2002).

1.10 Cultivation in various geographical locations.

Dunaliella, was first identified by Dunal a French scientist in 1838 and he described it as unicellular biflagellate alga living in concentrated brines (Dunl 1838). It was reported to be growing near Montpellier, the costs of Meditaranian costs of France. Later in 19^{th} century, algae similar to the one reported by Dunal was observed by other biologist in hyper salinity lakes near Crimea (Butschinsky 1897), Algeria (Blanchard 1891), Lorrine, France (Florentin 1899) and Romania (Bujor 1900).

Nutritional requirement of *Dunaliella* was first studied in depth by Gibor (1956) and Johnson et al., (1968). They have found that salt requirement of *Dunaliella* depends upon the strain and it is in the range of 5-12 %. It was found that actual salt concentration in the environment from which strain had been isolated was always much higher than the salt concentration found to be optimal in laboratory conditions. From this it was confirmed that *Dunaliella*, which grew in marine conditions not necessarily means that it is optimal for cultivation. This lead to idea of cultivation of algae in simulated environmental conditions where it can perform still better and it need not struggle with competitors for existence. First quantitative occurrence of the algae was reported in lakes of Dead Sea, cell density of 4×10^4 cells ml^{-1} was observed on surface water (Kaplan and Friedmann 1970). It was also observed that high content of magnesium and calcium ions known for inhibition of *Dunaliella* (Bass-Becking 1931). It was also observed that temperature and pH play a major role in growth of the algae, small green motile cells known as 'chlorospores' use to turn to red motile cells known as 'erythrospore' in winter after dilution with rain and this formation was directly proportional to salt concentration (Nicolai and Bass Becking 1935). *Dunaliella* is one of the most environmentally tolerant eukaryotic organism known and can cope with a salinity range from seawater (= 3% NaCl) to NaCl saturation (= 31% NaCl), and a temperature range from <0 °C to >38 °C (Ginzburg 1987; Borowitzka and Borowitzka 1989).

Based on the above scientific observation and environmental utilization *Dunaliella* is mainly cultivated for commercial utility in Israel, Australia, USA and also in part of India and China, where the commercial production have begun very recently. In Australia algae is mostly exported and used in nutritional supplements and food processing.

Australia is the world leader in *Dunaliella* sales and technology, with two companies recording sales of $10 million per annum. Two major companies producing algae are Western Biotechnology Ltd. Perth, Western Australia (5 ha each production pond and total area of production is 50 ha) and Betatene Ltd. Melbourne, Victoria (production is much bigger than other company). They grow the algae in very large and shallow (approx. 20 cm deep) ponds constructed either on the bed of hypersaline coastal lagoon, or formed by artificially expanding a lagoon (Curtain et al., 1987; Borowitzka and Borowitzka, 1989). Production rate and nutrient requirement depends upon the environmental condition and varies throughout the year. Paddlewheel-driven raceway ponds are used in Israel and USA for the cultivation of *Dunaliella*. In California, aquaculturalists "rotate" farming *Dunaliella* with brine shrimp. The cultivation is highly preferred in the area where environmental conditions favors the growth for more than 8-9 months to make it economically viable (Oren 2005).

Alternate method is closed culture systems at which seems to be too expensive at this time. The advantages of closed systems such as the tubular photobioreactors are that contamination by other species of *Dunaliella* and protozoa can be virtually eliminated (Chaumont 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. This also reduces harvesting costs. On the other hand, closed systems require pumping of the culture for circulation, and *Dunaliella* species are very sensitive to shear damage. As well as this, there is also the high capital cost of these systems and a higher operating cost. Therefore open system is invariably preferred and is adopted in conditions favorable for *Dunaliella* as a source for carotenoids production.

1.11 Need for β- carotene for developing world.

Research indicates that Natural β- carotene possesses numerous benefits for the human body. Since it cannot be synthesized in human body, should be consumed from food and other ingredients. Human body converts β - carotene to Vitamin A via body tissues as opposed to the liver, hence avoiding a build up of toxins in the liver. Vitamin A is essential for the human body in that it assists the body's immune system and helps battle eye diseases, such as cataracts and night blindness, various skin ailments such as acne, signs of aging, and various forms of cancer (Agarwal and Rao 2000).

Food deficient of β- carotene, leads to deficiency of vitamin A as there are limited direct dietary sources of vitamin A. It plays an important role in the early embryonic development of all mammals, and in proper functioning of the immune system, the rod cells in the retina of the eye and mucous membranes throughout the body

Requirement of β- carotene plays a vital role in children. Some of the facts about vitamin A deficiency are,

- Roughly 400 million people in the world are at risk of Vitamin A deficiency.
- 100-200 million children were affected by Vitamin A deficiency in 2000 and recently it has reached 124 as reported by UNICEF.
- 1.0 to 2.5 million deaths per year of preschool children -upto 30% of total deaths in that age group - could potentially be averted by bringing Vitamin A deficiency under control worldwide (http://www.who.int/nut/vad.html).

In early 1980s, researchers studying Vitamin A deficiency in Indonesia observed that young children diagnosed with mild night blindness were at a significantly higher risk of dying from other diseases in the next three to four months. The greatest vitamin A deficiency occurs in South and Southeast Asia, where 70% of the children under five are affected (Gary 2000).

Recommended dietary allowance of vitamin A is around 800 μg for normal adult and 1300 μg for lactating woman. In case of children it range from 375-700 μg. Since 6 μg of β-carotene is considered as one retinal equivalent, the requirement in terms of β -carotene is 4.8 mg for adults, 7.8 mg for lactating mother and in the range of 2.25-4.2 mg for children. The same is not provided in the daily food in case of children of south and southern Asia and some of African countries due to poverty and related problems. In order to overcome these problems there is a need for renewable and sustainable source of β-carotene.

In this direction various scientific approaches have been attempted to produce β-carotene rich food ingredient of plant and microbial origin. Production of rice rich in β-carotene is one such successful biotechnological approach, which resulted in ‘golden rice’. Genetic engineering of some of vegetables, fruits as well as large-scale production of micro algae via biotechnology are some of success stories in this direction.

1.12 Biotechnological efforts to meet β- carotene requirements.

Carotenoids are intracellular components of plant cells and can be extracted into medium by fermentation process. In order to adopt this technology selection of biomass, amount of total carotenoids and in that quantity of interested carotenoids are factors which limit the process and make it economically not viable.

Other biotechnological approaches includes, increasing the biomass production containing β- carotene. This can be done in selected host but the amount of biomass enhanced remains only as academic interest as it will not be economically viable for commercial utility (Ausich 1997). Like all secondary metabolites biosynthesis of carotenoids in cells is governed by various enzymes. Use of recombinant technology can help in altering the levels and activity of enzymes, which in turn help to increase the productivity of carotenoids in plant cells. Utilization of this technique has some requirements like, availability of the gene responsible for the synthesis of carotenoids and availability of the host system, which have those genes incorporated and expressed in a stable manner through many generations of the cells (Ausich 1997).

Carotenoids are isoprenoids compounds, having seven biochemical conversion steps which are involved in conversion of precursor of isoprenoid unit 'acetyl CoA' into C_{15} molecule fernesyl diphosphate. All the genes involved in this have been isolated and characterized (Armstrong and Hearst 1996). The gene for conversion of fernesyl diphosphate to C_{20} compound geranylgeranyl diphosphate and then into first specific precursor of carotenoids phytoene have been isolated and characterized. A number of different carotenoids biosynthetic genes encoding enzymes that converts phytoene to lycopene, ζ- carotene, α-carotene, β- carotene and enzymes that further coverts β-carotene in to zeaxanthin, canthaxanthin, astaxanthin, capsorubin and capsanthin have been isolated and charecterized (Armstrong and Hearst 1996). Using these genes, various research have been undergone to develop protocols to clone and transfer to various hosts. *Phycomyces blacken* is a fungus which produces β- carotene, a protocol has been developed in which DNA from one strain could be introduced in a stable manner into different strains by micro injection (Clerda et al., 1993). Similar attempts have been successfully made to introduce DNA into the astaxanthin producing yeast *Phaffia rhodozyma* (van Ooyen and Van Ooije 1994). One which has seen maximum utility and enjoyed market among the genetic transformants of β- carotene is golden rice.

1.13 Golden rice and β - carotene.

The term "golden rice" was coined by a Thai businessman who is active in initiatives aimed at reducing the birth rate, a major cause of the food security problem (Potrykus 2001).

The first Golden Rice encodes phytoene synthase (PSY), which utilizes the endogenously synthesized geranygeranyl-diphosphate to form phytoene, a colorless carotene with a triene chromophore (Burkhardt et al., 1997; Ye et al., 2000). The second and more recent version all rely on the expression of two transgenes which encodes the bacterial CRT1, a carotene desaturase that introduces conjugation by adding four double bonds. This rice is known to contain upto 20-23 times more of β- carotene (Figure-8). The genes used for the creation of golden rice were, ***psy*** (photoene synthase), ***lyc*** (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).

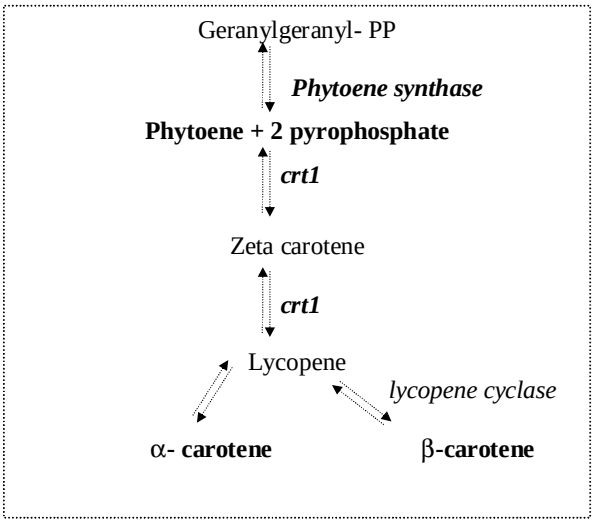


Figure 8. A simplified overview of the carotenoid biosynthesis pathway showing the enzymes expressed in the endosperm of golden rice.

Eventhough it is undergoing number of Intellectual Property Rights issue around the world, with an expectation to help vitamin A deficiency, agricultural aspects of cultivation of golden rice are under consideration for its effective utility.

Another biotechnological approach for effective production of β- carotene is through micro algae *Dunaliella,* which can produce highest amount of β- carotene along with other carotenoids under manipulated condition. Details of the cultivation and utility of algal is the aim of present research work.

In view of requirement in demand for β- carotene and possibility of utilization of algal source for large-scale production. This study was undertaken with the following objectives. The aspects of safety and efficacy of the product both biomass and isolated carotenoids is also borne in mind to add value to production and utilization of algal biomass in an eco friendly manner.

1.14 Objectives of the study.

- « Collection of germplasm of *Dunaliella* sp. from various sources.
- « Optimization of media, culture conditions, nutrient and environmental factors for the growth and production of β- carotene.
- « Identification of different carotenoids and isomers of β- carotene.
- « Downstream processing for the production of carotenoids and separation in various vehicles including edible oils.
- « Stability studies of various carotenoid fractions.
- « Antioxidant properties and bio-efficacy of various isomers of β- carotene using *in vitro* and *in vivo* models.

2.0 Material used for experiments.

2.1 Micro algae *Dunaliella* species.

Dunaliella salina (No 19-3) was obtained from Sammulung von Algen kulturen, Pflanzen, Physiologische Institute, Universitat Gottingen, Gottingen, Germany. *Dunaliella bardawil* was collected from the Sambar salt lake of Rajasthan, India.

2.2 Glasswares.

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

2.3 Chemicals used for experiments.

All the media chemicals were of analytical grade obtained from either Hi media, Laboratories, Mumbai, Qualigens Fine Chemicals, Mumbai, or SISCO Research Chemicals, Loba 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, α,α diphenyl β- picryl hydrazine, Nitro blue tetrazolium were from Sigma Chemicals Ltd., St Louis, Missouri, USA.

Solvents used for study were of analytical and HPLC grade, obtained from Qualigens Fine Chemicals, Mumbai, Rankem Pvt Ltd., Mumbai., and E.Merk (India) Ltd., Mumbai, India.

Various authenticated edible oils used for experiments were purchased from local market.

Different diagnostic kits used for biochemical analysis were obtained from Span Diagnostics Ltd., Sachin, Beakon Diagnostic Pvt Ltd., Navsari and Identity Diagnostics Pvt Ltd., Bangalore, India.

Diet used for experimental animals was prepared as per CFTRI rodent diet formula and used for experiment.

All the experiments of cultivation were repeated minimum of three times in triplicates and the values reported mean if three best values obtained throughout the experiments.

Experiments on cultivation and chemical analysis was done both in *D.salina* as well as *D.bardawil*, however pilot scale studies were done using *D.bardawil*, indigenous strain. Biological activity was studied in both the strains by *in vitro* models and *D.salina* in case of *in vivo* system.

3.0 Maintenance and production of *Dunaliella*.

3.1 Maintenance of the germplasm.

The axenic cultures were maintained in AS-100 media reported by Vonshak [1986]. Chemical composition of the medium (AS-100) is shown in Table-13. Cultures were maintained in Erlenmeyer flasks as well as in agar slants. The medium was dispensed into 150-ml flasks and sterilized at 121^0 C at 20 psi for 15 minutes. *Dunaliella salina* and *Dunaliella bardawil* were inoculated under aseptic conditions in laminar airflow cabinet.

Table 13. Chemical composition of AS-100 media.

Chemicals	**Quantity ($g\ L^{-1}$)**	**Quantity (Moles)**
$MgSO_4$	2.44	10.0 mM
$CaCl_2$	0.3	2.0 mM
KH_2PO_4	0.05	0.4 mM
KCl	0.6	8.0 mM
$NaNO_3$	1.0	11.75 mM
NaCl	50.0	0.86 M
Tris buffer	1.0	8.25 mM
Trace metal solution ($10.0\ mL\ L^{-1}$)		
H_3BO_3	3.426	0.5 mM
$CoCl_2.6H_2O$	1.215	1.0 mM
$MnCl_2.4H_2O$	4.32×10^{-4}	7.0 mM
$Zn\ Cl_2$	31.5×10^{-3}	1.0 mM
$(NH_4)_6.Mo_7O_2.4H_2O$	31.19×10^{-3}	1.0 mM
$Conc.H_2SO_4$	1.0 mL	-
*Chelated Iron solution ($3.0\ mL\ L^{-1}$)		
Na_2EDTA	1.0×10^{-4}	0.09 mM
$FeCl_3.6H_2O$	8.1×10^{-3}	0.06 µM

** Preparation of Chelated Iron stock solution:*

500mL of glass-distilled hot water was added to 10g of Na_2EDTA and stirred well to make a solution. 0.81g of $FeCl_3.6H_2O$ was dissolved in 500mL of 0.1N HCl and was slowly poured to the hot EDTA solution, with stirring. This solution was cooled before addition to the media.

3.2 Standardization of medium for *Dunaliella* cultivation.

3.2.1 Growth of Dunaliella at different pH.

The effect of pH on the growth of *Dunaliella salina* was studied using wide range of pH status. Initial pH of the medium was adjusted to 6.0 to 9.5 with an increment of 0.5 units. The flasks were inoculated with equal initial inoculum density ($0.1 \pm 0.5 \times 10^6$ cells mL^{-1}).

3.2.2 Effect of salt concentration on growth of Dunaliella.

The cultures were subjected for growth in AS-100 medium containing different concentration of sodium chloride viz., 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0 M. and the cultures were observed for growth and carotenoid content till 12 days.

3.2.3 Effect of nitrate concentration on growth of Dunaliella.

To study the growth and pigmentation efficiency of *Dunaliella* cells, the cultures were washed twice with 1.5M NaCl solution and resuspended in AS-100 medium with different concentrations of $NaNO_3$ viz., zero, half and double that of the actual concentration of original medium (1.0 g L^{-1}).

3.2.4 Effect of sulphate concentration on growth of Dunaliella.

To study the effect of sulphate depletion on growth and pigmentation of *Dunaliella***,** cells of the stock cultures were washed twice with 1.5 M NaCl solution and resuspended in AS-100 medium with different concentrations of $MgSO_4.7H_2O$ viz, zero, half and double that of the concentration in original medium (2.44 g L^{-1}).

3.2.5 Effect of light on growth of Dunaliella.

To study the effect of light on growth of algae, it was cultivated under varying light intensities. White light (tubular light, Philips Pvt., Ltd., India.) of 1.0 and 2.0 Klux, halogen light of 8.0 Klux and also direct sunlight attaining maximum intensity of 25-30 Klux (ranging from 15-35 Klux) were applied. Cultures were exposed at two stages viz., initial growing stage i.e., after three days of inoculation and on 14^{th} day after complete growth referred to as *carotenogenesis* stage. Two separate sets of cultures were exposed to different light concentrations and were harvested after seven days of exposure to light. The biomass and carotenoid contents were estimated.

3.2.6 Effect of different carbon concentrations on growth of Dunaliella.

The major source of carbon is bicarbonate (supplemented as sodium bicarbonate) and direct supplementation of CO_2 in gaseous form. Algae were cultivated under different concentrations of sodium bicarbonate, viz., 0.01 to1.0 M (at 0.01 M increments).

A set of experiments was conducted by supplementing the cultures with CO_2 at the rate of 0.5-0.75Kg $inch^{-2}$ for different durations, viz., 15, 30, 45 min…. upto 4 hours per day.

3.2.7 Effect of different concentrations of micronutrients on the growth of Dunaliella.

In order to know the effect of various micronutrients, an experiment was conducted using different concentrations of zinc (as zinc chloride), as it is one of the major components of micronutrients. Effect of different concentration of total micronutrient solution was also studied using range of concentrations viz., 2.0 mL, 4.0 mL…10.0 mL. Effect of these on growth and carotenoids pigmentation was studied.

3.3 New medium for the growth of micro algae *Dunaliella*.

Based on the above results and considering the essential nutrients and ideal growth parameters a medium with modified chemical composition was standardized and referred as 'modified medium'. Here after the term 'modified medium' refers to the medium developed in our laboratory during the study. Essential composition of the medium is as shown below (Table-14).

Table 14. Chemical composition of Modified medium.

Chemicals	**Concentration (g L^{-1})**
$MgSO_4$	2.44
KH_2PO_4	0.05
KCl	0.6
$NaNO_3$	1.0
NaCl	50.0
$NaHCO_3$	4.0
Trace metal solution (Contents are shown in Table- 13)	10.0 mL L^{-1}
Chelated Iron solution (Contents are shown in Table-13)	3.0 mL L^{-1}

3.4 Carotenoids production in different media.

Growth rate and carotenoids accumulation in modified medium was compared with two well-known media used for cultivation of *Dunaliella* i.e., AS-100 medium - (Vonshak 1986) and BA - medium (Ben-Amortz et al., 1983). Compositions of AS-100 medium and BA- medium are shown in Table-13 and Table 15 respectively.

Table 15. Chemical composition of BA- medium.

Chemicals	**Concentration (g L^{-1})**
$MgSO_4$	1.23
KH_2PO_4	0.273
$CaCl_2$	0.44
KNO_3	0.5
NaCl	87.8
$NaHCO_3$	4.2
EDTA	1.2×10^{-3}
$FeCl_3$	8.0×10^{-3}

3.5 Measurement of growth and productivity.

a. Cell number measurement.

Growth of *Dunaliella* was measured in terms of cell number and counted using hemocytometer after fixing the cells by adding a drop of diluted hydrochloric acid (1% v/v). Cell count was expressed as number of cells mL^{-1}. In order to know the growth optical density (OD) was also measured at 660.0 nm in case of pilot scale studies.

b. Fresh weight (FW) and Dry weight (DW).

The fresh weight of algae was determined after centrifugation of the cells at 3000 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 at $60 \pm 2.0\ ^0C$ till a constant weight was attained on a glass petridish. Biomass was expressed as gram FW per litre of culture and gram DW per litre of culture.

c. Packed cell Volume (PCV)

Culture suspension (~10 mL) was taken into a graduated centrifuge tube and total volume of packed cell was determined after centrifuging the culture at 3000 rpm for 10 minutes. The PCV was expressed as percentage of the volume of culture in the tube.

d. Estimation of pigments

i. Chlorophyll content

Dunaliella cells were taken into a graduated tube and centrifuged at 5000 rpm for 15 minutes. The pellet was extracted in acetone by homogenizing using pestle and mortar and extract was kept in dark for 1 hour. This was subjected to vortexing for few minutes followed by centrifugation under low light at 3000 rpm for 10 minutes. The absorbance of supernatant was measured spectrophotometrically (Shimadzu-160A, Japan) at 645 nm and 661.5 nm against acetone blank. Concentration of chlorophyll a, b and total content were calculated by the equation of Lichtenhaler (1987) and expressed as (µg/mL).

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

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

$$\text{Total chlorophyll} = \text{Chl a} + \text{Chl b} = 7.05 \text{ X } OD_{645.0} + 18.09 \times OD_{645.0}$$

ii. Carotenoids content

Carotenoids were extracted in acetone as mentioned above and analyzed spectrophotometrically by measuring the absorbance at 470nm. Content of carotenoids was calculated according to method of Davis (1976) using extinction co-efficient 2500. Content of carotenoids were also expressed as pico gram cell $^{-1}$.

Carotenoids content (mg/vol.) = $(OD_{450} \times$ volume of the sample taken)/ 2500

3.6 Modification of culture conditions for carotenogenesis in outdoor conditions.

In order to induce accumulation of carotenoids, algal cells were subjected to various stress conditions like light, salt and nutrients (nitrates and phosphate) after growth period of 14 ± 2 days.

3.6.1 High light stress.

To study the pigmentation efficiency of *Dunaliella* cells in outdoor conditions, two weeks old culture was exposed to (a) temperature (25 ± 1^0 C) and light (2000 lux) controlled room under illumination with the cool white fluorescent lamps and (b) sunlight under light intensity in the range of 30 to 36 Klux, at a varying temperature of 25 to 33° C. The cell count and carotenoids contents were estimated using methods described earlier.

3.6.2 Nitrate deficiency stress.

To study the growth and pigmentation efficiency of *Dunaliella* cells under nitrate deficiency, cultures grown for two weeks were washed two to three times with 1.5M NaCl in water and resuspended in AS-100 medium by substituting sodium nitrate concentration with sodium chloride (1.0 g L^{-1}). Culture was observed for 7 days under direct sunlight at the end of which, the biomass and carotenoids contents were estimated.

3.6.3 Phosphate deficiency stress.

The stock cultures of *Dunaliella* were washed two to three times with 1.5 M NaCl solution and resuspended in AS-100 medium by substituting KH_2PO_4 with KCl (0.05 g L^{-1}). Culture was observed for seven days under direct sunlight and, the biomass and carotenoids contents were estimated at the end of seven days.

3.6.4 Salt stress.

To study the effect of depletion and enhanced content of sodium chloride on growth and pigmentation of *Dunaliella* cells, two weeks old cultures were exposed to increasing amounts of NaCl from 1.5M to 3.0 M. In another batch, the culture was diluted to bring down the content of sodium chloride to one third of original concentration. Both the set of cultures were monitored for cell count and β - carotene contents at regular intervals.

3.6.5 Effect of culture height on growth and carotenoids accumulation.

In order to know the optimum culture height for both production and carotenogenesis an experiment was done by growing *Dunaliella* cultures in different height. Fourteen days old cultures were spread in to height of 5, 10 and 15 cm in 1.0 m^2 circular tank under direct sunlight and culture production in terms of wet biomass weight g L^{-1} and carotenoids contents in terms of mg L^{-1}were measured.

3.7 Scale up studies.

Once the culture was stabilized for its growth in the laboratory under controlled conditions of temperature (18 ± 2^0C) and light intensity (2.0 ± 0.2 Klux 24 hours), scale up studies were carried out by cultivation of *Dunaliella* in 250mL flask, later in 500mL flasks and in 1L, 5L and 20L carboys, which was then transferred to glass rectangular tanks of 20 and 25 L culture capacity. During transferring of cultures from indoor to outdoor, initially carboy were closed with cotton plugs and the closures was removed slowly during 2^{nd} and 3^{rd} cycle followed by maintaining the culture in completely open carboys system (Schematic representation of scale up process depicted in Figure -10).

In continuation of scale up studies, *Dunaliella* culture was grown in circular cement tank of 500 L capacity with culture volume of 200 L. Initially AS-100 medium (Table-13) was used and the cultures were supplied with air mixed with CO_2 by means of a float. Culture tank was maintained in natural environmental conditions of temperature and light. Direct sunlight on the culture is avoided for the vegetative growth phase. Occasional mixing of culture was found necessary to prevent settling of cells at the bottom of the tank. Mechanical mixing was provided by means of rotating arms (30-cm length × 5-cm width) at 15-20 rpm. The schematic representation of a culture tank of 100 L culture capacity with a provision for agitation is depicted in Figure-11.

3.7.1 Cultivation in raceway ponds.

Utilization of raceway ponds for algal cultivation provides benefits over other mode of cultivation. Raceway cultivation provides benefits like, easy mixing of nutrients and can also provide uniform exposure of cultures for sunlight. Initial trials were done in raceway of 5.0 M^2 of culture capacity (schematic representation of pond design is as shown in Figure-8a) of 500 L for vegetative phase. Initially 250 L media was inoculated with 40 ± 2 L of green culture grown for 14-15 days, having cell count of >6.0 × 10^6 cells mL^{-1} under

50 % cut-off light (12-15 Klux under local condition) provided by agricultural shading net (Figure-10). This was grown till attain maximum cell count was attained, with mixing of culture using paddle wheel at 10-12 rpm, for three minutes, twice or thrice a day depending on the light intensity. Upon attaining the maximum cell count this was transferred into 15 M^2 raceway pond by diluting the culture with 1:1 water containing 2.0 % (w/v) sodium chloride and exposed to direct sunlight and growth was monitored along with carotenoids content of cells. The culture was harvested by flocculation after attaining maximum carotenoids.

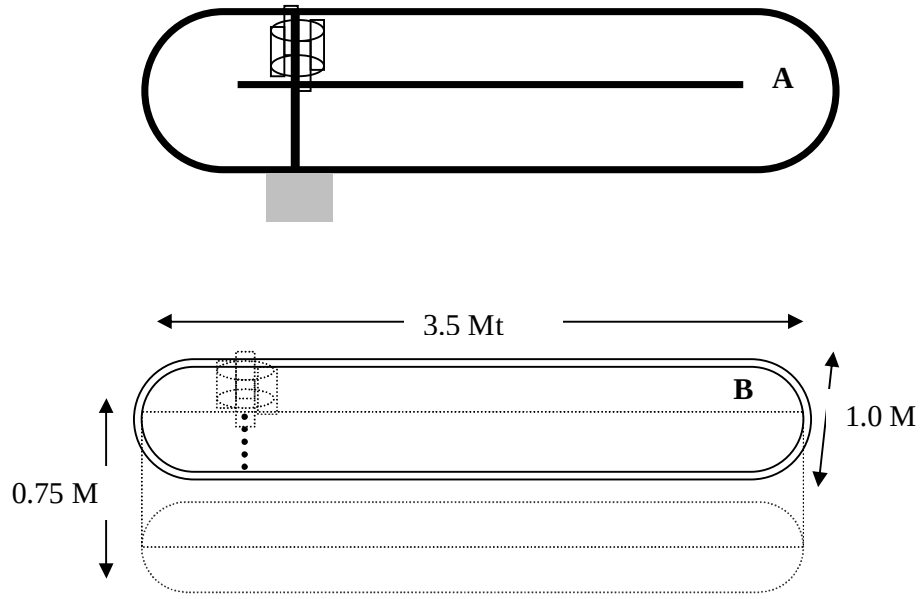


Figure 9. Model of raceway pond of 5.0 M^2, A- Aerial view and B- cross-section and dimension of pond.

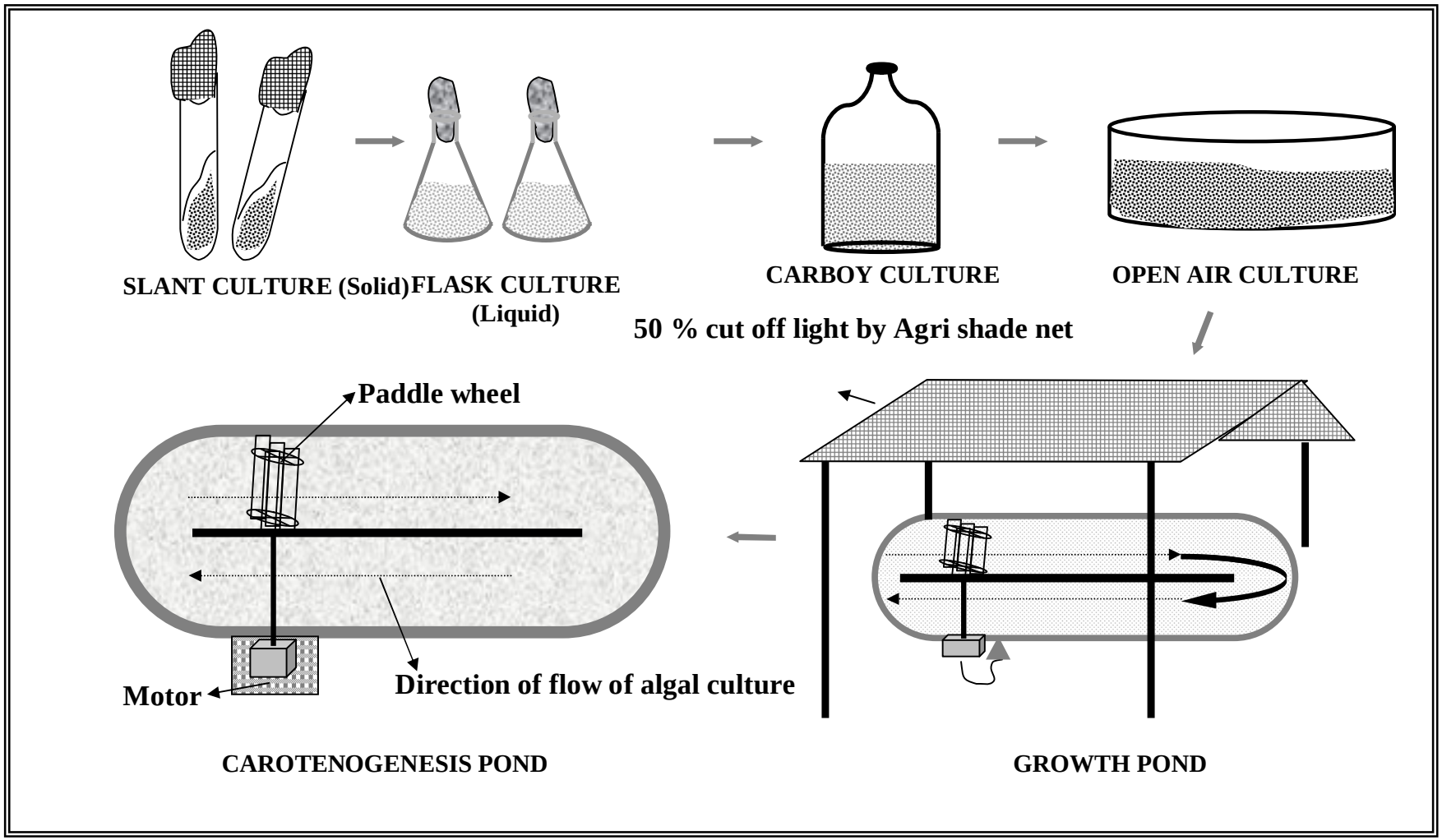


Figure 10. Schematic diagram for scale up process in production of *Dunaliella* biomass.

These cultures were transferred to raceway type pond of upto 5000L capacity with culture volume of 2000L. Cultures were grown in these systems for 15 days and once the maximum growth was attained it was transferred into carotenogenesis pond. During the growth, contamination with protozoa, diatoms and *Chlorella* was observed initially, particularly during rainy season and which could be controlled by the salt (2.0% w/v) and bicarbonate concentrations (1.0 g L^{-1}).

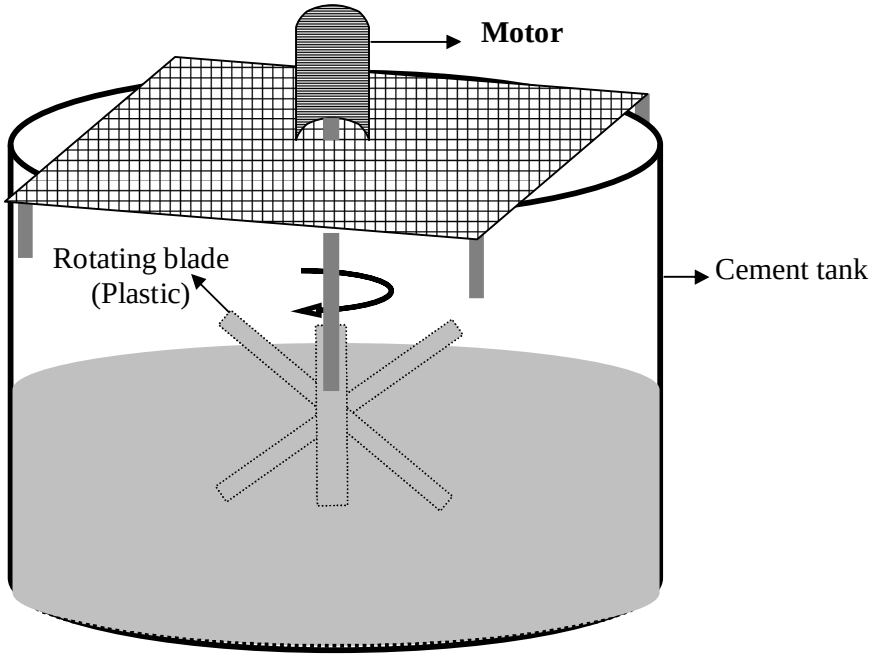


Figure 11. Schematic diagram of set up used for 100L capacity in cement tank for growth of *Dunaliella*.

a. Fertilizer based medium.

A simple medium containing commercial fertilizer ('SUPHALA' M/s Madras fertilizers and chemicals., Chennai, India., containing N: P: K in the ratio of 15:15:15) as a supplement of N, P, K was tried for the cultivation of *Dunaliella.* An experiment was conducted with varying concentration of essential nutrients viz., Suphala (0.5 to 2.0g L^{-1}), $MgSO_4$ (1.22 to 4.88 g L^{-1}), $NaHCO_3$ (0.5 to 2.0 gL^{-1}) and NaCl (25 to 100 g L^{-1}). Growth of cells and carotenoid accumulation were recorded for three cycles.

b. Growth in out door condition in production scale.

Based on the above experiments, a trial was conducted in order to grow *Dunaliella* in out doors in 500 L and 2,000L capacity raceway tanks at an average temperature of (25± 2^0C) and an average light of 25 ± 3.0 Klux at midday and ~ 15 Klux during rest of the

day. Initial growth phase was allowed under 50 % cutoff light provided by using agricultural polythene shade net (Figure - 8), which took 14-16 days for maximum growth. Further, this was subjected to carotenogenesis by exposing to direct sunlight after diluting with water containing 2.0 % common salt.

3.8 Harvesting of algal cells.

As the *Dunaliella* cells are 4-10 μm wide, 6-15 μm long and flagellate organisms with high motility, it is difficult to harvest these algae unlike other algal forms. Some of the techniques tried for harvesting includes, batch and continuous centrifugation and flocculation followed by filtration or centrifugation.

3.8.1 Batch Centrifugation.

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

3.8.2 Online centrifugation.

Online centrifugation was also used to study the efficiency in comparison with batch operation. A batch of 500 L culture was fed to online centrifuge (M/s Sharples, UK) at the rate of 8-10 L/hr, at average speed of 7500 ± 1500 rpm. Biomass which was retained in inner side of cylindrical tube was collected and supernatant was recycled.

3.8.3 Flocculation technique for harvesting of Dunaliella.

Flocculation was the other technique used to settle these motile algal cells reducing its separation from the culture medium. Various flocculants employed were, $Al_2 (SO_4)_3$, $FeCl_3$, and chitosan. pH variation was made to bring cells closer and aggregate to form bigger flocci, which can settle down due to gravity. In all the experiments, for initial trials, 100mL of the culture was used in a glass-measuring cylinder. An experiment was carried out with the addition of varying concentrations of $FeCl_3$ (0.1, 0.2 up to 0.5 0.75and 1.0mM), $Al_2 (SO_4)_3$ (0.2, 0.4, 0.6, 0.8, 1.0 and 2mM) and chitosan (0.01, 0.02, 0.03, 0.04, 0.05 & 0.10 mg L^{-1}) or by adjusting the pH of the culture to 9.5, 10, 10.5, 11.0, 11.5 and 12.0. The settled cells were collected and weight of the biomass along

with the β-carotene concentration was calculated to find out the effect of these flocculants on carotenoids.

3.9 Drying of *Dunaliella* biomass.

In order to study the relative effectiveness of various drying processes, different techniques were studied, so that the process that gives most stable product can be utilized for drying and storage of biomass. For this, oven drying, sun, shade, freeze and spray drying were adopted.

***Sun drying*:** This was done by spreading the wet biomass of *Dunaliella* as a thin layer of ~0.5cm thickness on a aluminium tray. Samples were exposed to direct sunlight (~27 ± 3 ^{0}C). Sample was analysed for moisture content and also for the content of pigments. Time taken for complete drying and total loss of carotenoids were recorded and percentage loss of pigments were calculated.

***Oven drying*:** This was done using hot air oven (Sanyo, Electric Biomedical Co., Ltd, Japan). Freshly harvested *Dunaliella* was spread as a thin layer in six inch petriplate and dried in oven at 45 ± 2^{0}C and the sample was analysed once in 30 minutes for the content of moisture and content of pigments. Time taken for complete drying and total loss of carotenoids were recorded.

***Shade drying*:** This was done in a similar manner as described above. However, the drying was done in shade avoiding direct sunlight (18 ± 2^{0}C).

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

Spray drying: Algal biomass was subjected for spray drying using Bowen spray drier (Bowen Eng Inc., New Jercy 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 ^{0}C and outlet was maintained at 80 ± 5.0^{0}C. Sample was fed using the blower at an air pressure of 2.2-2.5 kg in^{-2}.

One of the experiment was done using nitrogen as carrier of sample at 1.5-1.8 kg in^{-2} keeping the other conditions similar as above.

IR drying: Infrared drying was done using online drier with continuous and pulsed IR radiation (Indigenous drier fabricated by CFTRI equipped with 1.5 HP motor) to the biomass spread as 0.25 and 0.5 cm sheath on a glass support and the content of

carotenoids and moisture was analyzed at regular intervals. Temperature was maintained below 42 ± 2.0^0C in all the experiments.

3.10 Downstream processing of carotenoids from *Dunaliella* biomass.

3.10.1 Extraction in solvents.

Carotenoids were extracted from freeze-dried samples of *Dunaliella* biomass using mixture of polar and non-polar solvents. Five volumes of solvent was taken with the sample (5.0gm) in mortar along with glass powder and subjected for mechanical grinding for 2-3 minutes and the same was transferred into Borosil glass tubes and sonicated using MS-72 probe producing diameter 2.0 mm, with 70G horn of amplitude 200µ m_{ss} (Bendalin, Sonoplus ultrasonic Homogenizer, CE electronics, Berlin). This process was repeated 2-3 times or till the sample turned pale or white. Whole process was carried out under yellow light in order to minimize the loss due to photo degradation. This was centrifuged at 3000 rpm for 5 minutes and the supernatant was collected and absorbance was measured at 450 nm spectrophotometrically. The solvents used were acetone, methanol, petroleum ether, n- hexane, chloroform, isopropyl alcohol, ethyl acetate and n-hexane with isopropyl alcohol (1:1).

3.10.2 Extraction in edible oil.

Freeze dried, moisture free samples of *Dunaliella* were mixed with edible oil in the ratio of 1:5, vortexed for 3 minutes and kept under low light for 2-3 hours followed by extraction by pulsed sonication for 4 -5 minutes. Centrifuged at 5,000 rpm for 10 minutes and supernatant was decanted. Carotenoids content was measured immediately using OD_{450} nm, spectrophotometrically. Different edible oils used were coconut, gingilly, palm, olive, groundnut, rice bran, mustard and sunflower oil.

3.11 Stability of carotenoids in different edible oils under different storage conditions.

The total carotenoids were extracted using n-hexane and isopropyl alcohol (1:1). Concentrations of carotenoids were estimated spectrophotometrically by measuring absorption at 450.0 nm (Devis 1976). Known concentration of sample extract were dissolved in 100 mL each of edible oils (coconut oil, gingili also known and Sesame oil, palm, olive, groundnut, rice bran, mustard and sunflower oil) and divided to six parts each containing 15 mL and stored under different conditions mentioned below,

a. Without preservative and inert gas
b. With 0.01% BHA (preservative) under normal condition
c. Under nitrogen without BHA
d. With 0.01% BHA under nitrogen.

The samples under above category were each stored at three different conditions, viz., a) in normal light at room temperature (24 –29^0 C), b) 24 hour dark at room temperature, c) in dark at 4^{0}C (in refrigerator). In one of the sets, carotenoid extract was kept in glass container without oil under different conditions mentioned above for comparative purpose. All the samples were stored in 20 mL amber colored vials with rubber closure of pharmaceutical grade. In case of samples stored under nitrogen, samples were drawn using hypodermic needle and nitrogen was flushed from other needle simultaneously every week. All the analysis was done under dim yellow light in order to minimize the photodegradation.

3.11.1 Analysis of the samples for carotenoids content.

Samples of the above storage conditions were analysed for the content of carotenoids spectrophometerically. Absorbance at 450 nm was measured in hexane against the oil dissolved in same concentration to serve as blank. The concentrations of carotenoids were calculated by Devis' (1976) formula mentioned afore (3.5.d.ii).

3.11.2 HPLC analysis of samples.

HPLC of the samples was done in three months intervals to know the carotenoids degradation pattern using following conditions. HPLC system consists of Hewlett Packard, (Palo, Acto CA) equipped with a quaternary pump fitted with a zorbax C_{18} – silica (Hewlett Packard) analytical column (25cm x 4.6mm I.D 5µ particle size). The injection system (Rheodyne) used was of 20µl capacity. Detection was done by an HP 1250 series variable wavelength detector at wavelength of 450.0 nm. The gradient mobile phase consisted of acetonitirile and chloroform with a flow rate of 1.0mL min^{-1}. The elution program involved a linear gradient from 80 to 20% of acetonitirile for 0-5 min and 20 to 80% of chloroform for 5-15 min and again 80% of acetonitrile for 15-20 min followed by 5 min equilibrium. Total programme time was of 25 min. The compounds were quantified using HP chemstation software.

3.11.3 Kinetic analysis.

Degradation rate of β-carotene was determined based on the method described by Tang and Chen (2000). The correlation coefficient (R^2) was measured from the plot of logarithm of amount of β -carotene *verses* time. The degradation rate constant (day^{-1}) was calculated using the following formula:

$$R^2 = -\ln (CA/CA_0)/t$$

Where CA: the total concentration of β-carotene after storage; CA_0: the initial concentration of β-carotene and t: storage time.

4.0 Chemistry and Biochemistry of *Dunaliella*.

4.1 Identification and estimation of carotenoids and chlorophyll by spectrophotometry.

As explained before, spectrophotometric determination was done by measuring optical density of β -carotene at 450.0 nm and concentration was calculated using extinction coefficient as 2500. In order to know the fingerprinting of carotenoids and chlorophyll, spectrum was measured using wavelength of 200-800 nm in acetone (details of measurement is given in section *3.5.d*).

4.2 Identification of carotenoids by TLC.

Acetone and other solvent extracts of the *Dunaliella* cells were spotted on silica gel TLC readymade plates (Merck Ltd., 20 × 20 cm, 0.5mm thickness) and separated using different solvents systems as mobile phase, including, methanol, acetone, ethyl acetate, hexane and petroleum ether in different proportions. R_f values for each spot were calculated and individual spots were scraped from the TLC plate and extracted with acetone or chloroform, absorption spectra of the same was measured to confirm the compound.

4.3 Determination of carotenoids isomers.

The acetone extract as well as β - carotene separated by thin layer chromatography was subjected to isomerization using iodine at high intensity fluorescent light. This was achieved by exposing the carotenoids (1.0 mg mL^{-1}) in petroleum ether (40-60^{0}C) in quartz cells to fluorescent UV light (254 nm) for 3 hours. The exposed fraction was subjected for UV- Visible scanning for photometric absorption in the range of 200.0 - 800.0 nm. The *cis* peak absorbed at 340.0 nm was recorded.

Further, the same was confirmed by HPLC, which has shown the separation of *cis* from *trans* isomer. HPLC was conducted on a C_{18} silica column (Shimadzu, 3.6×300mm) using Shimadzu LC 10A system, using methanol: acetonitrile: dichloro methane (50:41:9) as mobile phase at a flow rate of 1.0 mL min^{-1}.

4.4 Separation of carotenoids by preparative TLC.

Solvent extract of *Dunaliella* was subjected to preparative TLC using activated silica gel plates (0.75 mm thickness, manually prepared) with various solvent systems like acetone: methanol, acetone: hexane, acetone: hexane: ethyl acetate in different proportion. Pigments were identified based on the reported retardation factor values.

4.5 Separation of carotenoids by column chromatography.

The total carotenoids from the freeze-dried biomass were extracted using acetone, ethanol and petroleum ether, all the extracts obtained were pooled. Partitioning of carotenoids between xanthophylls and carotenes was carried out using different solvents and separated carotene and xanthophyll fractions were subjected to column chromatography (Jungalwala and Cama, 1962). The carotene fraction was resolved on neutral alumina column (45×1.5cm), when the column was eluted (gradient elution) with light petroleum containing increasing amounts of diethyl ether. Xanthophyll fraction was resolved on methanol deactivated alumina column (45×1.5cm) and eluted with light petroleum containing increasing amount of acetone. Three mL each of eluted fractions were collected and their U.V and visible spectra were determined. Fractions showing similar spectra were combined and concentrated and subjected for further analysis.

4.6 Separation of carotenoids by preparative HPLC.

Extract of algae was analyzed by preparative HPLC, using ODS (Bonda pack) column with an isocratic solvent system consisting of Methanol: Acetonitrile: Dichloromethane (50:41:9) at a flow rate of 10mL min^{-1}. Content of carotenoids and chlorophylls were detected at 450 and 660nm respectively. Mixture was separated as three major peaks. These were collected separately and confirmed by UV-visible spectrophotometer.

4.7 Identification of different carotenoids by HPLC.

Different solvents systems were tried for HPLC using reverse phase methodology for separation and identification of carotenoids and xanthophylls of algae. Among the different solvents used only few produced reproducible and reliable separation. Some of the important ones are as described below.

Initially, estimation by HPLC was done on reversed phase silica C_{18} column (Shimadzu, 4.6×300mm) using Shimadzu LC 10A system, using methanol: acetonitrile: dichloro

methane (50:41:9) as mobile phase at a flow rate of 1.0 mL min^{-1}. Carotenoids and chlorophylls were detected at 450.0 and 660.0 nm respectively. The total carotenoids were identified with respect to area of standard all *trans* β- carotene.

Another method of HPLC was tried for identification using conditions similar to above with 100 % methanol as mobile phase.

Yet another method was followed by gradient elution technique. The system consisted of Hewlett Packard (Palo, Acto CA) equipped with a quaternary pump fitted with a zorbax C_{18} (Hewlett packard) analytical column (25cm x 4.6nm I.D 5μ particle size). The injection system (Rheodyne) used was of 20μl capacity. Detection was done by an HP 1250 series variable wavelength detector at wavelength of 450.0 nm. The gradient mobile phase consisted of acetonitirile and chloroform with a flow rate of 1.0mL min^{-1}. The elution program involved a linear gradient from 80 to 20% of acetonitrile for 0-5 min and 20 to 80% of chloroform from 5-15 min and again 80% of acetonitrile for 15-20 min followed by 5 min equilibrium. Total programme time was of 25 min, compounds were quantified using HP chemstation software. 10 μL samples were injected after dissolving the same in the mobile phase.

Apart from chloroform and acetonitrile other mobile phase were tried in order to separate the carotenoids like, methanol (100%), acetonitrile: methanol: dichloromethane, and methanol: acetonitrile (1:9) using silica C_{18} column.

4.8 Identification of carotenoids by MALDI (Matrix assisted laser desorption ionization).

The whole cell extract of n-hexane and isopropyl alcohol (1:1) was subjected for MALDI analysis. This was carried out for the acetone extract of both *D.salina* and *D.bardawil* using α- cyano 4 hydroxy-cinnamic acid (in 50% acetonitrile and 50% water containing 0.1% Trifluro acetic acid) as matrix (1:1). The instrument used was from KRATOS Analytical, at Molecular Biophysics Unit, Indian Institute of Science, Bangalore.

4.9 Identification of carotenoids by MS.

Extracts of algae in acetone, n-hexane: IPA and ethyl acetate were subjected to direct MS analysis (MS-Qtof.ultima, No.Qtof GAA 082, Waters Corporation, Manchester, UK). It was done by positive electro spray ionization (ESI) using time of flight (TOF) mode. Capillary voltage was 7000 V, fragmentation voltage was 70 mV, using drying temperature of 210 ^{0}C. Gas flow was (N_2) was 6.5 mL min^{-1}, m/z scan was at the range

of 100-1000 daltons, Scan rate was 1.0 seconds cycle^{-1}. Algal extracts (100 µL) in different solvents was injected directly into the system.

Standard all *trans* β- carotene was analyzed by both positive electro spray ionization as well as atmospheric pressure chemical ionization (APCI) in order to know the variation in Mass pattern and further used for quantification.

4.10 Identification of carotenoids by and LC-MS.

LC-MS was also done for acetone extracts of both *D.salina* and *D.bardawil*. The instrument used was Hewlett Packard 1100 MSD series mass spectrometry with Hewlett Packard HPLC system fitted with silica C_{18} column. The volume of sample injected was 6µl, the mobile phase used was 100 % methanol at the flow rate of 0.2 ml min^{-1} and detection wavelength was 450.0 nm. MS was done using positive ion spray mode and the mass spectra were recorded under electron impact ionization at 70 eV electron energy with a mass range from 100 - 1000 at a rate of one scan second^{-1}.

Another set was done using Waters 2695 series LC fitted with MS-Qtof.ultima (No.Qtof GAA 082, Waters corporation, Manchester, UK) system. Liquid chromatography was performed using C_{18} column (Waters, 4.6 × 250mm), using methanol: actonitrile: dichrolomethane (70:20:10) at flow rate of 0.6 mL min^{-1} with photodiode detector. MS was done using positive electro spray by Time of flight (9.10 kV) technique and mass spectra was recorded under electron impact ionization at 70 eV electron energy and mass was recorded in the range of 100-1000.

4.11 Estimation of proximate composition of *Dunaliella* .

4.11.1 Moisture content.

Moisture was estimated by drying a known biomass of the algae in hot air oven at 70 ± 2^{0}C to constant weight. This was cooled in desiccator and weighed. The loss in weight was expressed as percentage of moisture (AOAC, 1997).

4.11.2 Ash content.

A known weight of the algal material was initially charred on a tared silica crucible and placed in a muffle furnace at 400- 450 ^{0}C for 6 hours till the charred material became white. The dish was allowed to cool to room temperature in a desciccator and reweighed. The difference in weight was taken as total ash content (AOAC, 1997).

4.11.3 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 residue was filtered and washed with distilled water and quantitatively transferred to a previously weighed silica crucible. The crucible was dried at 110^0C for 3 hours and weighed again. The difference in weight before and after drying was expressed as crude fiber content.

4.11.4 Estimation of protein.

Protein content of the biomass was calculated using total nitrogen content, which was estimated by micro-Kjeldhal method (Pearson, 1982). A known amount of algal sample was digested in concentrated sulphuric acid with catalyst (copper sulphate, potassium sulphate and selenium dioxide) for 6-8 hours, until the solution became clear. 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 standard N/70 hydrochloric acid. The nitrogen content was expressed as crude protein by multiplying with factor 6.25 (Sadasivam and Manikam, 1992).

4.11.5 Estimation of lipids.

Known amount of sample was taken and extracted with low boiling petroleum ether (40-60^0C) in a soxhlet extraction apparatus for 8-10 hours. The solvent was evaporated and the lipids were estimated gravimetrically (AOAC, 1997).

4.11.6 Estimation of carbohydrate.

Known amount of algal biomass was suspended in 2.0 mL distilled water followed by drop wise addition of concentrated sulphuric acid while keeping the sample in cold water for half an hour and then for another half an hour at room temperature. The reactants contained 0.5 mL of sample to which 0.3 mL of 3% (w/v) phenol and 1.5 mL of concentrated sulphuric acid was added rapidly. After cooling, the brown colour developed was measured at 490 nm against reagent blank. Glucose (5-25 µg) was used as standard. The values were expressed in terms of percentage dry weight. The amount of sugars was calculated from standard graph (Pearson, 1982).

4.12 Biotic elicitation studies.

4.12.1 Microbial Cultures and preparation of elicitor extracts.

All the fungal strains were obtained from department of Fermentation Technology and Bioengineering, CFTRI, Mysore, India. All the fungi were grown in 250-mL flasks

containing 50 mL of potato dextrose agar medium and were incubated at 37 ± 2 ^{0} C. Twenty days old cultures were autoclaved and the fungal mat was separated by filtration. The mat was then washed several times with distilled water. The water extracts of the fungi were prepared similarly as explained above and were used for the study in different concentrations.

4.12.2 Measurement of growth and carotenoid content.

Both *D.salina and D.bardawil* were grown in modified medium in 500 mL flask containing 100 mL culture each for 14 days at 23 ± 2 ^{0} C temperature and 24 hours light of 2.5-3.0 Klux in triplicate. The growth was recorded by centrifuging 10.0 mL of culture at 8000 rpm for 10 min and measuring the wet biomass weight. The biomass was further extracted with n-hexane: Isopropyl alcohol (1:1) for carotenoid estimation. The carotenoids were analyzed by measuring the absorbance at 450 nm and 645 and 661.5 nm respectively for chlorophyll a and b (Devis 1976). The carotenoids content was further estimated by using gradient HPLC using afore mentioned system by gradient elution of acetonitrile and chloroform.

The stock solutions of microbial extracts (25 mg dry cells equivalent mL^{-1} of aqueous sodium chloride 5.0 %) of fungal origin were administered to culture of *Dunaliella* at 2.5, 5.0 and 10.0 % level, which are equivalent to 0.05, 0.1 and 0.2 mg mL^{-1}.

4.13 Atorvastatin intervention studies on production of carotenoids in *Dunaliella*.

Both *D.salina* and *D.bardawil* were grown in the modified medium using initial pH of 7.0. Different concentrations of statin was (5, 10 and 25 mg L^{-1}) added on 3rd, 6th, and 9th day. The carotenoids were extracted in n-hexane: isopropyl alcohol (1:1) and quantified spectrophotometrically using the formula given by Devis (1976) measuring the optical density at 450.0 nm and further confirmed by gradient HPLC. Squalene was extracted using n-hexane and quantified by GC-MS.

4.13.1 Analysis of carotenoids by HPLC.

The carotenoids content was further estimated by using Hewlett packard system fitted with Zorbax C_{18} (Hewlett Packard) analytical column (25cm x 4.6nm I.D 5µ particle size). Solvent system consisted of gradient elution with acetonitrile and chloroform as mentioned previously.

4.13.2 Analysis of squalene by GC-MS.

Lyophilized biomass of *Dunaliella* species was sonicated for a total period of 30 minutes (intermittently 20 sec pulsing) in hexane, centrifuged and the supernatants were pooled

and concentrated. The hexane extract was purified by column chromatography on silica gel by eluting with hexane. The sample was analyzed using ELITE -5 capillary columns (0.5 x 30.0mtrs) with Helium as carrier gas. The oven temperature was kept initially at 130°C for 5 min and then increased to 200 °C at the rate of 8°C min^{-1}, at which the temperature of the column was maintained for 2 min and then increased upto 280°C at the rate of 5°C min^{-1}, at which the temperature of the column was maintained for 15 min. The injector port and the detector temperatures were 240°C and 250°C respectively. The mass spectra were recorded under electron impact ionization at 70 eV electron energy with a mass range from 40- 600 at a rate of one scan $second^{-1}$. Squalene was identified by comparing the fragmentation pattern with authentic standard (Sigma, USA). The squalene content was identified by comparing spectrum with NIST library.

4.13.3 Assay of lycopene cyclase.

Protein concentrations of different fractions were estimated by the method of Bradford (1976). Protein concentrations were also estimated from SDS/PAGE by comparison with the staining intensity of standard markers form Gene-I Biotechnology Ltd., Bangalore. Relative amount of expressed lycopene cyclase band on staining SDS/Polyacrylamide gels was determined using flatbed scanner with densitometric software. Further lycopene cyclase assay was done using HPLC. Samples were prepared in soybean lipids containing 20% of L-α-phosphotidylcholine. The lipid was first dissolved in chloroform and solvent was removed by stream of nitrogen gas followed by addition of 50 mM of Tris/maleate buffer (pH 6.5), sonicated for 30 minutes in dark at 0^0 C to form a suspension and to this suspension lycopene was added.

The assay mixture contained 150 µL of lipid suspension including the substrate, NADP (5mM). Enzymes were added (~ 25 µg of protein) in 59 mM Tris/maleate buffer, pH 6.5 and incubated in dark at 30^0C for 5h. Reaction was terminated by adding 1.5 mL of 6% methanolic KOH followed by heating at 60^0 C for 20 min. The remaining substrate and the product formed were extracted from the aqueous incubation mixture with diethyl ether: petrol (1: 9 v/v). The reaction product was analyzed by HPLC with C_{18} column (5µm) using acetonitrile: methanol: propanol (85:10:5) with UV detector at 440.0 nm (Schnurr et al., 1996).

5.0 Biological activity of *Dunaliella* and its carotenoids.

5.1 *In vitro* antioxidant activity of crude extracts of *Dunaliella* .

Initially in order to know the overall antioxidant ability of *Dunaliella* ethanolic extracts of both vegetative (green) and carotenoids accumulated (red) cells were subjected to antioxidant activity assay by well-known models namely, β-Carotene linoleic acid model system (b-CLAMS) and DPPH (α,α diphenyl β - picryl hydrazine) (Singh et al., 2002). Ethanol extract was obtained by grinding the dried *Dunaliella* in solvent followed by sonication and centrifuged at 3000 rpm for 10 minutes and the supernatant was used for assay. These extracts were subjected for by following protocol and results were compared with that of butylated hydroxy anisole (BHA) a well-known antioxidant.

5.1.1. β-*Carotene linoleic acid model system.*

Aliquots of *Dunaliella* ethanol extract and BHA solution were added to separate tubes according to concentration for assay and volume was made up to 500 µl with ethanol. 4 ml of β-carotene linoleic acid emulsion (0.4 mg β -carotene, 40mg of linoleic acid and 0.4 mg of tween-20 made as emulsion by mixing with rotation under vacuum) was added to each tube. Absorbance of all samples were taken at 470 nm at Zero time and tubes were placed at 50^0 C in water bath, Measurement of Absorbance was continued at an interval of 15 minutes, till the colour of β carotene disappeared in the control reaction (t = 180 min). A mixture prepared as above without β carotene emulsion served as blank and mixture without extract served as control. Dose response of antioxidant activity for various extracts was determined at different concentrations (Murthy et al., 2002)

The antioxidant activity (%AA) of extracts was evaluated in terms of bleaching of β carotene using the following formula (Hidalgo et al., 1994).

$$\% AA = 100[1- (A^0 – A^t)/ A^o 0 – A^o t)]$$

Where % AA = Antioxidant activity, A^0 = Zero time absorbance of sample, A^t = Absorbance of sample after incubation for 180 min, A^0 0 = Zero time absorbance of control, A^t 0 = Absorbance of control after incubation for 180 min.

5.1.2 α,α *diphenyl* β *- picryl hydrazyl model.*

Aliquots of *Dunaliella* ethanol extract and standard (ascorbic acid) were taken in different test tubes. To this 5.0 ml of methanolic solution of D.P.P.H (100µM) was added, shaken well and the mixture was allowed to stand at room temperature for 20

minutes. The blank was prepared as above without extract. The readings were noted at 517 nm. The absorbance of blank was first noted at 517nm. The changes of absorbance of the samples were measured (Blios 1958).

Scavenging activity was expressed as the inhibition percentage calculated

using the following formula,

$$\% \text{ Radical scavenging activity} = \frac{\text{Control}_{\text{Abs}} - \text{Sample}_{\text{Abs}}}{\text{Control}_{\text{Abs}}} \quad \mathbf{X} \quad \mathbf{100}$$

5.2 Successive extraction of *Dunaliella* constituents.

Known amount of (25.0 gm) of algal biomass were taken in Soxhlet extractor and subjected for successive solvent extraction in the order of polarity, i.e., n-hexane, chloroform, ethyl acetate, acetone, methanol and water. The extraction was done for 8 - 10 hrs each till siphon was colorless. After each solvent extraction the samples were dried in vacuum before extraction in the next solvent. The above extracts were dried under vacuum (40 ± 2^0C) and known concentrations were dissolved in methanol and used for following assay and chemical analysis.

5.2.1 Radical scavenging assay using α, α- diphenyl-β-picryl hydrazyl (DPPH).

The free radical scavenging activity of successive extracts of both *D.salina* and *D.bardawil* (100 and 250 ppm) were measured by α, α- diphenyl-β-picrylhydrazyl radicals using the method of Murthy et al. (2002) as mentioned above. Results were expressed as % radical scavenging activity.

5.2.2 Reducing power assay.

The total reducing power was determined by the method of Duh and Yen (1997). Different concentrations of extracts of *D.salina* and *D.bardawil* in 1 mL of methanol were mixed with 2.5 mL of potassium ferricyanide (1% w/v) and the mixtures were incubated at 50^0C for 20 min. After incubation 2.5 mL of trichloroacetic acid (10% w/v) was added to the mixture and was centrifuged at 5000 rpm for 10 min. Finally, 2.5 mL of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of $FeCl_3$ (0.1% w/v), and the absorbance was measured at 700 nm. Ascorbic acid was used as positive control. Increase in the absorbance of reaction mixture indicated the reducing power of the samples.

5.2.3 LDL peroxidation.

Effect of different solvent extracts of *Dunaliella* on prevention of peroxidation of lipids were measured by using various lipid sources such as egg lecithin, human erythrocytes and lipids from rat brain as well as kidney. Peroxidation in all the lipid medium were measured in terms of concentration pink chromophoric adduct formed by reaction between thiobarbituric acid added and melondialdehyde formed due to peroxidation of lipids, which was induced by metal ions (Duh and Yen 1997).

5.2.3.1. Egg lecithin peroxidation assay.

Lipid peroxidation inhibitory activities of various extracts of algae were measured according to the method of Duh and Yen (1997). Egg lecithin (3 mg mL^{-1} phosphate buffer, pH 7.4) was sonicated for 5 minutes using Bandelin ultrasonicator at 200 μm_{ss} amplitude. The test samples of different concentrations (100 ppm and 250 ppm) were added to the 1 mL of the liposome mixture; control was without test sample. Lipid peroxidation was induced by adding 10 µL $FeCl_3$ (400 mM) and 10 µL L-ascorbic acid (200 mM). The mixture was incubated at 37^0C for 1 hour. The reaction was stopped by adding 2 mL of 0.25 N HCl containing 15% TCA and 0.375% TBA and the reaction mixture was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 532 nm. Butylated hydroxyanisole (BHA) was used as a standard for comparison. The inhibition ratio (%) was calculated using the following formula

$$\textbf{Inhibition ratio (\%)} = (A\text{-}A_1)/A \times 100\ \%$$

Where A was the absorbance of the control and A_1 was the absorbance of the test sample

5.2.3.2 Human Erythrocyte Peroxidation assay.

Blood was collected from human volunteers in heparinized tubes at health center of the Institute. It was centrifuged at 2500 rpm for 20 min and supernatant discarded. The cells were suspended in equal volume of 0.9 % saline and centrifuged at 2500 rpm. The sedimented cells was washed 3-4 times with saline and centrifuged. One mL of cells was suspended in 39 mL of phosphate buffer and centrifuged at 12,000 rpm for 30 min, washed with buffer for 3-4 times till cells were white and stored at -20^0 C for further use. One mL aliquots of white cells in 0.15 M KCl or 0.025 M Tris-HCl, pH 7.5 were incubated with different concentrations of test samples in the presence of 10 µM of $FeSO_4$ at 37^0C for 5 min. The reaction was started by adding 0.1 mM ascorbic acid and the tubes were further incubated at 37^0C for 30 min. The reaction was stopped by adding 2mL of TBA reagent (0.375% TBA and 15% TCA in 0.2 N HCl). The reaction mixture

was boiled for 15 min, cooled, centrifuged and absorbance of the supernatant was measured at 535 nm. The inhibition ratio (%) was calculated using the formula explained in previous section (Kulkarni et al., 2004).

5.2.3.3 Rat kidney lipid peroxidation.

The kidneys of normal rats were dissected and homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce 1/10 homogenate (Liu and Ng 2000). The homogenate was centrifuged at 14,000 rpm for 15 min. Lipid peroxidation assay was carried out by the method of Murthy et al. (2002). One ml aliquots of homogenate were incubated with different concentrations of test samples in the presence of 10 µM of $FeSO_4$ and 0.1 mM ascorbic acid at 37^0C for 1 hour. Reaction was stopped by the addition of 1 mL trichloroacetic acid (TCA, 28% w/v) and 1.5 mL thiobarbituric acid (TBA, 1% w/v) in succession and the solution was then heated at 100^0C for 15 min. After centrifugation to remove the precipitated protein, the colour of the malondialdehyde (MDA) - TBA complex was detected at OD 532 nm. Butylated hydroxyanisole (BHA) was used as a standard. The inhibition ratio (%) was calculated using the formula explained before.

5.2.3.4 Rat brain lipid peroxidation.

The brain of normal rats were dissected and homogenized in ice-cold Tris-HCl buffer (20 mM, pH 7.4) to produce 1/10 homogenate (Liu and Ng, 2000). The homogenate was centrifuged at 14,000 rpm for 15 min. Lipid peroxidation assay was carried out by the method of Murthy et al. (2002). One ml aliquots of homogenate were incubated with different concentrations of test samples in the presence of 10 µM of $FeSO_4$ and 0.1 mM ascorbic acid at 37^0C for 1 hour. The reaction was stopped by addition of 1 ml trichloroacetic acid (TCA, 28% w/v) and 1.5 ml thiobarbituric acid (TBA, 1% w/v) in succession and the solution was then heated at 100^0C for 15 min. After centrifugation to remove the precipitated protein, the colour of the malondialdehyde (MDA) - TBA complex was detected at OD 532 nm. Butylated hydroxyanisole (BHA) was used as a standard. The inhibition ratio (%) was calculated using the formula explained in section before.

5.3 Estimation of nutritional quality of *Dunaliella* cells *in vivo*.

Albino rats of either sex of the Wister strain weighing 180-220 g were used for the studies. The animals were grouped into three groups containing six animals in each group, first group served as Normal without algal biomass treatment, the second and third group received *Dunaliella* whole algal cells at 2.5 and 5.0 g kg^{-1} respectively

(which is approximately equivalent to 70 and 140 mg of total carotenoids kg^{-1}). Biomass was fed by forced feeding using oral catheter after making a fine powder and suspending in water, once a day for 14 days.

Weights of animals were measured on 7^{th} and 14^{th} day. On 14^{th} day animals were sacrificed after anasthetized using diethyl ether, blood was collected in a vial containing 10 % EDTA (anticoagulant) and stored in cold temperature until further analysis. Another part (~1.0 mL) was collected without any anticoagulant and serum was separated by keeping the tube in slanting position and serum which was separated as upper layer was separated and used for analysis of protein. Weight of vital organs viz, liver, heart, brain, spleen, kidney, adrenals, thymus, lungs and testis from male animals were noted. Blood was subjected for analysis of % hemoglobin, white blood cells (WBC) and serum protein manually. Other parameters like red blood cells (RBC) count, Mean corpuscular haemoglobin (MCH), Mean corpuscular haemoglobin concentration (MCHC), Mean corpuscular volume (MCV) were analysed at Bahgavan pathology laboratory, Mysore, using automatic hematocrit analyzer (ABX micro blood count analyzer).

5.3.1 Estimation of haemoglobin content.

Haemoglobin content of different animals was measured by cyanomethhaemoglobin method using Drabkini's reagent (Beacon diagnostic ltd., Navsari, India). In alkaline condition haemoglobin and its derivatives were oxidized in presence of potassium ferricyanide and converted into methemoglobin, which react with potassium cyanide to form purple red colored cynmethhemoglobin, which were monitored colorimetrically at 546 nm. Content of the samples were calculated comparing with the optical density of standard and expressed as % Hb content.

5.3.2 Estimation of white blood cells (WBC) count.

This was done at pathology department, CFTRI, health center. WBC count was done by diluting 20 µl of blood with diluting fluid and allowing it to react for 2-3 minutes and counting was done using hemocytometer and results were expressed as number of cells ml^{-1} of blood.

5.3.3 Estimation of serum protein.

Serum separated from blood was analysed for protein content using method of Lowry et al., (1951). Protein content was calculated using standard curve prepared using Bovine serum albumin and expressed as mg of protein per dL of serum.

5.4 Estimation of antioxidant activity of whole cells *in vivo*.

Albino rats of either sex of the Wister strain weighing 180-220 g were used for the studies. The animals were grouped into five groups, the first group served as Normal fed with commercial diet without treatment of toxin, the second group named as control received normal diet and was administered with toxin (CCl_4). The third and fourth groups were treated with *Dunaliella salina* biomass of 2.5 and 5.0 g kg^{-1} respectively by oral route after making a fine suspension of algal cells in water. Fifth group was treated with synthetic all *trans* β-carotene orally at dose of 100 mg kg^{-1} (dissolved in olive oil) for 14 days. The animals of 1^{st} to 4^{th} groups were simultaneously administered with olive oil until 14 days. The animals of 2^{nd}, 3^{rd}, 4^{th} and 5^{th} group were given a single oral dose of CCl_4 (1:1 in olive oil) at dose of 2.0 g kg^{-1} b.w. 6hr after the last dose of administration of biomass / β-carotene / olive oil at the 14^{th} day. After 24 hr animals were sacrificed and liver from each animal was isolated to prepare the liver homogenate. 5 % (w/v) liver homogenate was prepared with 0.15M KCl & centrifuged at 800 g for 10 min. The cell free supernatant was used for the estimation of lipid peroxidation, catalase, peroxidase and SOD catalase assay. Biochemical markers were analysed spectrophotometrically as explained below.

5.4.1 Catalase assay.

The catalase assay was carried out as per the method of Aebi (1984). One mL of liver homogenate from group 1-5 was taken with 1.9mL of phosphate buffer in different test tubes (125mM pH 7.4). The reaction was initiated by the addition of 1mL of hydrogen peroxide (30mM). Blank without liver homogenate was prepared with 2.9mL of phosphate buffer and 1 mL of hydrogen peroxide. The decrease in optical density due to decomposition of hydrogen peroxide was measured at the end of 1 min against the blank at 240 nm. Units of catalase were expressed as the amount of enzyme that decomposes 1μM H_20_2 per minute at 25^0C. The specific activity was expressed in terms of units per milligram of proteins.

5.4.2 Estimation of SOD.

The assay of SOD was based on the reduction of nitroblue tetrazolium (NBT) to water insoluble blue formazan as per the method of Beauchamp and Fridovich (1976). To 0.5 mL of liver homogenate 1 mL of 125mM sodium carbonate, 0.4mL of 24μM NBT, and 0.2 mL of 0.1mM EDTA were added. The reaction was initiated by adding 0.4mL of 1mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560nm followed

by recording the absorbance after 5 min. The control was simultaneously run without liver homogenate. Units of SOD activity were expressed as the amount of enzyme required for inhibiting the reduction of NBT by 125%. The specific activity was expressed in terms of units per milligram of proteins.

5.4.3 Estimation of peroxidase.

The peroxidase assay was carried out using the method of Nicholas (1962). To 0.5 mL of liver homogenate 1mL of 10mM KI solution and 1mL of 40mM sodium acetate solution were added. The absorbance of potassium periodide was read at 353nm, which indicates the amount of peroxidase. Twenty micro liters of hydrogen peroxide (15mM) was added, and the change in the absorbance after 5min was recorded. Units of peroxidase activity were expressed as the amount of enzyme required to change the OD by 1 unit per minute. The specific activity was expressed in terms of units per milligram of proteins.

5.4.4 Lipid peroxidation activity.

Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct, a pink chromogen, which can be detected spectrophotometrically at 532nm by the method of Buege and Aust (1978*)*. Liver homogenate (0.5mL) and 1mL of 0.15M KCl were taken. Peroxidation was initiated by adding 250µL of 0.2 mM ferric chloride. The reaction was run at 37^0C for 30 min. The reaction was stopped by adding 2mL of an ice-cold mixture of 0.25N HCl containing 15% trichloroacetic acid, 0.30 % TBA, and 0.05 % BHT and was heated at 80^0C for 60 min. The samples were cooled and results were expressed as MDA equivalent, which was calculated by using an extinction coefficient of 1.56 $x10^5 M^{-1}$ cm^{-1}. One unit of lipid peroxidation activity was defined as the amount of TBA that converts to TBARS. The specific activity was expressed in terms of units per milligram of protein.

5.4. 5 Determination of proteins.

Protein was determined using the method of Lowry et al., (1951) Protein content was calculated using standard curve prepared using Bovine serum albumin and expressed as mg of protein per dL of serum.

5.5 Estimation of hepatoprotective activity of whole cells *in vivo*.

Heparinized blood was collected from animals of above experimental groups viz., Normal, Control, 2.5 and 5.0 g kg^{-1} of *Dunaliella* fed, 100 mg kg^{-1} synthetic all *trans* β-carotene and serum was separated by centrifugation and used for the analysis of heptoprotective biochemical markers as below.

***5.5.1 Serum glutamic oxalacetic transaminase* (SGPT).**

This was estimated by monitoring pyruvate formed when α- ketoglutarate reacts with L-alanine, meadiated by SGPT. Pyruvate is made to react with 2, 4- Dinitrophenyl hydrazine to form hydrazone producing brown colour in alkaline medium which was monitored calorimetrically at 505.0 nm. The SGPT content was calculated using standard graph with pyruvate as standard using diagnostic kit (SPAN diagnostic, Surat, INDIA).

***5.5.2 Serum glutamic pyruvic transaminase* (SGOT).**

This was estimated by monitoring oxaloacetate formed when α- ketoglutarate react with L-aspertate, meadiated by SGOT. Oxaloacetate is made to react with 2, 4- Dinitrophenyl hydrazine to form hydrazone producing brown colour in alkaline medium, which was monitored calorimetrically at 505.0 nm. The SGOT content was calculated using standard graph with oxaloacetate as standard using diagnostic kit (SPAN diagnostic, Surat, INDIA).

***5.9.5.3 Serum alkaline phosphatase* (SALP).**

Serum ALP converts phenyl phosphate to inorganic phosphate and phenol. The phenol formed is made to react with 4, amino antipyrine in presence of oxidizing agent potassium ferricyanide to form a orange - red complex and this was measured spectrophotometrically at 505 nm (Kind and King 1954). The SALP content was calculated using standard graph with standard provided in diagnostic kit (SPAN diagnostic, Surat, INDIA).

5.5.4 Serum protein.

Serum protein was determined using the method of Lowry et al., (1951) Protein content was calculated using standard curve prepared using Bovine serum albumin and expressed as % mg of protein in serum.

5.5.5 Serum albumin.

This was estimated spectrophotometrically by method of Wooten (1964). Albumin was made to react with bromocresol green in succinate buffer and the characteristic color developed was measured colorimetrically at 630.0 nm using diagnostic kit (SPAN diagnostic, Surat, INDIA).

5.5.6 Histopathological studies of hepatocytes.

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.

5.6 Estimation of antioxidant activity of carotenoids *in vivo*.

Albino rats of either sex of the Wister strain weighing 180-220 gm were used for the studies. The animals were grouped into five groups, the first group served as Normal without treatment of toxin, the second group named as control received normal diet and was administered with toxin (CCl_4). The third and fourth groups were treated with carotenoids of *Dunaliella* (125 and 250 $\mu g\ kg^{-1}$). This is based on the recommended dose for human i.e., 50 mg $days^{-1}$ for average person. Fifth group was treated with synthetic β-carotene orally at dose of 250 $\mu g\ kg^{-1}$ (dissolved in olive oil) for 14 days. The dose was selected based on the recommended β-carotene dose. The animals of 1^{st} and 2^{nd} groups were simultaneously administered with olive oil until 14 days. The animals of 2^{nd}, 3^{rd}, 4^{th} and 5^{th} group were given a single oral dose of CCl_4 (1:1 in olive oil) at dose of 2.0 g kg^{-1} b.w. 6hr after the last dose of administration of carotene / olive oil on 14^{th} day. After 24 hr animals were sacrificed and liver from each animal was isolated to prepare the liver homogenate. 5 % (w/v) liver homogenate was prepared with 0.15M KCl & centrifuged at 800 g for 10 min. The cell free supernatant was used for the estimation of lipid peroxidation, catalase, peroxidase and SOD catalase. All the biochemical markers of antioxidant activity were estimated by protocols as explained in section 5.4.

5.7 Estimation of hepatoprotective activity of carotenoids *in vivo*.

After 24 hr animals of above experiment (section 9.6) were sacrificed and blood was collected in heparinized tubes from each animal by cardiac puncture (~3.0 mL). Serum was separated by centrifugation and used for the analysis of heptoprotective biochemical markers, namely SGPT, SGOT, SALP, Serum albimun and protein by protocols as explained in section 9.5. Histopathogy of liver from different treatment animals was also performed as explained afore.

5.8 Statistical analysis.

All the experimental analyses were done in triplicate. Result values were expressed as mean $\pm$ SD in case of *in vitro* experiments. In case of *in vivo* analysis values were expressed as mean $\pm$ SD and one-way analysis of variance (ANOVA) was used and the test was used for comparison of mean values. All tests were considered to be statistically significant and highly significant at $p < 0.05$ and 0.001 respectively.

The studies have been conducted to meet the objectives set for the thesis, this includes:

i. Cultivation of *Dunaliella* biomass *in vivo* as well as outdoor cultivation
ii. Induction of carotenogenesis.
iii. Process for large scale production
iv. Downstream processing
v. Analysis and quantification of carotenoids and proximate compositions
vi. Biological activity of *Dunaliella* biomass and extracted carotenoids

Background:

Dunaliella is a halotolarant, autotropic, marine algae. A number of factors, influences the growth of the algae, which includes, concentration of Sodium chloride required to maintain the osmotic balance. Its cell membrane is highly permeable and it allows water flux to equalize intracellular and extra cellular osmotic pressure (Oren 2005), pH of the medium, source of carbon, light (essential for photosynthesis of algae) and essential nutrients including sulphate, phosphate and nitrate are the factors which influences the growth and physiology of the organism. Studies were conducted to understand the influence of the above-mentioned factors for obtaining maximum biomass. A study was also conducted to know the effect of zinc, which is required for some of the vital enzymes, which mainly include antioxidant enzymes, like SOD required for carotenoids production and are essential for various vital physiological processes of micro algae.

Upon production of green biomass, various attempts were made to study the possible factors, which can trigger carotenoids accumulation. This was attempted examining various factors like, salt, nutrients and light required for carotenogenesis.

6.0 Cultivation of *Dunaliella* biomass *in vivo* as well as outdoors cultivation.

6.1 Maintenance of the germplasm.

Both, *Dunaliella bardawil* strain and *Dunaliella salina* strains were maintained on agar slants of AS-100 composition. Stock cultures were transformed to liquid culture in Erlenmeyer flask of 250 mL containing 40-50 mL AS-100 medium and later with modified medium (Composition is as shown in Table-14). Sub culturing was done at 30 days intervals and culture was incubated at 18.0 ± 2.0^{0}C under light intensity of 1.5-2.0 Klux. Slant culture and liquid culture of *Dunaliella salina* maintained are shown in Figure-12. All the experiments were conducted using *Dunaliella salina* and some of the parameters were compared with that of *Dunaliella bardawil*.

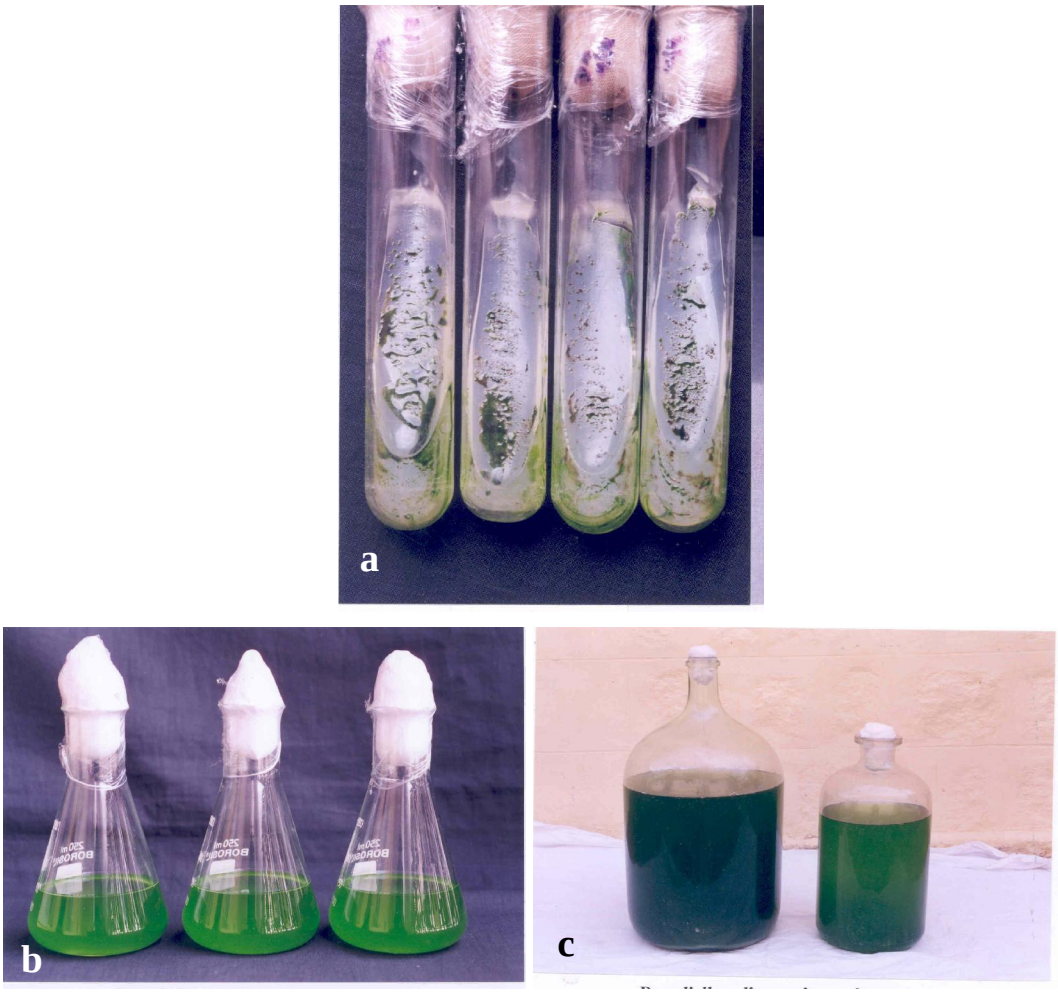


Figure 12. Maintenance of *Dunaliella salina* in (a) agar slant (b) liquid culture of flask and (c) carboy cultures (10.0 L and 5.0 L).

6.2 Standardization of medium for Dunaliella cultivation.

6.2.1 Growth of Dunaliella at different pH.

Availability of nutrients and its utilization in plants and algae mainly depends on the pH in which it is grown. *Dunaliella*, which is found generally in marine environment, is adopted to pH ranging from 5.8-6.2 under cultures conditions, concomitant with growth of algal cells, there will be depletion of some of media chemicals, like carbon, which result in enhancement of pH (section 3.2.1). When pH increases beyond 9.5, it was observed that aggregations of cells takes place leading to forced flocculation. Hence it was essential to understand the pH at which growth will be maximum. The cultures were subjected to a range of initial pH as explained in materials and method and growth was monitored till 30 days. Results have shown that initially upto 6 days, all the cultures grew almost similarly possibly due to sufficient availability of constituents required for growth (Figure-13). However steady growth was evident till 30 days in all the cultures at initial pH 7.0, 7.5 and 8.0, wherein pH 7.5 was found optimal. Thus further initial pH of 7.5 was employed for further work. The cells of culture with initial pH of 9.0 and 9.5 have shown reduced growth after 10 days. The pH of culture grown for 14 days was enhanced by 2.0 ± 0.2 units and found to be 9.2 ± 0.5 in all the conditions. Growth pattern of algal cells in a range of pH are as shown in Figure-13. The cell growth was measured in terms of cell count, cell wet biomass weight and optical density at 660.0 nm. Growth rate in terms of biomass weight will not be constant as it is associated with salts and the same was removed by washing with water, however the values were not consistent hence cell count was employed for expressing the growth in small scale and wet weight of biomass L^{-1} in case of pilot scale. It was found that 0.1 unit of Optical density is equivalent to 0.2×10^6 cells mL^{-1} in turn equivalent to 0.15 gL^{-1} of wet biomass or 0.022 g L^{-1} of dry biomass. This was standardized after taking several trails of reading with medium, which was transparent.

6.3 Effect of salt concentration on growth.

Dunaliella being halophile, it was necessary to determine the optimal NaCl levels for culture of the algae. The structure of the cell membrane is the one, which makes it more comfortable to adapt to range of salt content of 6-23% w/v, (Oren 2005). Even though algae can survive in a range of sodium chloride enriched medium, it can multiply well in particular range of sodium chloride concentration. Among the range of initial sodium chloride levels (1.0, 1.5, 2.0, 2.5, 3.0 and 3.5) applied, sodium chloride concentration of 1.5 M showed maximum growth as measured in terms of optical density as seen in case of indoor culture of 200 mL grown in flasks (Figure -14). Sodium chloride of 1.5 M (87.0 g L^{-1}) concentrations was used for further experiments.

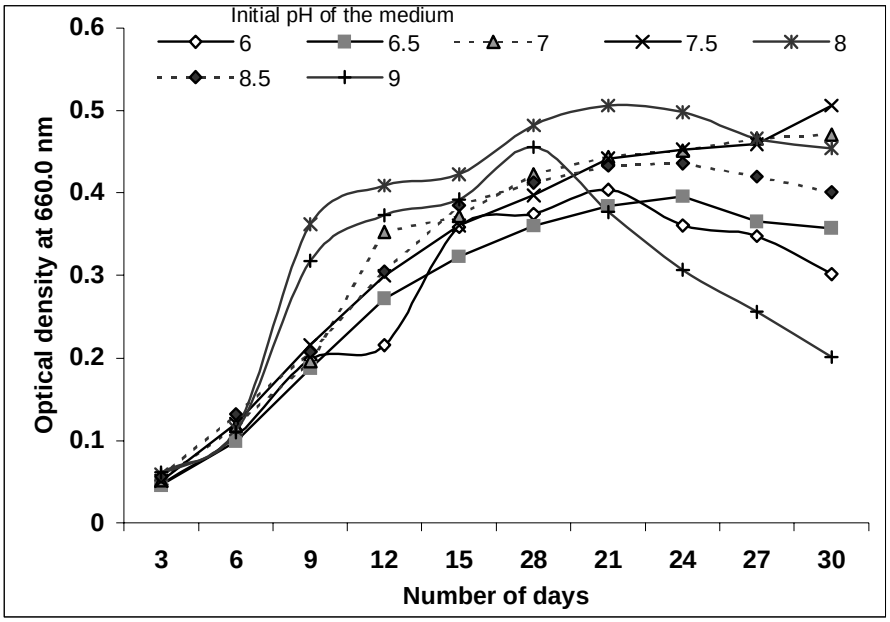


Figure 13. Growth of *Dunaliella salina* in response to various media pH.

[Cultures were maintained in AS-100 medium with different initial pH and growth rate was monitored by measuring optical density at 660.0 nm, values are mean of three values (1unit of Optical density = 0.2×10^6 cells mL^{-1})].

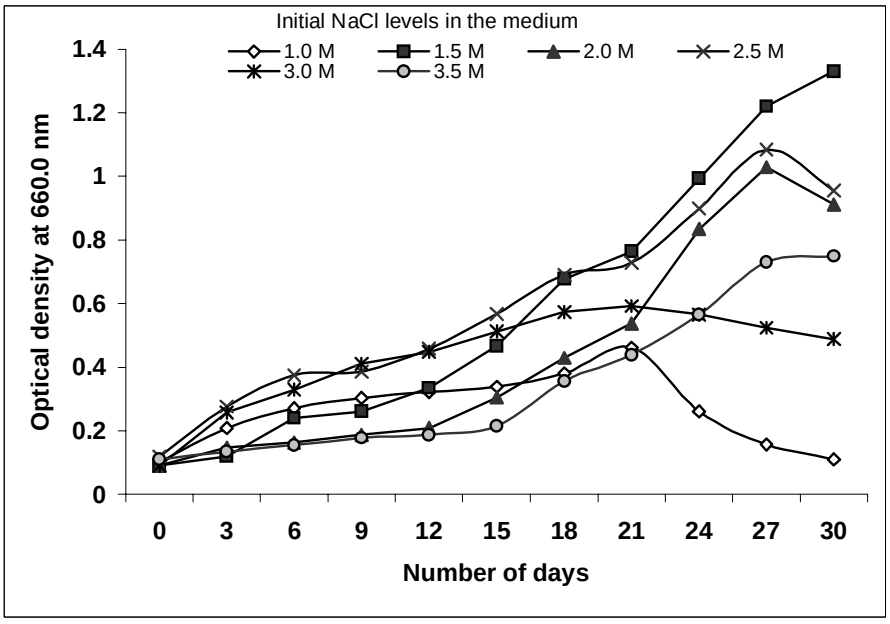


Figure 14. Growth of *Dunaliella salina* in response to different initial concentrations of sodium chloride.

[Cultures were maintained in AS-100 medium with different initial sodium chloride content , growth rate was monitored by measuring optical density at 660.0 nm, values are mean of three values (1unit of Optical density = 0.2×10^6 cells mL^{-1})].

6.4 Effect of nitrate concentration on growth.

Nitrate is the major source of nitrogen for algae (Borowitzka and Borowitzka 1988), essential for the formation of the protein, amino acids and other vital components of plants and algal

cells. Nitrate was employed at the concentration of 1.0 gL^{-1}, equivalent to 164 mg of nitrogen (11.71 mM) by BA and AS-100 medium (Composition in Table 15 and 14). Lowering the nitrogen content to 5.85 mM (half of the original content of AS-100 medium) keeping other salts in normal concentration has shown decline in growth as recorded on 12^{th} day. In this treatment average cell count of 0.4 cells mL^{-1} was obtained compared to normal level of nitrogen (11.71mM) which shows average cell count of 2.44×10^6 cells mL^{-1} on the corresponding day. Enhancement of nitrate to double (23.42 mM of nitrogen) did not shown any significant enhancement of growth over the 11.71 mM level as seen in indoor culture conditions. With these observations concentration equivalent to 11.71 mM in terms of nitrogen (1.0 gL^{-1} in terms of sodium nitrate) was employed for further set of experiments.

6.5 Effect of sulphate concentration on growth.

Phosphate is necessary for various key bio-molecules like, ADP, ATP, NADP and other enzymes, which play vital role in the algal cells. As the concentration of phosphate was very low i.e., 50.0 mg L^{-1} in the form of potassium dihydrogen orthophosphate (0.36 mM in terms of phosphorous), the variation in the content could not give any appreciable changes in terms of cell count, hence the concentration of 0.36 mM was used for the study.

Sulphate is an essential component of the nutrients, which helps in formation of certain amino acids and sulpholipids, which constitute the growth factors. Normal content of sulphate used as per AS- 100 medium is 2.44 g L^{-1} in terms of magnesium sulphate, which corresponds to ~ 10 mM of Sulphate. Cultures of cells in sulphate free and 5.0 mM of sulphur media have shown average cell count of 0.6×10^6 cells mL^{-1} and 1.14×10^6 cells mL^{-1} respectively, against average of 2.44×10^6 cells mL^{-1} of original AS-100 medium. However, increase in the content of sulphate to double (20.0 mM of sulphur) resulted in average production of 2.04×10^6 cells mL^{-1}, which was slightly lesser compared to that of AS-100 medium as seen in indoor culture conditions at 200 mL culture volume grown in flasks. Therefore the original concentration equivalent to10 mM of sulphur was adopted for further study.

6.6 Effect of light on growth of Dunaliella.

Light is essential to reactions of photosystem I and of photosystem II a key process for photosynthesis. It can induce phosphorylation of light harvesting chlorophyll a/b binding proteins LHCII (Liu and Shen 2004). Light intensity influences the content of chlorophyll as well as carotenoids production. Low intensity of light is known to slow the growth of micro algae (Ben Amotz and Avron 1983).

In order to determine the optimum level of light required for growth, *Dunaliella salina* cells were grown under different intensities of light. For this study cultures were grown in a Erlenmeyer flask of culture capacity of 1.0L both in indoor and outdoor conditions. Light intensity of 1.0 and 2.0 Klux (provided by white fluorescent light of 96 W of tubular light of 1.5 and 4.0 feet length) was adopted in indoor cultures. In these treatment algae exhibited linear growth rate till 15 days attaining average cell count of 0.72×10^6 cells mL^{-1} in AS-100 medium and that of 1.0 Klux has shown 0.56×10^6 cells mL^{-1}. In case of high light (8.0 K lux and direct sun light) there was constant decline in cell content, 8.0 Klux had shown average cell count of 0.6×10^6 cells mL^{-1}. In case of direct sunlight (25-

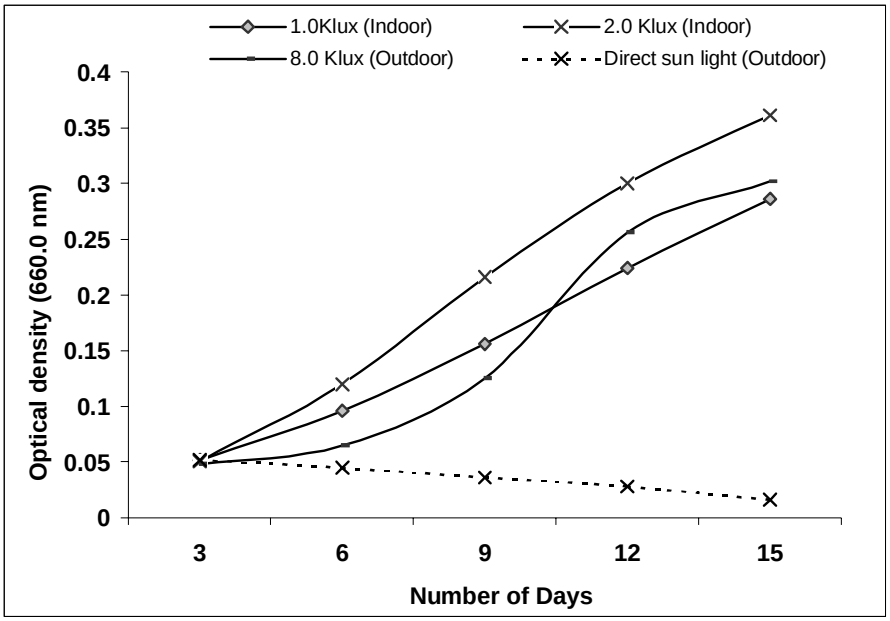


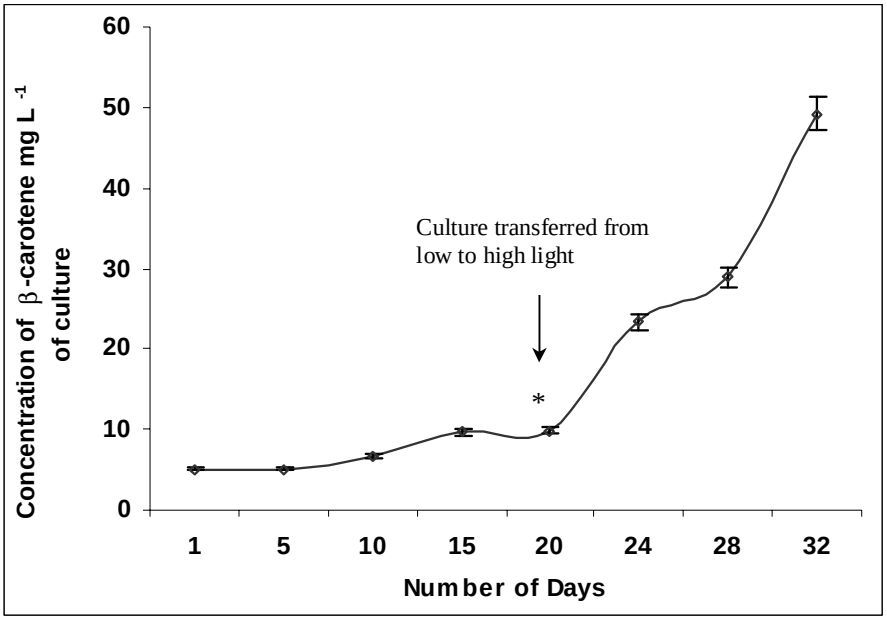
Figure 15. Growth of *Dunaliella salina* in response to various intensity of light.

[Cultures were maintained in AS-100 medium with different intensity of light and growth rate was monitored by measuring optical density at 660.0 nm, values are mean of three values (1unit of Optical density = 0.2×10^6 cells mL^{-1})].

30 Klux) cell number almost declined constantly from 6th day, reading 0.32×10^6 cells mL^{-1} as measured on 6th day. It is clear that the cells need low light for vegetative phase (for a duration of 14 – 20 days) depending on the culture conditions (Figure-15).

With data indicating that *Dunaliella salina* grows well at light intensity of 2.0 Klux and requires high light for carotenoids accumulation, another experiment was conducted, in which the cells were grown for 15 days under low light of 4.0 ± 1.0 Klux and later exposed to direct sunlight of 25-30 Klux intensity. This treatment shown a improved growth till 15 days and there was increase of carotenoids from 5.1 to 9.7 mg L^{-1} indicating the exponential increase in carotenoids accumulation. Carotenoids content of *Dunaliella salina* in growth phase under low light and carotenoids accumulation phase at high light is as shown in Figure -16.

Figure 16. Accumulation of carotenoids after exposing cells from low light (2.0 Klux) in



vegetative stage to direct sunlight (25-30 Klux).

[*Dunaliella salina* cultures were maintained in AS-100 medium at low light till 20 days and transferred to direct sunlight for high light and carotenoids content was monitored for next 12 days, values are mean of three values].

6.7 Effect of different carbon concentration on growth of Dunaliella.

Dunaliella being photoautotropic is capable of utilizing external source of carotenoids provided either in the form of chemicals like HCO_3, gaseous form like CO_2 or from the environment for their survival as carbonic anhydrase is present extra cellularly, which catalyses the conversion of HCO_{3-} to CO_2 indicating that the alga can utilize HCO_{3-} (Brown et al., 1987). *Dunaliella salina*, has shown the higher requirement of inorganic carbon sources as it is grown in high salinity, where there is lesser solubility of inorganic carbon. In order to know the effect of carbon source and amount of inorganic source required, an experiment was done utilizing carbon dioxide and sodium bicarbonate as inorganic source. Supplementing the culture with external supply of carbon above in the form of gaseous CO_2 at the rate of 0.5-0.75

Kg inch^{-2}, enhanced the rate of growth of 8.2×10^6 cells mL^{-1} on 16th day compared to culture supplemented with sodium bicarbonate (2.0 gL^{-1}), which has shown the cell count of 7.6×10^6 cells mL^{-1}. In carbon dioxide supplemented medium growth was significantly high till the 8th day but decreased slowly there after till 16 days. However the growth of cells in 4.0 gL^{-1} of sodium bicarbonate was the highest among the treatments, which showed 10.6×10^6 cells mL^{-1} on 16th day. Hence further experiments were conducted using $NaHCO_3$ at the concentration of 4.0 gL^{-1} (Figure-17 and Figure-18).

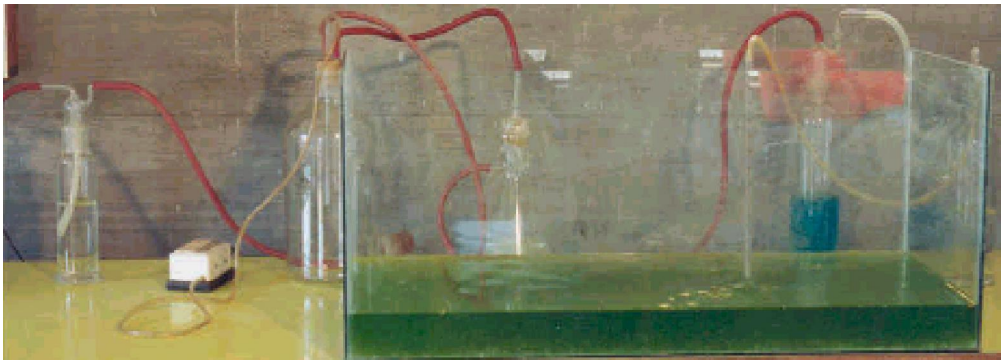


Figure 17. Experimental setup showing the carbon dioxide supplementation for cultures of *Dunaliella salina.*

[*Dunaliella salina* cultures were maintained in AS-100 medium at 2.0 Klux light, carbon dioxide was supplemented at the rate of 0.5-0.75 Kg inch^{-2}].

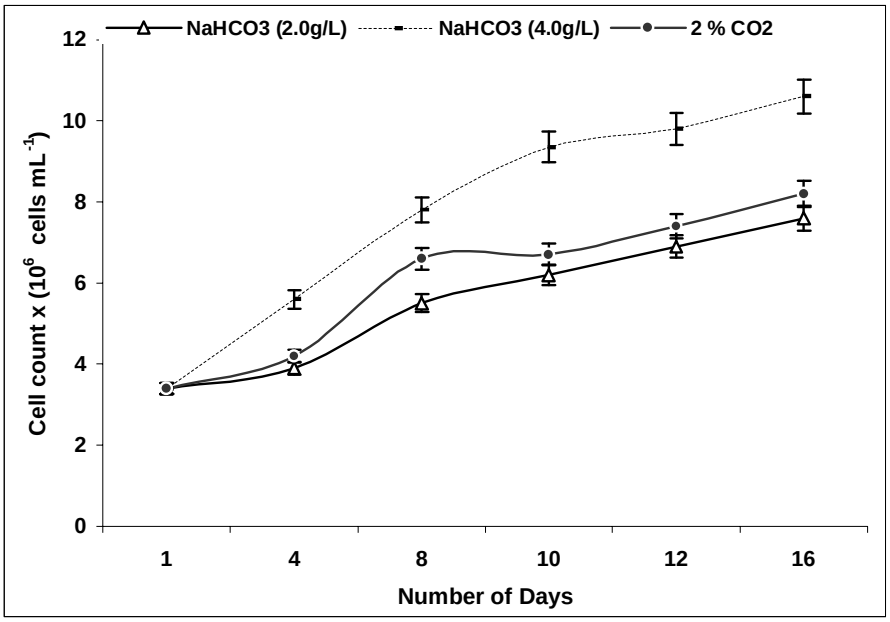


Figure 18. Effect of carbon dioxide supplementation with that of supplementation of sodium bicarbonate.

[*Dunaliella salina* cultures were maintained in AS-100 medium and different bicarbonate levels and external carbon dioxide was supplemented at 18 ± 2.0^{0}C with 2.0 Klux light intensity, cell count was measured by haemocytometer after making the cells immotile with 0.1% HCl. Values are mean of three replicates along with standard deviation].

6.8 Effect of different concentrations of micronutrients on the growth of Dunaliella salina.

Various micronutrients like, Co, Zn, Mn, Mo, B are essential for various physiological functions of algae as that of plants. Among these Zinc appears to be very vital for *Dunaliella* as it is associated with various antioxidant enzymes, like SOD hence the effect of the same on the growth of *Dunaliella* were studied in the form of zinc chloride. Zinc is known to induce phytochelatin in *Dunaliella*, a heavy metal binding protein which helps in detoxification (Tsuji et al., 2003) and hence it is important to know the concentration of zinc chloride required for better growth. A range of Zinc chloride starting from zero (without zinc) to 0.07, 0.14, 0.21, 0.28, 0.35 µM were used in terms of zinc. Among the levels used, 0.03 mg L^{-1} zinc chloride (0.21 µM) was found to be the ideal concentration for the growth of algal cells, which resulted in a cell count of 4.98×10^6 cells mL^{-1} on 18th day. Concentration more than 0.21 µM zinc had no beneficial effect, showing 4.6 and 4.36 $\times$ 10^6 cells mL^{-1} in case of 0.28, 0.35 µM concentration respectively. On the other hand growth was lower at concentrations less than 0.03 mg L^{-1} zinc chloride. The growth was reduced to almost 50 % as seen on 18th day, when cells were depleted of zinc (Figure-19).

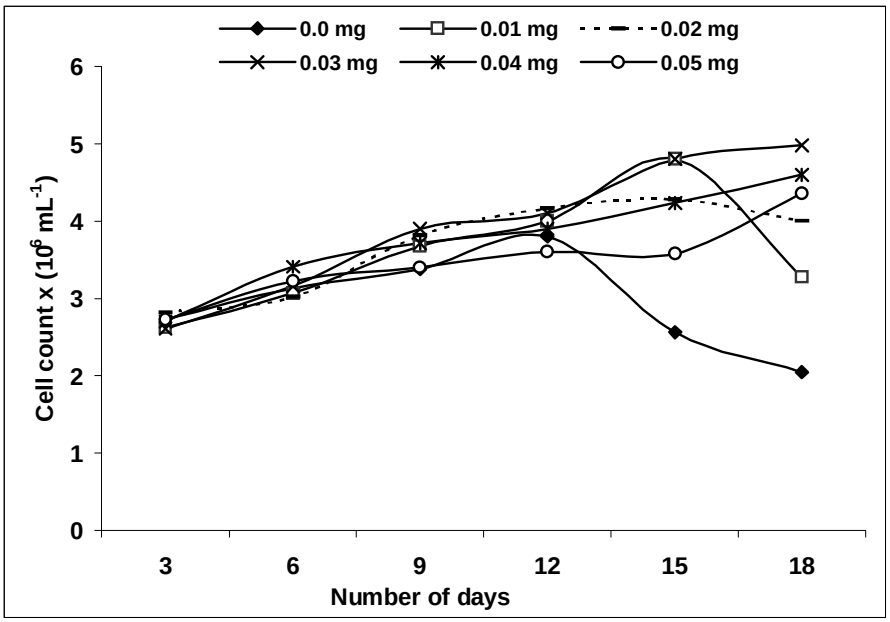


Figure 19. Effect of zinc concentration on the growth of *Dunaliella salina.*

[*Dunaliella salina* cultures were maintained in AS-100 medium and different concentration of zinc were supplemented in the form of zinc chloride. The culture was maintained at 18 ± 2.0^{0}C with 2.0 Klux light intensity, cell count was measured by haemocytometer after making the cells immotile with 0.1% HCl. Values are mean of three values along with standard deviation].

An experiment conducted using different concentrations of micronutrients revealed inconsistent results and no significant difference in the growth rate was observed in-group with 8.0 and 10 mL L^{-1} micronutrients concentration. However, in groups fed with less than 8.0 mL L^{-1} have shown reduction growth rate of algae, which was >0.2 × 10 cells mL^{-1} compared to 2.05×10 cells mL^{-1} in case of control fed with 10 mL L^{-1} micronutrients as employed in AS-100 and other medium as on 14th day.

6.9 Effect of different media on growth and carotenoid content of Dunaliella salina.

Upon determining the requirement of various nutrients and that of light and carbon sources, a medium was formulated with modified composition of chemicals and designated as '*modified medium*'. *Dunaliella* cultures were maintained in these media viz., medium A (AS-100 medium, Vonshak 1986), medium B (Ben-Amotz et al., 1983, medium C (modified medium) and monitored for growth and carotenoids content for 30 days. The initial pH was adjusted to 7.5 in all the cases, and flasks were inoculated with the same initial cell count of ~0.1 × 10^6 cells mL^{-1}. In case of Modified medium higher growth (9 × 10^6 cells mL^{-1}) and carotene production (9 µg mL^{-1}) was observed compared to other two media (Figure -20 and 21).

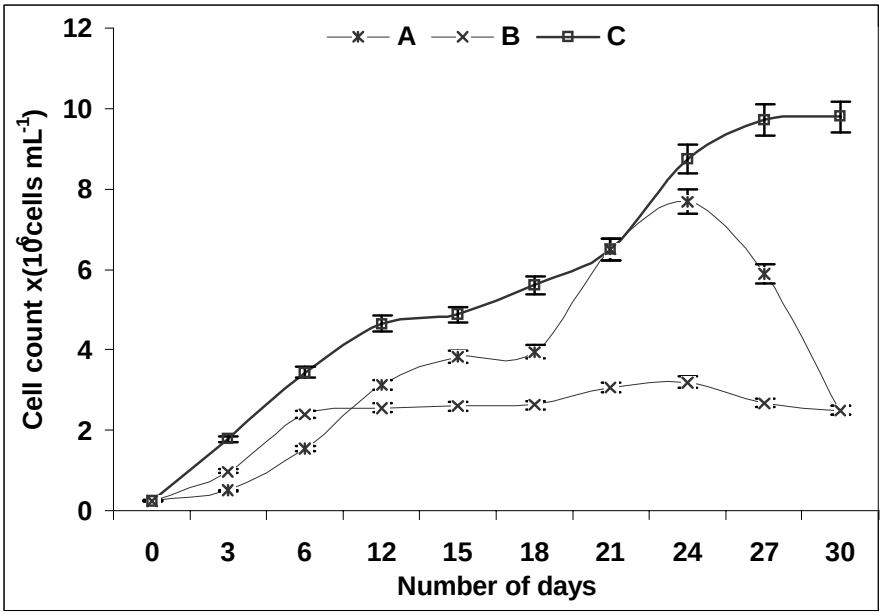


Figure 20. Effect of different media on growth of *Dunaliella salina.*

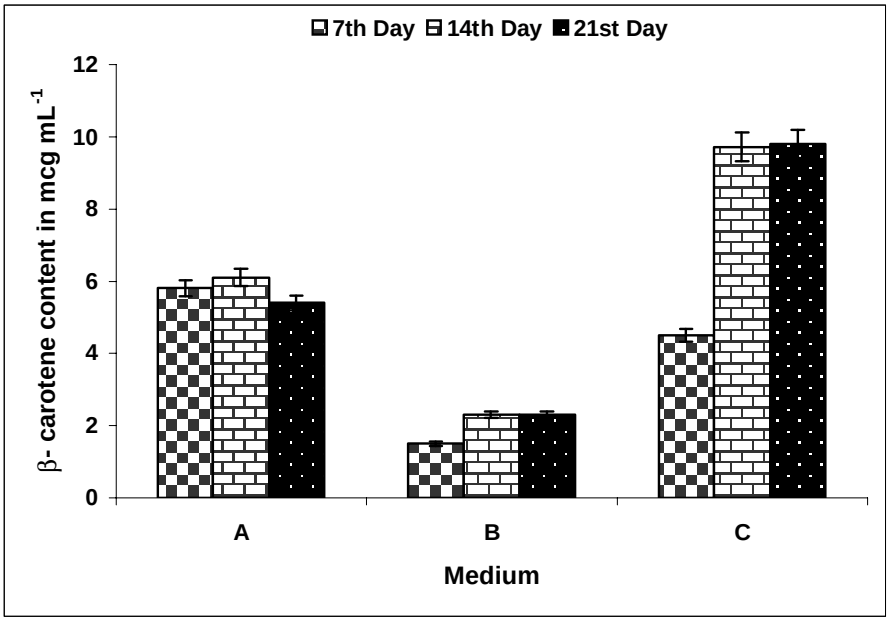


Figure 21. Effect of different media on β-carotene production. A- AS-100 medium, B- Ben Amotz (1983) medium and C - Modified medium.

[*Dunaliella salina* cultures were inoculated with different medium were maintained at 18 ± 2.0^0C with 2.0 Klux light intensity, cell count was measured by haemocytometer and carotenoids content was measured spectrophotometrically. Values are mean of three values along with standard deviation].

Salient features:

A simplified medium known as '*modified medium*' was developed for cultivation of *Dunaliella salina.* Composition of the *modified medium*' was 10.0 mM $MgSO_4$, 8.0 mM KCl, 0.4 mM, KH_2PO_4, 11.75 mM $NaNO_3$, 0.47M of $NaHCO_3$, 0.86 M NaCl along with micronutrients (10.0 mL L^{-1}) and chelated iron solution (3.0 mL L^{-1}). Cultures grown using this medium were compared with internationally reported values as a benchmark. Productivity of biomass and carotenoids of *Dunaliella salina* biomass was clearly higher compared to that of globally reported value as shown below (Table -16).

Table 16. Comparison of production of algal cells with global benchmark.

Content	**Values obtained***	**Values reported in the literature**	**Reference** (US patent No)
Cell count (cells mL^{-1})	$10.0 \pm 0.5 \times 10^6$	10×10^6	4,115,949
β-carotene (mg g^{-1} dry wt.)	Up to 25	10-12	4,115,949
Chlorophyll (mg g^{-1} dry wt.)	25.0 ± 2.5	13-21	4,115,949
Cell mass			
Wet	2.0mL L^{-1}	2.0 mL L^{-1}	4,115,949
Dry	0.25 gL^{-1}	0.2g L^{-1}	

* *Modified medium,* values are maximum reported in literature.

ii Induction of carotenogenesis.

Background:

The major step towards commercial production is to make the algal cells accumulate maximum content of carotenoids. This step will decide the fate of whole production process. *Dunaliella salina* is known to accumulate carotenoids as a primary response to various stress conditions, like light (Lers et al., 1990), salt and nutrient stress (Ben Amotz and Avron 1983). In this connection some of the experiments were done to induce stress and the results are reported below.

6.10 Modification of culture conditions for carotenogenesis.

In order to know the effect of light stress on the growth and carotenogenesis, *Dunaliella salina* cells were grown in *modified medium* for 14 days with initial pH of 7.5, cultures of green cells were subjected to further growth for 14 days (a) in temperature (25 ± 1^0 C) and light (2000 lux) controlled room under illumination with the cool white fluorescent lamps and (b) in sunlight under light intensity in the range of 30 to 36 Klux, at a varying temperature of 25 to 33° C in outdoor conditions.

Results of the study shows that the growth of culture constantly increased as evident from the cell count in case of low light, which attained average cell count of 8.9×10^6 cells mL^{-1} at the end of 30^{th} day of total culture period. Whereas the growth in case of highlight (outdoor conditions) provided to culture was very slow, subsequent to growth phase (Figure -22). However the carotenoids contents were in the reverse order, in which low light had shown 6.64 µg carotenoids mL^{-1} of culture, whereas in high light it was 2.5 folds higher reaching a carotenoids content of accounting to15.6 µg mL^{-1} of culture (Figure- 23). Direct sunlight exposed culture appeared pale yellowish green colour from the day two of exposure and slowly developed into orange yellow colour indicating carotenoids accumulation (Figure- 24). This study indicates that there is requirement of low light for growth period and high light stress for carotenogenesis, and the same was employed for further scale up studies.

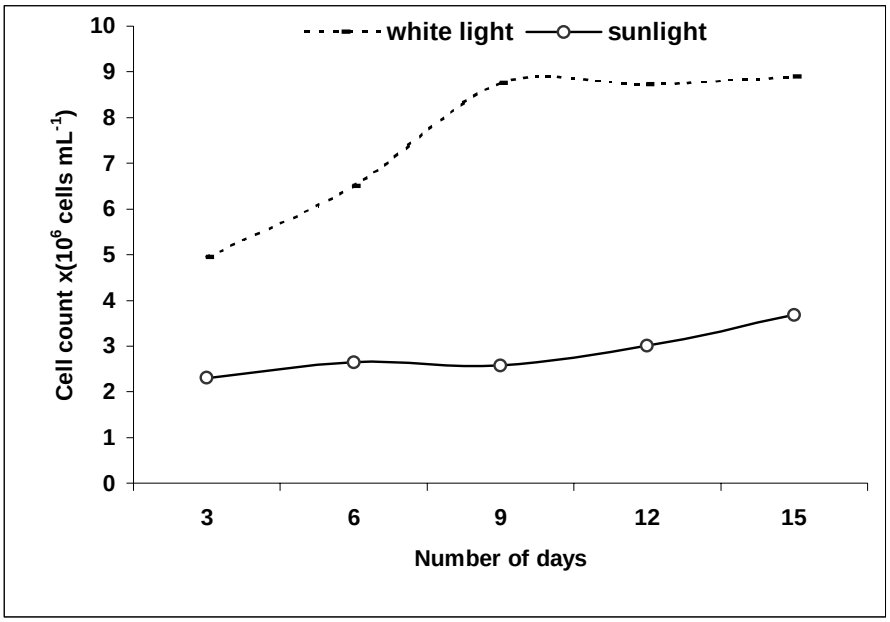


Figure 22. Effect of light on growth of *Dunaliella salina.*

[Cultures were incubated at 18 ± 2.0^{0}C with white (2.0Klux) or direct sunlight (25-30kLux) at ambient temperature of 24± 4^{0}C , cell count was recorded].

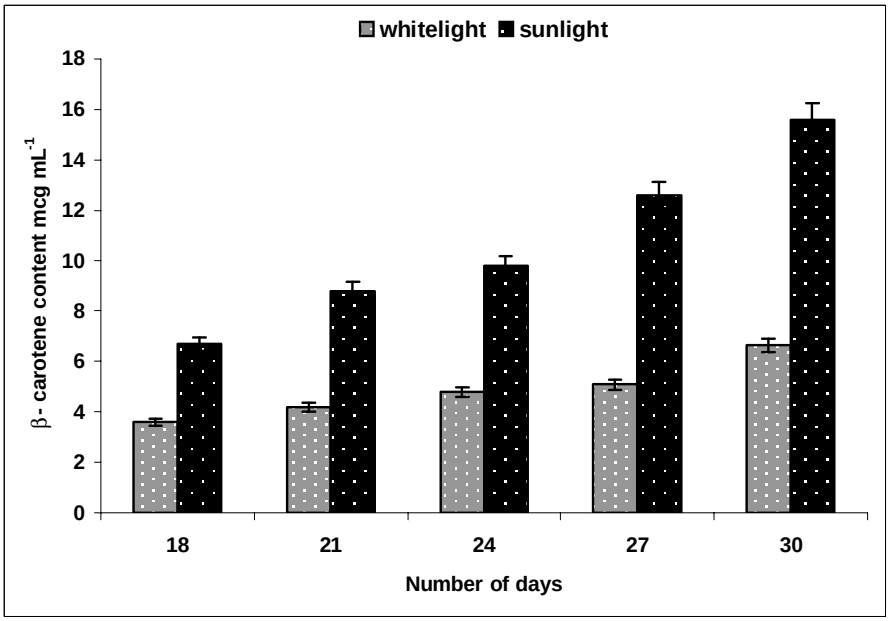


Figure 23. Effect of light on β- carotene production in *Dunaliella salina.*

[*Dunaliella* cultures grown in modified medium were incubated at 18 ± 2.0^{0}C with white (2.0Klux) or direct sunlight (25-30kLux) at 24± 4^{0}C].

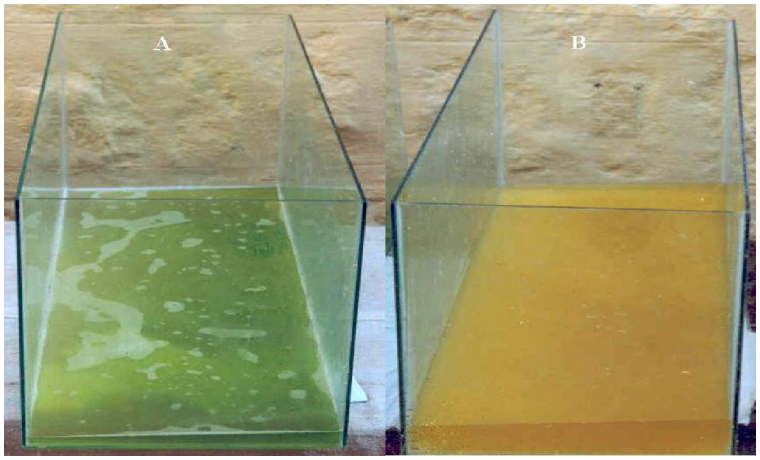


Figure 24. *Dunaliella* culture exposed to low light (A) and direct sun light (B) as they are seen after 15 days of exposure.

6.11 Nitrate deficiency stress.

Nitrate limitation is known to affect the cell division and decrease the content of chlorophyll and enhance the β- carotene production (Ben Amotz et al., 1983). The results of the study of nitrate stress during second phase i.e., after 14 days of growth has shown enhancement of carotenoids content by 3.2 folds in modified medium under high light intensity. From the average initial content of carotenoids of 1.86 μg mL^{-1} the accumulation enhanced to 5.96 ± 0.62 μg mL^{-1} under the higher light intensity (Figure -25).

6.12 Sulphate deficiency stress.

Sulphate has also shown similar trend of results as that of nitrate. The results of this study has shown that with the decrease in the availability of sulphate after growing cells for 14 days in modified medium, content of the β-carotene increased from average of 1.86 μg mL^{-1} to 4.09 μg mL^{-1} (Figure-25).

6.13 Phosphate deficiency stress.

Decrease in the availability of phosphate for 14 days old culture grown in modified medium did not show any significant variation in the overall growth and carotenoids accumulation. (Figure-25).

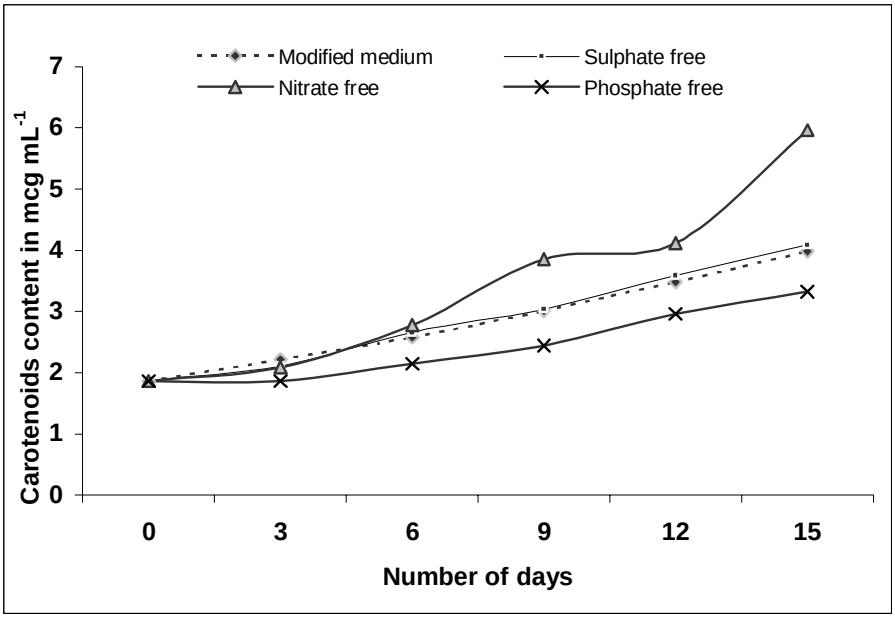


Figure 25. Effect of different nutrient stress on carotenoids production in comparison with modified medium.

[*Dunaliella* cultures grown for 14 days with modified medium were inoculated into different stress inducing medium and were maintained at 18 ± 2.0^0C. Number of days on x-axis denotes the time after transferring to stress media].

6.14 Salt (Sodium chloride) stress and culture height.

Since *Dunaliella* is a halotolarent form it was intresting to study the effect of salt stress on carotenogenesis. To know the effect of salt stress on growth of cultures, two weeks old cultures were subjected to increasing concentration of salt by 2.0 % w/v (0.35 M concentration) along with enhanced light intensity. Enhanced levels of sodium chloride has not shown marked change in the cell productivity, however it resulted in increase in β-carotene content upto 2.2 % (w/w) on dry weight. Moreover, there is no much beneficial effect on vegetative stage beyond 1.5 M concentration of sodium chloride as seen in AS-100 medium earlier. Hence increment of 2.0 % w/v (0.35 M) of sodium chloride for induction of carotenoids was found to be ideal and the same was adopted for further studies.

Culture height also play a vital role in production and carotenoid accumulation of *Dunaliella*. Culture depth will affect the factors like availability of external carbon dioxide and sunlight for photosyntheis. Culture height is of great significance in carotenogenesis phase as it has not shown any significant change in vegetative stage. In case of *Dunaliella*, maintenance of culture at 1.0 cm thickness at direct sunlight (25-30 Klux) has shown to be not efficient as

cells were almost bleached on 12th day , which may be possibly due to high light induced heating of culture (after attaining >35^{0}C during 1-3pm). However culture height of 5.0 cm is found to be ideal as noted from the studies carried out in cultures grown in circular cement tanks. This has also shown maximum carotenoids accumulation. However the increasing height of culture to 15cm did not affect the growth but the carotenoids content was found to be less, since nearly 60% reduction was found in comparison to 5.0 cms depth (Table-17a and 17b).

Table 17a. Effect of culture height on biomass and carotenoids production in *Dunaliella salina.*

Culture height (in cm)	Biomass in gram of cells L^{-1} of culture		
	5th Day	**10th Day**	**15th Day**
1.0	2.49	1.72	Bleaches
5.0	2.55	2.63	3.72
10.0	2.57	2.88	3.68
15.0	2.52	3.09	3.65

[Biomass in terms of wet biomass in g/L, values are average of three experimental readings]

Table 17b. Effect of culture height on carotenoids production in *Dunaliella salina* Carotene content (mg/L).

Culture height (in cm)	**5th Day**	**10th Day**	**15th Day**
1.1	0.48	1.18	-
5.0	0.56	1.59	1.61
10.0	0.29	1.8	0.98
15.0	0.22	0.7	0.85

[Light intensity was of 20-25 Klux, carotenoids content in terms of mgL^{-1}, values are average of three experimental readings]

Salient feature:

The results have shown that light and nutrients stress are the efficient method for induction of carotenoids. Carotenoids induction can be achieved upon cultivation of cells for 14 -18 days in vegetative phase and subsequently transferring the culture into carotenoids induction conditions. It was also found that growing cells under low light (2-8 Klux) in ' *modified medium*' (composition in table-14) to attain maximum cell density in the range of 8-10 $\times$ 10^6 cells mL^{-1} by 12-14 days, followed by dilution of culture to 1:1 with 2.0 % w/v (0.35 M) sodium chloride can help by lowering of nitrogen, phosphorous and sulphur. At the same time providing high salt stress was found to be beneficial for maximum production of carotenoids. However high productivity of carotenoids can be obtained by transferring the culture to direct sunlight keeping 5.0-7.0 cm thickness of culture depth. This method has yielded 25.0 mg carotenoids g^{-1} dry weight, which is highly significant compared to that of reported value of 10-12 mg carotenoids g^{-1} dry weight as reported in an US patent (No.4,115,949).

iii. Process for large-scale production.

Background:

Successful scale up of algal culture is a prerequisite to achieve commercial utility. Major problems associated with scale up study is natural contamination of microbes and accidental contamination of other algae and other forms in outdoor conditions. This is a major problem in most of the algal cultivation like, *Spirulina, Hematococcus* etc., The constituents of media can support life of the other microbes if the micro algal culture density is subcritical. *Dunaliella* has an advantageous edge over other algal forms, as it will grow in basic pH up to 9.0 moreover being a marine origin, it is cultivated in a sodium chloride concentration of 5-7 % (w/v), which may not support other contaminants. We have employed circular cement tanks and raceway pond cultivation for the large scale up cultivation, as they are highly suited for the cultivation economically. Some of the findings of this study are as below.

6.15 Scale up studies.

Cultures were scaled-up by stepwise transfer of stock culture from 100 mL Erlenmeyer flask to raceway pond of 2000 L culture capacity. A detailed flow diagram showing various stages of cultures for scale up is shown in Figure-9, under chapter-2. Maximum cell count attained in carboy culture was upto $9.2 \pm 0.5 \times 10^6$ cells mL^{-1}. These cultures were maintained (at least four container), each stage by sub-culturing once a month as backup for scale up.

Cultures from carboy were transferred to circular tanks of 1.5 m diameter (Figure-26), after 15 days cultures from these circular tanks were transferred into raceway ponds of 5 m^2 capacity containing 500 L medium. The performance of 500L culture in outdoor condition was evaluated in terms of cell multiplication. During the outdoor cultivation, the temperature was in the range of 20 to 28°C with light intensity of 12-15 Klux ensured by 50% cutoff of direct sunlight using shade net as shown in Figure-27b. The cultures were continuously maintained in the vegetative phase of the alga in the outdoor conditions under shade (12-15 Klux) throughout year. Further these cultures were transferred to direct sunlight condition for induction of β -carotene production after dilution (1:1) with 2.0 %w/v sodium chloride. Maximum cell count of 158×10^4 cells mL^{-1} was achieved. When the cells were exposed to high light (25-30 Klux), cells accumulated carotenoids of upto 2.8 % (w/w) (Figure- 28).

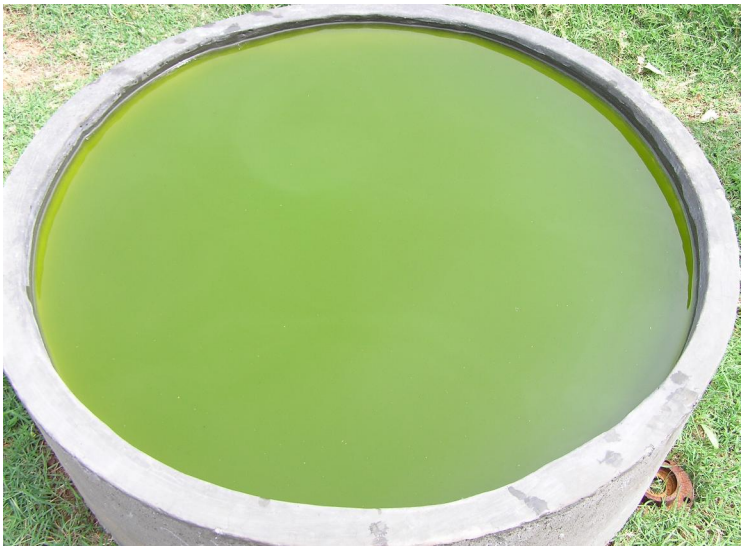


Figure 26. Cement tank (1.5 m diameter and 0.3 m height) showing vegetative cultures of *Dunaliella salina.*

Figure 27. *Dunaliella salina* cultures in vegetative stage (500 L) grown under cut off light intensity of 12-15 Klux (A) and carotenogenesis stage (1000 L) as induced by nutrient and light stress (B).

6.17 Growth in raceway pond.

As explained earlier, cultivation of algae in raceway pond will have certain advantages in terms of high productivity and ease of control of atmospheric conditions. This method is used in Israel, USA, China and in Chile for commercial utilization of *Dunaliella* (Ben Amotz 2003). It is also used for *Spirulina* throughout the world as reported by various researchers. Cultivation in raceway pond has shown the better efficacy with a possibility of recycling of the medium. Hence this method was tried for *Dunaliella* cultivation, in which few cycles of growth were studied in raceway pond in order to know the optimum condition to be provided for maximum multiplication of cells. Outdoor cultivation of *Dunaliella* was continued at pilot scale (upto 2000L) for extrapolation to commercial utilization with the *modified medium.* Initially for 4-6 days, The culture was in lag phase. Cultures showed exponential growth during 6-14 days (Figure-29). This was further subjected to carotenogenesis by diluting the culture and spreading liquid culture as a thin layer of 5-10 cm thickness.

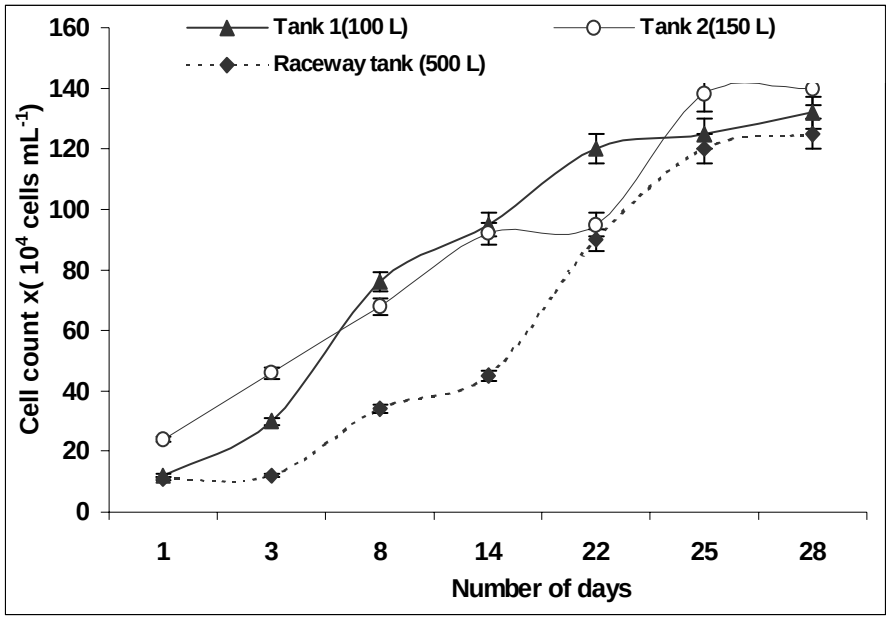


Figure 28.Growth curve showing growth of *Dunaliella salina* cells in circular and raceway ponds.

[*Dunaliella* cultures grown for 28 days with modified medium in out door conditions in circular cement tanks and raceway ponds of 500L temperature was in the range of 24 ± 4.0^0C and light intensity was in the range of 12-25 Klux. Cell count was measured by haemocytometer, values are mean of three values].

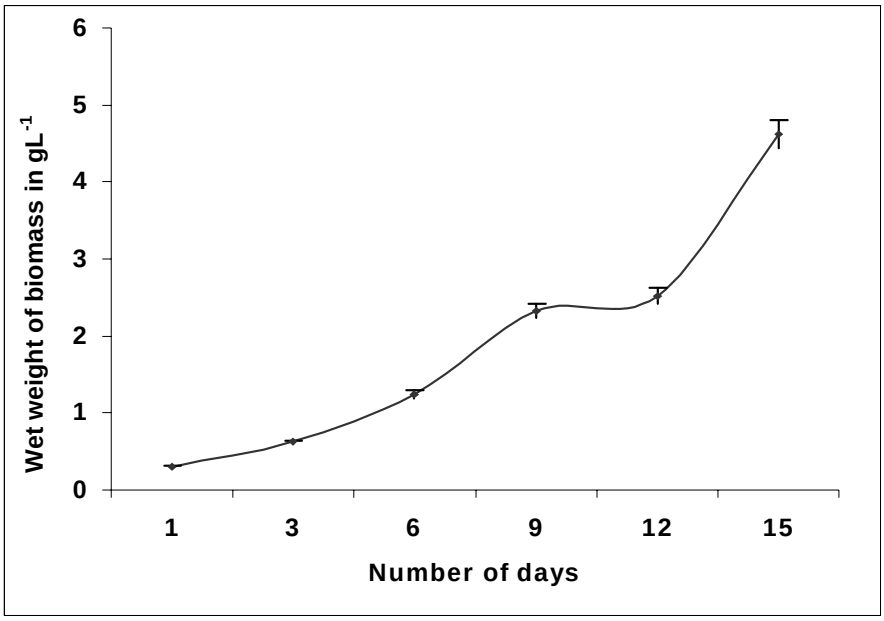


Figure 29. Growth of *D.salina* in out door raceway pond as measured by wet biomass production per litre of culture.

[*Dunaliella* cultures grown for 28 days with modified medium in out door conditions in raceway ponds of 500L, temperature was in the range of 24 ± 4.0^0C and light intensity was in the range of 12-25 Klux. biomass content was measured after centrifugation of culture at 2000 rpm for 10 minutes].

6.16 Growth in fertilizer based medium.

Media cost plays a vital role in making the algal technology economical, as yield per liter of *Dunaliella* is low compared to other algal forms like, *Spirulina*. In order to reduce the cost of medium an alternate source for nitrogen, phosphorous and potassium was tried in the form of agricultural fertilizer (N: P: K :: 15: 15: 15) which was used in place of sodium nitrate, potassium dihydrogen orthphosphate and potassium chloride. Agriculture fertilizer mix was supplemented at 0.5, 1.0, 1.5 and 2.0 g L^{-1} concentration. Among the levels used 1.0 g L^{-1} fertilizer was found to support significant growth compared to AS-100 medium in 50 L culture grown in 1.0 m diameter cement tank. Utilization of the fertilizer based medium for large-scale continuous cultivation in outdoor conditions was not effective. There was reduction in growth rate compared to *modified medium* (Figure-30).

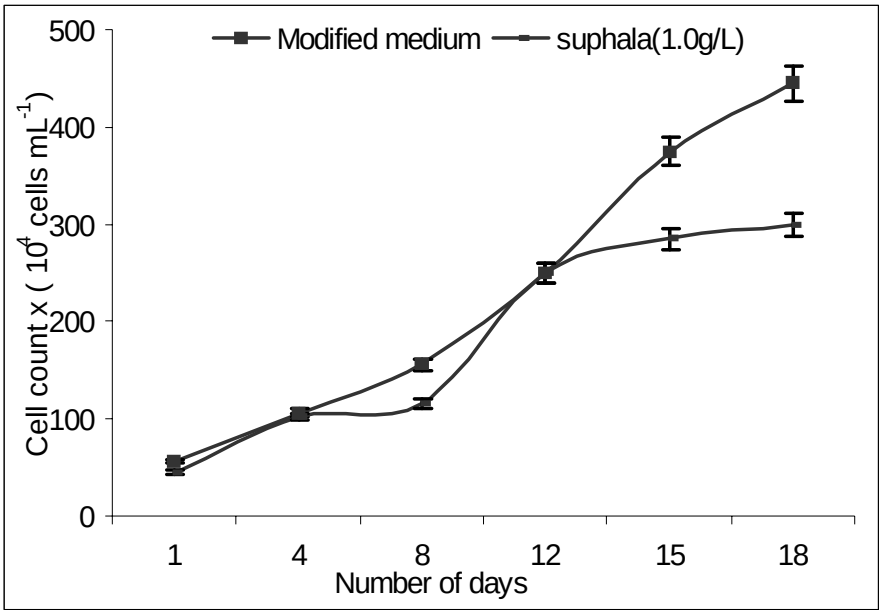


Figure 30. Growth comparison of *Dunaliella salina* in modified medium and suphala medium.

[*Dunaliella* cultures grown for 18 days with modified medium and fertilizer based medium in outdoor conditions in 5.0 L, temperature was in the range of 24 ± 4.0^0C and light intensity was in the range of 12-25 Klux. . Cell count was measured by haemocytometer, values are mean of three values and their standard deviation].

Salient feature:

Successful cultivation of *Dunaliella salina* in large scale was achieved with stepwise scale up from stock culture in to raceway pond. Cultures could be successfully scaled up. Maximum of 10.0 ± 0.5 x 10^6 cells mL^{-1} was achieved in both, circular cement tank (100 L culture) as well as in raceway pond (500 L), which was significantly more than internationally reported growth values. Much higher cell density ~12.0 x 10^6 cells mL^{-1} was achieved in case of carboy cultures. The content of β - carotene obtained by both circular tanks and raceways were also above the values reported in literature (i.e., > 2.5% w/w). Under the outdoor conditions in Mysore 50% cutoff light (intensity of 12-15 Klux) for growth period and exposure to salt and nutrient stress coupled to 24-30Klux light exposure for carotenogenesis was favorable.

iv. Downstream processing

Background:

Upon standardization of the culture conditions for the production of biomass rich in carotenoids, it is equally important to harvest the cells effectively. As cells are small and

motile, it is difficult to harvest cells by simple techniques of filtration. Moreover after downstream processing of biomass for recovery of carotenoids, their degradation must be minimal. Factors which are known to cause degradation of carotenoids are light, temperature and oxygen.

Various energy efficient and effective methods were tried for harvesting of the cells. We adopted high-speed centrifugation as one of the method, both online and batch centrifuges were tried. Apart from these, flocculation was tried as an alternate method for separation of biomass, which reduces the culture volume for centrifugation.

6.18 Flocculation of biomass.

Flocculation was shown to be efficient for micro algal harvesting for effective process, which increase the effective particle size and hence ease sedimentation, centrifugal recovery and filtration (Elmaleh et al., 1991). This is based on neutralization of surface charges, which prevents repulsion of cells; hence flocculants used will be multivalent cations or cationic polymers. Ideal flocculant should be inexpensive, nontoxic and effective at lower concentration. In addition, flocculants used should not interfere with further processing of the product. Some of the multivalent salts employed for the purpose are aluminum sulfate, ferric chloride, ferric sulfate, polyferric sulfate (PFS) and polyacrylamide (Grima et al., 2003). Multivalent metal salts have been used widely to flocculate algal biomass in wastewater treatment process (Lincon 1985) Alum is effectively used as flocculant for *Scenedesmus* and *Chlorella* (Golueke and Ostwald 1965). Controlling of pH is used as an effective method for the harvesting *Dunaliella tertiolecta*, pH was maintained in the range of 8.6 to 10.5 using NaOH and more than 90.0% was flocculated at pH of 10.1 (Horiuchi et al., 2003).

In the present study *Dunaliella salina* was flocculated using different concentration of $Al_2(SO_4)_3$ in the concentration of 0.2, 0.4, 0.6, 0.8, 1.0 and 2mM (Figure-34), $FeCl_3$ at the concentration 0.1, 0.2 up to 0.5 0.75and 1.0mM (Figure- 32), chitosan in the concentration of 0.01, 0.02, 0.03, 0.04, 0.05 & 0.10 mg L^{-1} (Figure- 33) and by using pH alteration.

Among the different chemical flocculants used $Al_2(SO_4)_3$ at the concentration of 0.4 and 0.6mM (Figure-34) and pH 10.5 (Figure-31) was found to be more effective in flocculating the algae.

The supernatant obtained after flocculation was recycled after adjusting the pH. There was no change in the concentration of β-carotene in cells harvested by flocculation technique. Flocculation of algal cells in laboratory scale is shown in Figure-35. Content of aluminum did not affect the growth of algal cells upon recycling the flocculated medium in subsequent cultures. Flocculation using alum in the concentration range of 0.75-0.80 g L^{-1} was used in further experiments. Flocculation reduces the volume of culture to be harvested by 90-93%.

The value of Floc-volume ratio h/h_0, (corresponds to concentration of recovered cells) was found to be 0.05 and 0.08 in case of 0.4 and 0.6mM $Al_2(SO_4)_3$ respectively after 60 minutes, the same was 0.05 in pH 10.5.

This reduction in the culture volume will render the further process of harvesting by centrifugation economical. The cost of electricity will be reduced proportionately by up to 80.0 % as the flocculation needs a pumping of supernatant median containing minimum cells content to other tank and makes the process faster and economic.

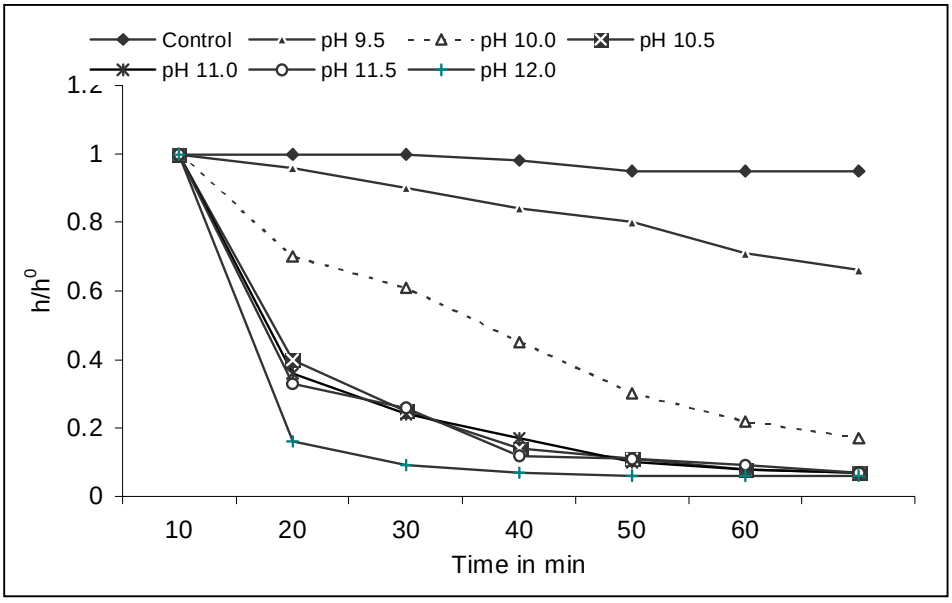


Figure 31. Flocculation efficiency of *Dunaliella salina* by pH alteration.
[*Dunaliella* cultures grown for 14 days were taken in a measuring cylinder and pH was adjusted with 2.0% sodium hydroxide and allowed to settle for an hour and volume of settles cells were read once in 10 min and expressed as floc-volume ratio]

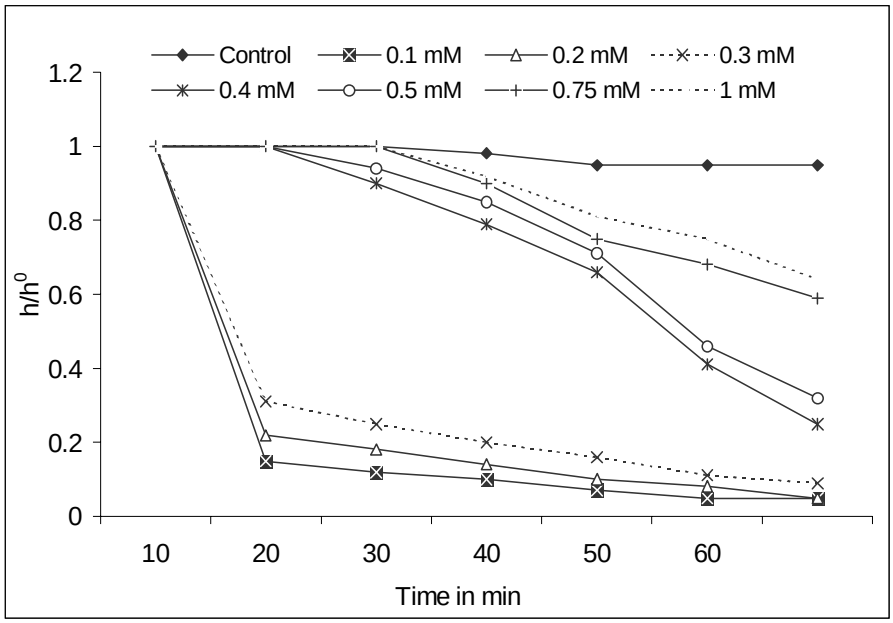


Figure 32. Flocculation efficiency of *Dunaliella salina* by Ferric chloride.

[*Dunaliella* cultures grown for 14 days were taken in a measuring cylinder and different concentration of Ferric chloride was added and mixed well by shaking and allowed to settle for an hour and volume of settles cells were read once in 10 min and expressed as floc-volume ratio]

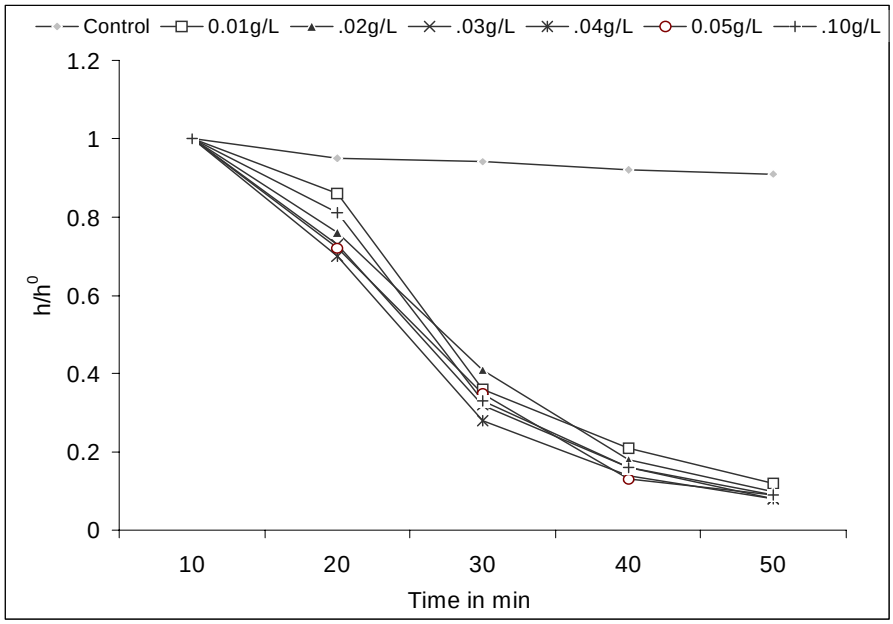


Figure 33. Flocculation efficiency of *Dunaliella salina* by Chitosan.

[*Dunaliella* cultures grown for 14 days were taken in a measuring cylinder and different concentration of Chitosan was added and mixed well by shaking and allowed to settle for an hour and volume of settles cells were read once in 10 min and expressed as floc-volume ratio]

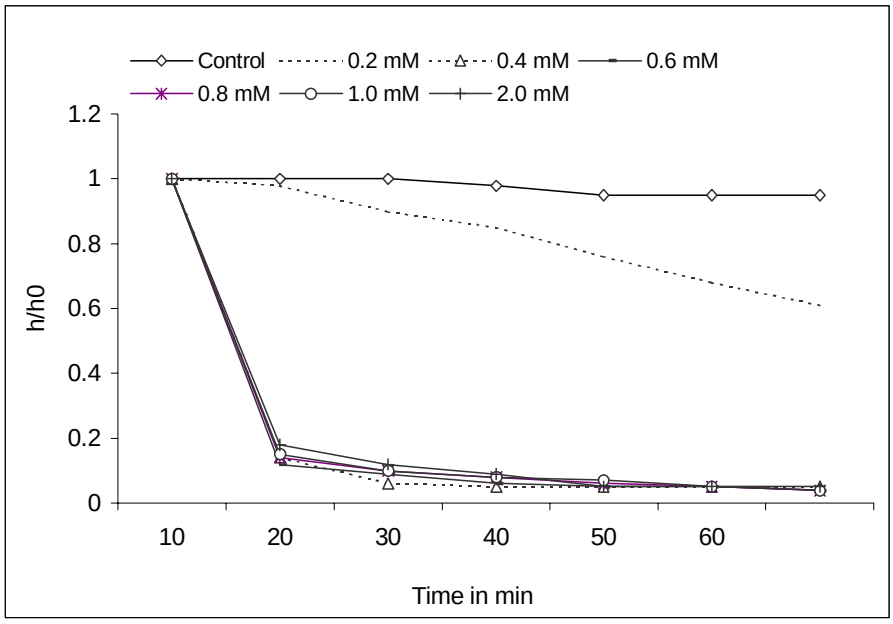


Figure 34. Flocculation efficiency of *Dunaliella salina* by Aluminum sulphate.

[*Dunaliella* cultures grown for 14 days were taken in a measuring cylinder and different concentration of Aluminum sulphate was added and mixed well by shaking and allowed to settle for an hour and volume of settles cells were read once in 10 min and expressed as floc-volume ratio]

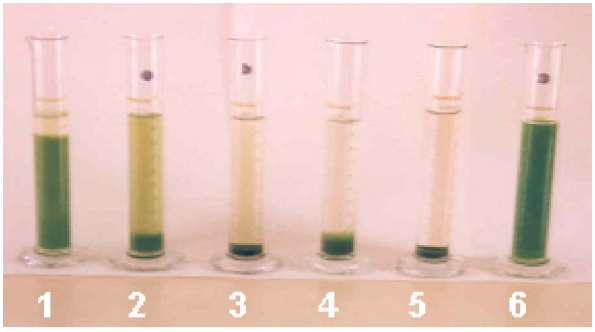


Figure 35. Effect of various flocculation techniques, specimen 3 showing the flocculation of algae by aluminum sulphate.

[1. pH 9.5; 2. Ferric chloride (1.0 mM); 3. Aluminium sulphate (2.0mM); 4. Chitosan (0.10 mg L^{-1}); 5. pH 10.5 and 6. Control (without flocculant)].

6.18.1 Harvesting of biomass by centrifugation.

High-speed centrifugation has been successfully used for separation of various microbes, like yeast and some of the fungus. Most of the algal forms are harvested from suspension by centrifugation. Guidelines for use of centrifugation for separation of algal biomass was given by Chisti and Moo-Young (1991). Though centrifugation is a rapid process it is energy

intensive. Some of the examples include Disk stake centrifuge (Westfalia) used for *Scenedesmus* containing 12 % solids, which consumes electricity of 1.0 kW h^{-1} for 1000 L^{-1} h^{-1} separation. Decanter bowl centrifuge is also used for *Scenedesmus* and *Coelastrum proboscisеum* containing upto 22% suspended solids, which consumes 8.0 kW h^{-1} 1000 L^{-1} (Grima et al., 2003). This has advantages in terms of efficiency, however the consumption of electricity will add up to the cost. In the present study approximately 1700 L culture needed to be centrifuged to yield 1.0 kg wet biomass, which constitutes ~125-150.0 g dry biomass yielding 3.5-3.75 gm of carotenoids. Direct centrifugation also had limitation for large scale as only 2400 L can be handled in a centrifuge per day (available with us) using online centrifugation system (100 L hr^{-1}). In online centrifugation, feeding was at the rate of 100 L hr^{-1} and biomass with ~85-87.5 % moisture could collected in a cylindrical tube. The biomass adhering to inner wall of rotor was removed manually. The average culture handling in the equipment used is in the range of 2200 to 2400 L per day and electricity consumption is at the rate of 2.0 HP. The same in case of batch centrifuge was in the in the speed of 450-480 L per day per equipment, however the maximum biomass that could be accumulated each time was 0.25-0.30 kg. After the run of the rotor needs to be detached for collection of biomass. Comparison of batch and online is as shown below.

Table 18. Power requirement for processing of 1000L of *Dunaliella salina* culture.

Centrifugation	1,000 L of culture processing	
	Time requires	Electricity
On line	10.0 hr	15.0 kW
Batch	100.0 hr	74.57 kW

6.19 Drying of Dunaliella biomass.

Drying is an important step in algal cultivation, as it helps in storing biomass for longer time, which can be used for various purposes. Drying methodology should be economical, should be able to remove maximum moisture without affecting the nutritional and biochemical qualities of algae. Retention of moisture of more than 2.0 % may lead to growth of microbes, like fungus, mold or other forms. Drying methods that are used for algae are, spray drying, freeze drying, drum drying and sun drying. Spray dying, freeze-drying and drum drying of β-carotene rich *Dunaliella* is reported to produce satisfactory results in terms of uniformity of biomass powder and stability of β- carotene in biomass (Ben Amotz and Avron 1987). Spray drying is generally used for high value products (>\$1000 ton^{-1}), but it can cause significant deterioration of some algal components like, pigments.

In this study different drying techniques used for *Dunaliella* are shade, sun, oven, lyophilization, IR drying, spray drying, which was done as explained in section 3.9 in materials and method. In contrast to earlier reports all the techniques employed resulted in loss of certain amount of carotenoids. However the loss was comparatively less in case of IR drying (Table-19). Utilization of nitrogen as carrier gas did not shown any beneficial effect on preventing carotenoids loss during spray drying. Nutritional quality of the biomass was unaffected by drying of sample. Proximate composition of the spray dried algal biomass is as shown in Table-20.

Table 19. Effect of different drying techniques on carotenoids content of the *D.bardawil*.

Method of drying	**Approximate time (hr)**	**%Loss of carotenoids**	**Electricity consumed in kW**
Shade	45.0	38.90 ± 1.5	Not applicable
Sun	7.0	43.10 ± 0.5	Not applicable
Oven	5.0	54.50 ± 1.6	1.1
Lyophilization	8.0	31.72 ± 0.75	1.5
IR drying	0.75	50.50 ± 1.6	6.0
Spray (air as carrier of sample)	0.50	36.50 ± 1.8	1.5
Spray (Nitrogen as carrier)	0.50	19.65 ± 0.45	1.5

Table 20. Proximate analysis of the spray dried powder.

Parameter	Amount present (w/w)
Protein content	19.5 ± 1.2
Fat	7.2 ± 0.35
Carotenoids	1.7 ± 0.01
Carbohydrate	24.5 ± 1.1
Moisture	2.1 ± 0.2
Total Ash	43.3 ± 2.0

6.20 Downstream processing of carotenoids from Dunaliella.

Cell disruption is necessary for recovery of intracellular products from micro algae (Mendes-Pinto et al., 2001). High-pressure homogenizers are widely used to disrupt various micro algal forms. Agitation of microalgal biomass in presence of glass and ceramic beads in bed mills has been used to disrupt cells of *Scenedesmus obliquus*, *Spirulina platensis* (Chisti and Moo-Young 1986). Ultrasonication of suspended microalgal cells can be used to disrupt small amount of biomass, but this cannot be applicable for large scale (Bermejo Roman et al., 2001). Generally microalgal cells with hard cell walls (*Haematococcus*) require high impact process of cell disruption like, mechanical disruption or treatment with acid / alkali / enzymes. *Dunaliella* lacks a polysaccharide wall and is a natural protoplast enclosed by a thin elastic membrane (Ben-Amotz and Avron 1983). Hence it may not require such hard disruption process, however gentle shaking or vibration should be able to extract all the constituents. Solvent extraction of algal constituents is widely used to extract metabolites like, astaxanthin, β- carotene and essential fatty acids. Solvents like, hexane, ethanol, chloroform, diethyl ether are employed for extraction of fatty acids such as ecosapentanoic acid (Grima et al., 2003).

6.20.1 Extraction in organic solvents.

Among the different solvents used for extraction of carotenoids from dry biomass of *Dunaliella*, n-hexane: isopropyl alcohol and ethyl acetate could extract maximum carotenoids. Methanol extract had chlorophyll contaminants. Acetone, chloroform, ethyl acetate and n-hexane: isopropyl alcohol (1:1) extracted carotenoids and xanthophylls. Content of relative percentage of carotenes extracted (compared to maximum extractability in ethyl acetate till biomass becoming colorless) is as shown in Table- 21.

Table 21. Extractability of carotenoids in different solvents.

Solvent used	Relative % extractability
Acetone	68.42
Chloroform	23.69
Methanol	31.57
n- hexane	65.78
n-hexane : IPA (1:1)	97.36
Ethyl acetate	100.0

** Results are with reference to ethyl acetate*

6.20.2 Extraction in edible oils.

Extraction of carotenoids by dry biomass of *Dunaliella* using edible oils was tried in order to have oil based carotenoids formulations. Results reveal maximum extractability of carotenoids in palm oil followed by olive oil (initial content of carotenoids in oil was subtracted by a correction factor for baseline avoidance). Amount of carotenoids extracted by various edible oils are shown in Table-20. Total content of carotenoids in the biomass as seen by solvent extraction was 22.0± 2.0 mg $100g^{-1}$. The results showed that only 21 % of these carotenoids were extractable in oil by repeated extraction process, especially in palm oil (Table-22).

Table 22. Extractability of carotenoids in different oils.

Oil	Amount of carotene extracted (mg $100g^{-1}$)
Coconut	3.244
Gingilli (Sesame)	2.948
Ground Nut	3.110
Mustard	3.072
Olive	4.622
Palm	4.846
Rice bran	4.046
Sun flower	4.328

* Carotenoids of biomass was 2.0 ± 0.2 % w/w

6.21 Stability of carotenoids in different edible oils under different storage conditions.

Stability of carotenoids is the most crucial in the process of providing good biological value for the compounds in the form of various formulations. Carotenoids being tetraterpenes with number of unsaturated carbon atoms, they are known for lose these bonds to become colorless

which may inhibit biological activity. This process may take place in presence of oxygen, elevated temperature or light. In this direction various researches have shown the utility of stabilizers, like BHA, and inert atmosphere, which can help to greater extent. Hence an attempt was made to provide maximum stability for extracted biomass in different oil matrices used for cooking purpose.

Carotenoids exhibited highest stability in olive oil at room temperature (24 ± 2.0^0C) without the addition of any stabilizing agents (Figure-36). In case of olive oil more than 50 % of carotenoids were stable at the end of nine months. In case of palm oil, loss was around 60.0 %, mustard oil exhibited a loss of 68.2 % where as other oils showed loss of more then 80.0 % at room temperature at the end of nine months. Filling the overhead space with nitrogen gas offered protection to carotenoids degradation to a limited extent. The same has shown 27.4% loss in case of olive oil and 29.3% in case of palm oil. Total removal of oxygen could be beneficial. Addition of BHA at the concentration of 0.01% (Figure-37) showed nearly similar protection of carotenoids as that of nitrogen flushing (Figure-37). The combination of nitrogen and BHA did not significantly improve the stability. Without additive and nitrogen flushing carotenoids were found to be degraded completely within six months.

In case of storage at room temperature in dark, palm oil exhibited 30.10 % loss of carotenoids without stabilizer and without nitrogen flushing (Figure-36), 27.0% loss was observed when stored under nitrogen (Figure-37). However, BHA reduced the loss to 17.8 % (Figure- 38). Storage of BHA treated samples under nitrogen atmosphere did not offer higher protection when compared to BHA alone (Figure-39). In case of olive oil total loss of carotenoids treated with BHA was as low as 9.98%, palm oil also showed similar pattern.

Results of the cold storage have shown similar results with increase in overall stability. Sunflower oil provided least stability with 44.4% loss without additives after nine months (Figure-40), the same has shown 28.3 % when stored under nitrogen (Figure-41), 25 % loss when stored with BHA (Figure -42), and 24.1% in case of combined storage conditions (Figure-43). Olive oil has shown 20.86%, 14.94 & 8.53 % loss in case of normal, BHA and in combination of BHA with nitrogen respectively. Palm oil has shown 21.36, 14.86 & 10.11 % loss in case of normal, BHA and combination of BHA with nitrogen respectively. Coconut oil exhibited much change in stability in various conditions. This has shown 29.1 % loss in case of normal, 24.93 % in case of combination of BHA with nitrogen (Figure-43).

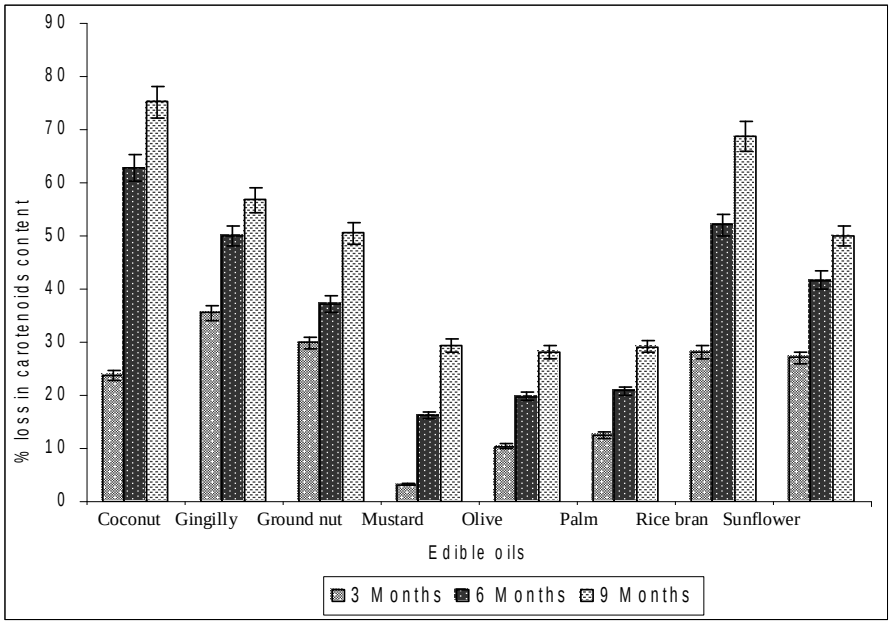


Figure 36. Percentage loss of carotenoids in different oils in room temperature in dark.

[Carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oils and stored at room temperature and these oils were analysed for the carotenoids content]

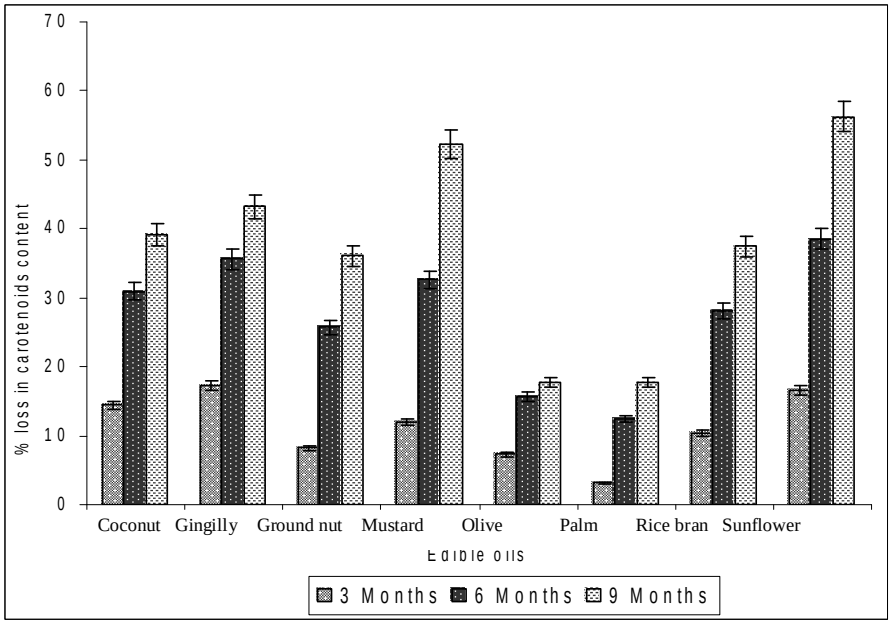


Figure 37. Percentage loss of carotenoids in different oils in dark with nitrogen overhead.

[Carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oils and stored in dark with displacement of overhead space with nitrogen and these oils were analysed for the content spectrophotometrically once a week till nine months and results were expressed as % loss in comparison with original content]

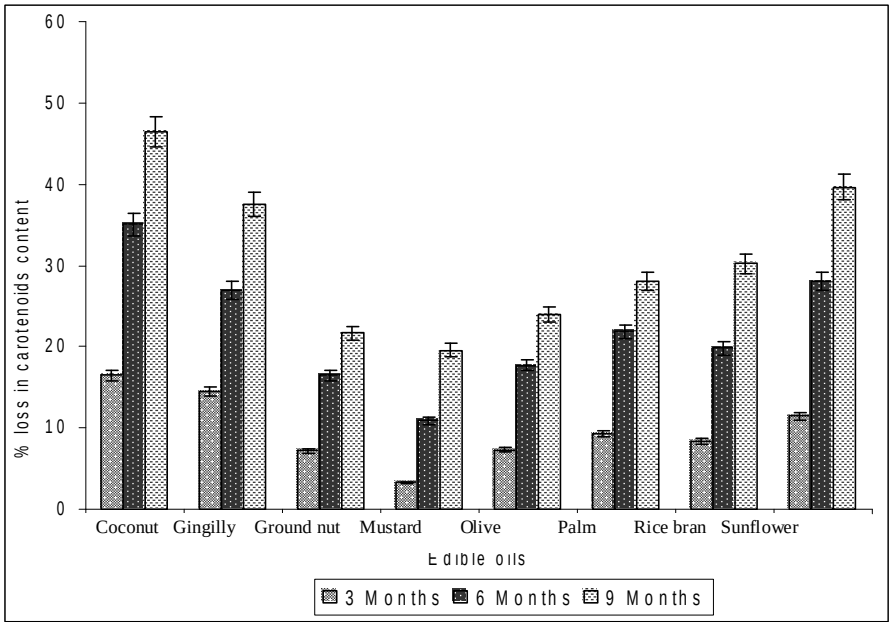


Figure 38.Percentage loss of carotenoids in different oils in dark with BHA.

[Known amount of carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oils and stored in dark with BHA and these oils were analysed for the carotenoids content]

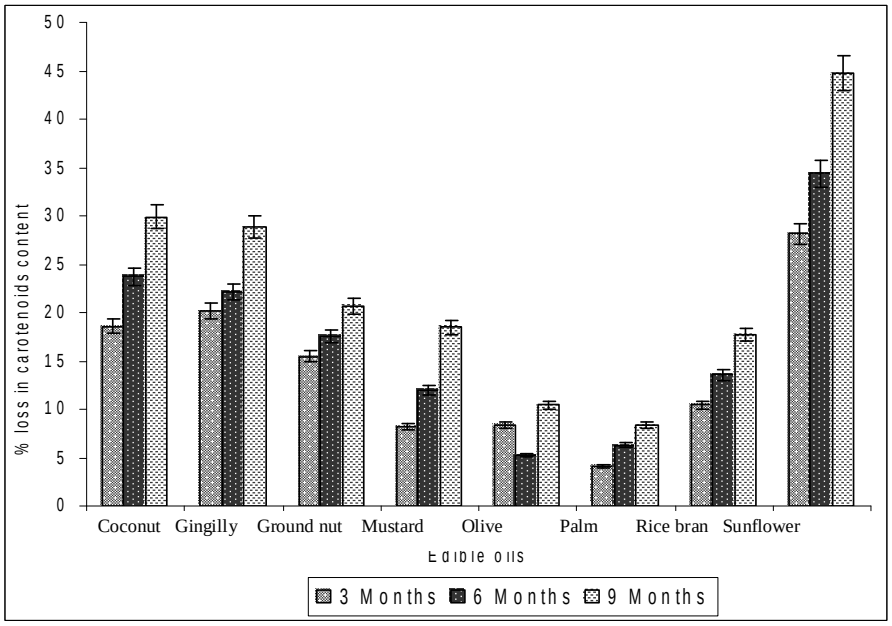


Figure 39. Percentage loss of carotenoids in different oils in dark with nitrogen in combination with BHA.

[Carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oils and stored in dark with nitrogen as well as BHA and these oils were analysed for the content of carotenoids and results were expressed as % loss in comparison with original contents]

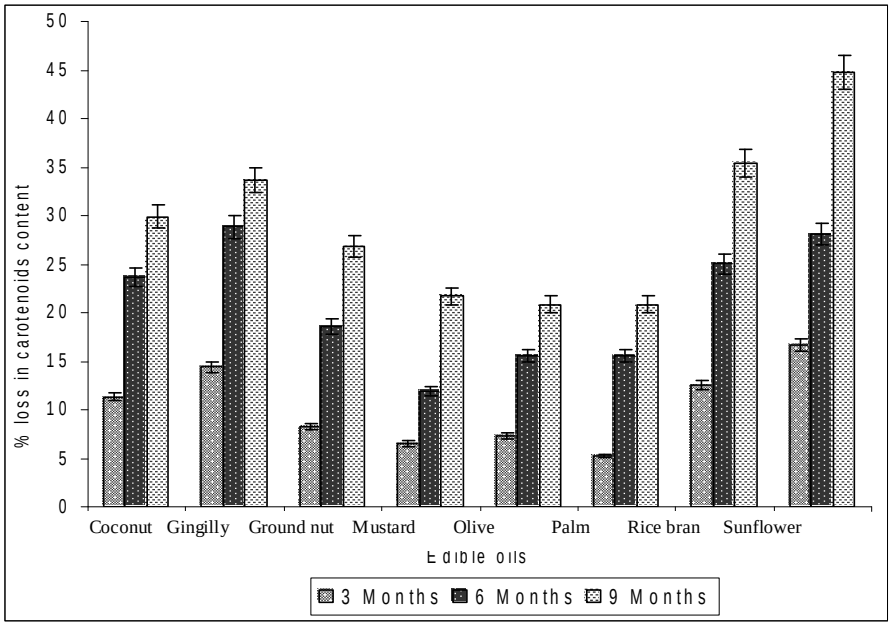


Figure 40. Percentage loss of carotenoids in different oils in cold temperature (4^0C).

[Carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oil and stored in cold temperature of 4^0C in dark and these oils were analysed for the carotenoids content in comparison with original values]

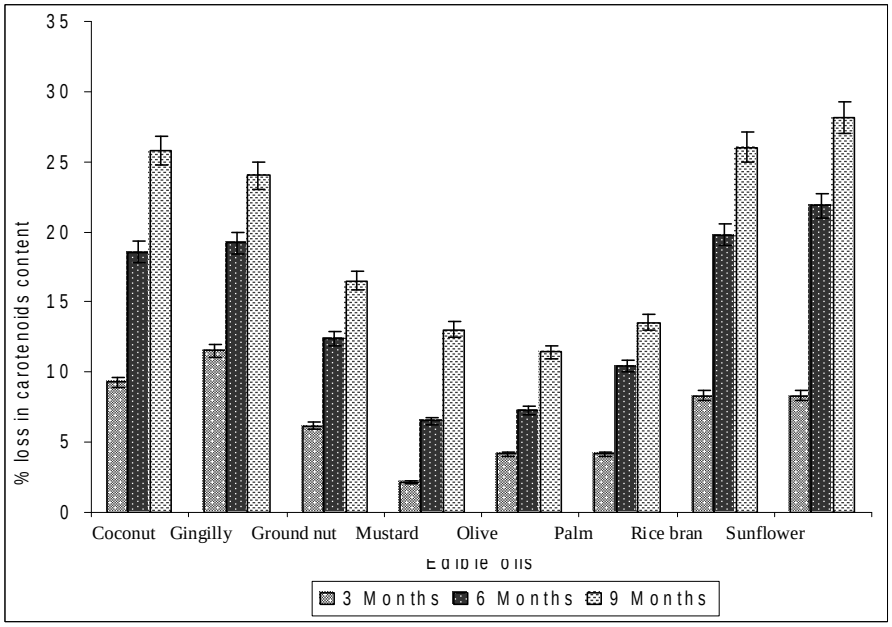


Figure 41.Percentage loss of carotenoids in different oils in 4^0 C under nitrogen.

[Carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oils and stored in cold temperature of 4^0C under nitrogen and these oils were analysed for carotenoids content once a week till nine months and results were expressed as % loss]

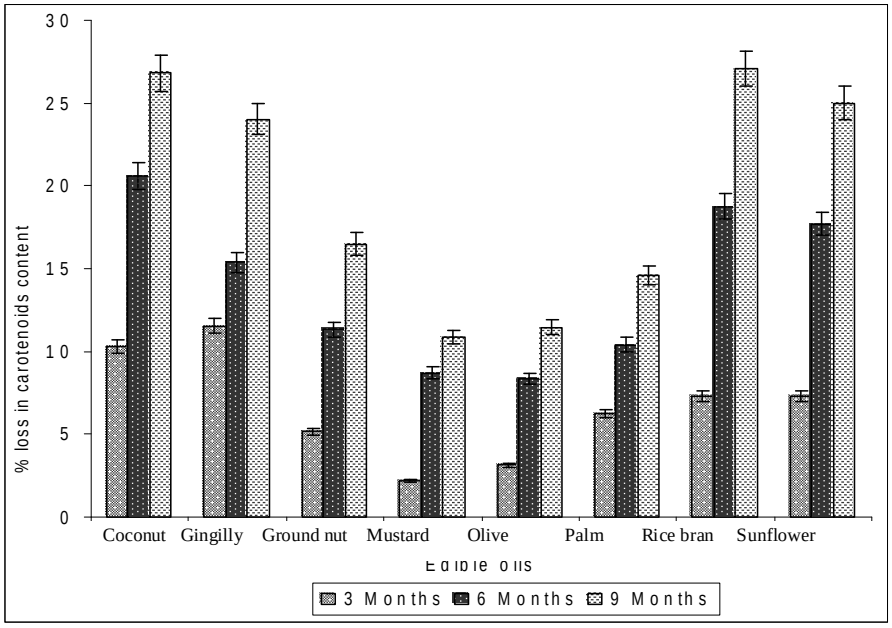


Figure 42. Percentage loss of carotenoids in different oils in 4^0 C with BHA.

[Carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oils and stored in cold temperature of 4^0C with BHA and these oils were analysed for carotenoids content once a week till nine months and results were expressed as % loss in comparison with original content]

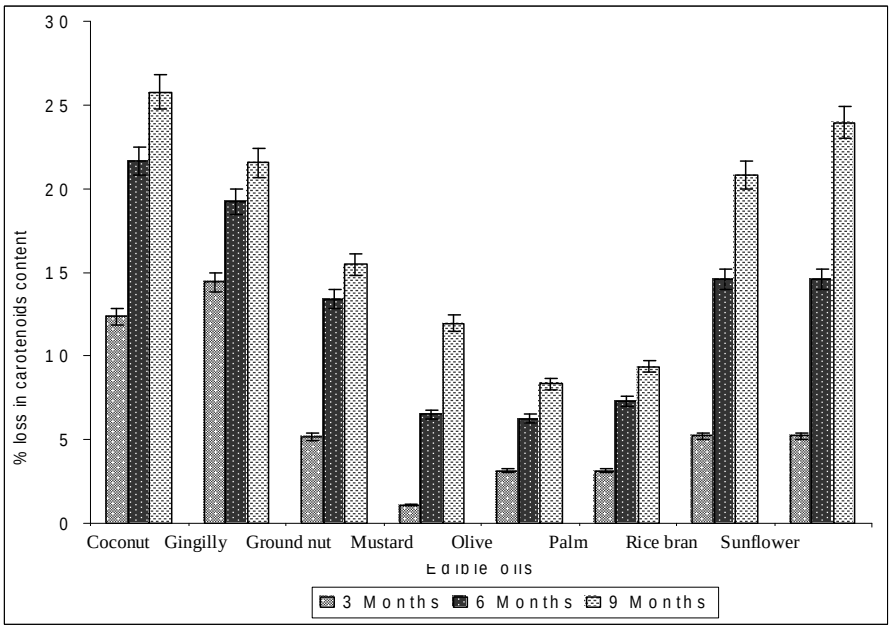


Figure 43. Percentage loss of carotenoids in different oils in 4^0 C with BHA under nitrogen overhead.

[Carotenoids of *Dunaliella* extracted in solvent was dispersed in edible oils and stored in cold temperature of 4^0C with BHA along with nitrogen and these oils were analysed for the content of carotenoids and results were expressed as % loss in comparison with original content]

HPLC chromatograms depict the degradation of carotenoids, which is linear and there was proportional degradation in all the carotenoids as shown by decrease in the intensity of peaks (Figure-44) and the results were correlated with that of spectrophotometric data.

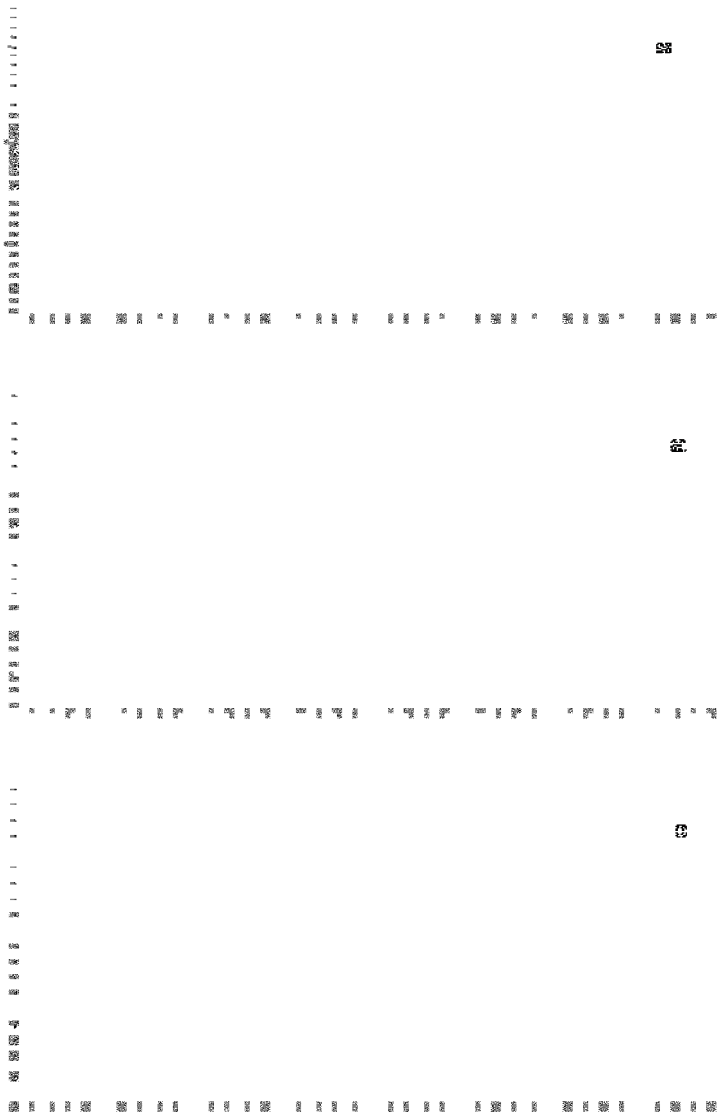


Figure 44. HPLC of β- carotene standard (s) and samples stored fir six months in oil. (a) Mustard oil in dark, at room temperature without stabilizer and (b), Groundnut oil at 4^0C in dark without stabilizer.

The kinetic analysis as shown by degradation rate constant day^{-1} (R^2) indicated that the degradation is very high when stored in the absence of oil matrix (0.6 to 2.6 x 10^{-3} mg day^{-1}). A comparison of different oil matrices showed maximum degradation of carotenoids when stored in rice bran oil. The degradation was minimum under cold storage conditions i.e., in the range of 0.3 to <1.0 x 10^{-3} mg day^{-1} (Table-23).

Table 23. R^2 Values for different oils in various storage conditions.

Oils	Room Temp. 12 hr light				Room Tem 24 hr dark (24 ± 2.0 ^{0} C)				4^0C in Refrigerator			
	Normal	With N_2	BHA	BHA+ N_2	Normal	With N_2	BHA	BHA+ N_2	Normal	With N_2	BHA	BHA+ N_2
Coconut	4.55	3.61	1.40	2.65	3.36	1.20	1.49	0.83	0.83	1.15	0.79	0.91
Ginjilly	2.39	2.53	1.72	1.91	2.0	1.36	1.12	0.83	0.98	4.55	0.85	0.94
Ground nut	1.94	1.89	0.98	1.67	1.66	1.06	0.58	0.55	0.73	0.74	0.42	0.51
Mustard	1.53	2.22	0.73	1.68	0.82	1.75	0.53	0.48	0.58	0.38	0.28	0.34
Olive	1.53	0.77	0.80	0.77	0.81	0.48	0.67	0.25	0.57	0.47	0.23	0.41
Palm	2.76	0.84	0.86	0.72	0.86	0.47	0.80	0.22	0.58	0.54	0.24	0.38
Rice bran	4.97	3.40	1.24	1.65	2.79	1.13	0.88	0.46	1.06	0.82	0.58	0.78
Sunflower	2.62	1.90	1.52	1.65	1.66	1.98	1.21	1.47	1.41	0.91	0.66	0.79
Without oil	∞	2.98	1.82	1.75	1.99	1.30	1.06	0.81	1.40	1.37	0.88	0.53

[Value x10^{-3}]

Salient feature:

Results of this section reveals that Utilization of flocculants (alum) followed by centrifugation/ filtration serves as effective and energy conservative process for harvesting the cells. All drying techniques were associated with significant loss of carotenoids, how ever its difficult to extract carotenoids from wet biomass as moisture interfere in separation and stability, for drying IR is suitable method, which shows minimum loss of pigments. Carotenoids from *Dunaliella* can be extracted completely using solvents and edible oils cannot take up the carotenoids beyond 4.8 mg $100g^{-1}$, maximum being in palm oil. Among the solvents used ethyl acetate and mixture of n-hexane and isopropyl alcohol have shown excellent extractability. To make carotenoid concentrate for commercial purpose extraction in solvent and resuspending in oil, after making it free from solvents appears to be an efficient procedure. Further it was clear that edible oils when used as matrix would provide stability to a greater extent. Both palm and olive oils can serve as best matrix when used along with BHA or stored under cold temperature (4.0 ± 1.0^{0}C).

v. Analysis and quantification of carotenoids and proximate compositions.

Background:

This section mainly focus on separation, identification and quantification of carotenoids of algae, proximate composition analysis and possible elicitation of biomass and pigments by biotic and abiotic elicitors.

The algal biomass obtained by above-mentioned methodology, were subjected for quantification of carotenoids and other pigments. These pigments were separated by using preparative TLC and further confirmed by HPLC using β- carotene and lutein standards. Many reported methods of HPLC protocols are able to resolve only few of the carotenoids present in algae on C_{18} silica column. Hence in order to get maximum separation of pigments a gradient elution methodology was standardized. The total pigments extract was subjected to column chromatography using different solvents on silica, alumina (neutral), and magnesium oxide, and compounds thus separated were identified by absorption maxima in various solvents.

Further the carotenoids and oxycarotenoids (xanthophylls) were confirmed by mass spectroscopy using positive ion spray mode and also by LC-MS technique as described in detail here under. Algal biomass was also subjected to analysis of proximate composition as biomass can serve as an additive for feed to live stocks, which had similar proximate composition except for the carotenoids content. An attempt was made to enhance carotenoid content by using biotic elicitors and agents interfering in pathway of carotenoids, producing higher contents.

The algal biomass was subjected to chemical analysis in terms of pigments and other constituents. This was done in order to know the quality of carotenoids produced by our technology. As carotenoids are complex mixtures associated with fatty acids and having almost similar physicochemical properties, it is very difficult to separate and identify, hence number of analytical techniques, like spectrophotometry, analytical and preparative TLC, column chromatography, preparative and analytical HPLC, MALDI, MS and LC-MS were adopted to separate and confirm them.

6.22 Identification and estimation of carotenoids and chlorophyll by spectrophotometry.

Both carotenoids and chlorophyll were identified using their characteristic absorption maxima. Carotenoids exhibited three peaks, a major peak at 426 nm and two shoulder peaks at 420 and 435 nm and a peak at 660 nm for chlorophyll (Figure-45). Content of both carotenoids and

chlorophyll were measured at 450 and 660 nm with standard extinction coefficient values as explained afore in materials and methods.

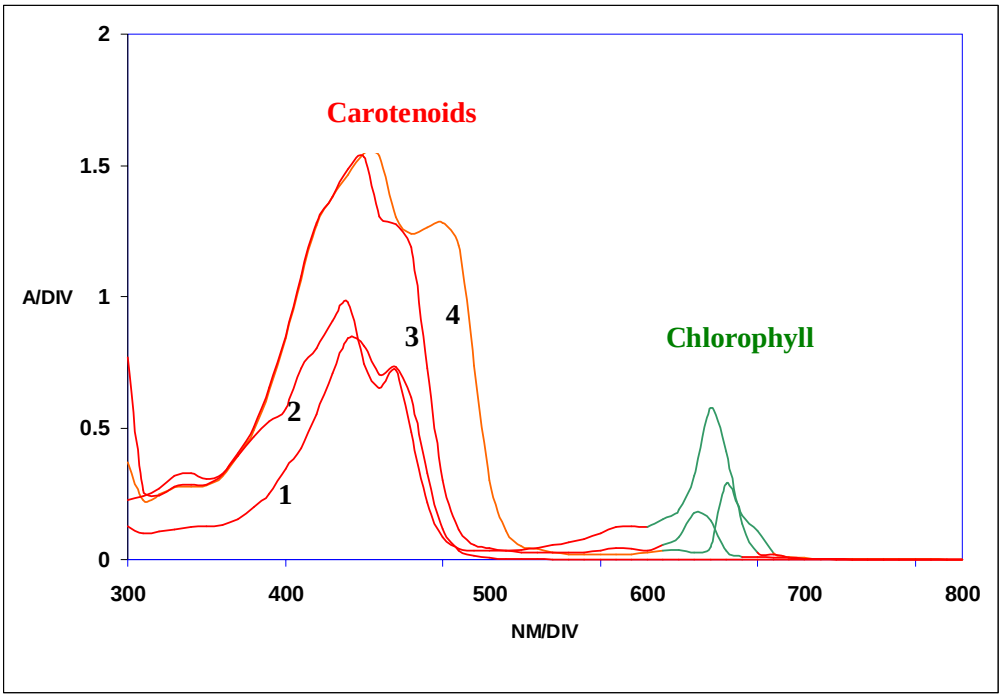


Figure 45. Spectra showing the presence of carotenoids and chlorophyll in different solvent extracts.

[Spectra were recorded using 0.01% solution in respective solvents, 1-synthetic β- carotene, 2-methanol, 3- n-hexane: Isopropyl alcohol, 4- ethyl acetate]

6. 23 Identification of carotenoids by TLC.

On TLC n- hexane and isopropyl alcohol (1:1) extracts of *Dunaliella salina* resolved in to 3 distinct bands in acetone and n-hexane (8:2) solvent system. R_f value of separated band being 0.55 (yellow), 0.16 (yellowish green) and 0.06 (pale yellow). Major spot (R_f -0.55) was confirmed as β- carotene by comparison with standard β- carotene.

In acetone: hexane: ethyl acetate (20:79.5:0.5) solvent system carotenoids resolved into eleven spots. The extract of each band obtained in TLC were analyzed in which 1, 2, 3 and 4 showed similar profile of carotenoid peaks except they differed in their relative quantities of β- carotene (Figure-41), fraction one was found to be rich in β- carotene (Table-24).

Table 24. R_f values of the different fractions as resolved by acetone: hexane: ethyl acetate (20:79.5:0.5)

Sl No	**Possible compound**	**R_f**
1	Synthetic β- carotene	0.93
2	β- carotene	0.93
3	Un identified	0.83
4	Un identified	0.77
5	Un identified	0.63
6	Zeaxanthin	0.54
7	Chlorophyll a	0.318
8	Chlorophyll b	0.274
9	Lutein	0.225
10	Unidentified	0.170
11	Unidentified	0.121
12	Unidentified	0.055

TLC was also carried out, with various solvent extracts of *Dunaliella salina,* and all the solvents have shown similar elution pattern except for chloroform extract, in which β-carotene is the major carotenoid along with few other carotenoids (Figure-46 and 47). It was found that n-hexane and n-hexane: Isopropylalcohol extract had maximum β- carotene in comparison with other solvents.

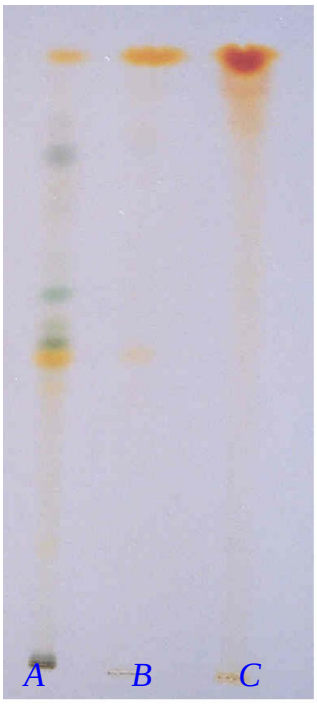


Figure 46. Photograph of TLC plate (A. Standard all *trans* β- carotene B. Extracts from *Dunaliella* carotenoids accumulated cells, C. Extracts from *Dunaliella* vegetative stage.

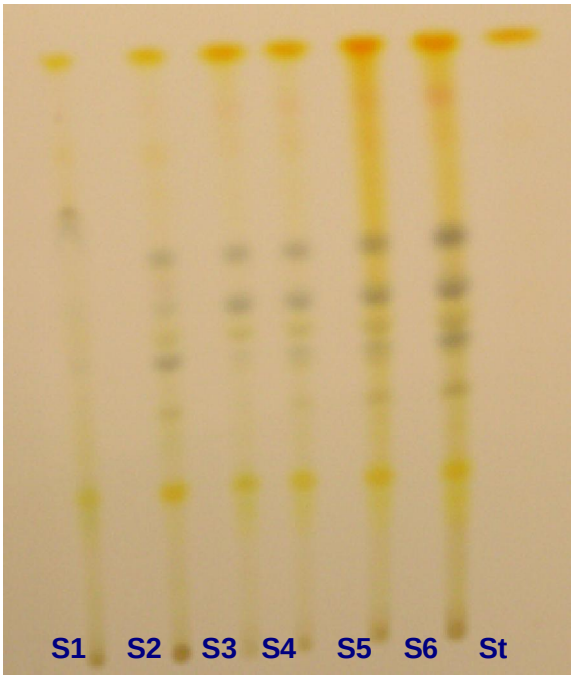


Figure 47. TLC showing the presence of carotenoids extracted using different solvents. [S1- chloroform, S2-methanol, S3-acetone: n-hexane (1:1), S4- acetone, S5- n hexane, S6- n hexane: Isopropyl alcohol (1:1), St – standard β- carotene]

6.24 Identification of isomers of carotenoids.

β-carotene after isomerization (Ai) showed a *cis*-peak between 330-360nm, which was not seen before isomerization (Bi). This indicated that the major β-carotene present in *Dunaliella salina* is of *trans*-configuration. This was confirmed using standard β - carotene, (ICN Chemicals, *trans* β-isomer) which also showed similar peak on isomerization (Figure-48). This was further used for identification of *cis* isomers, which is an indicator of natural source of β- carotene.

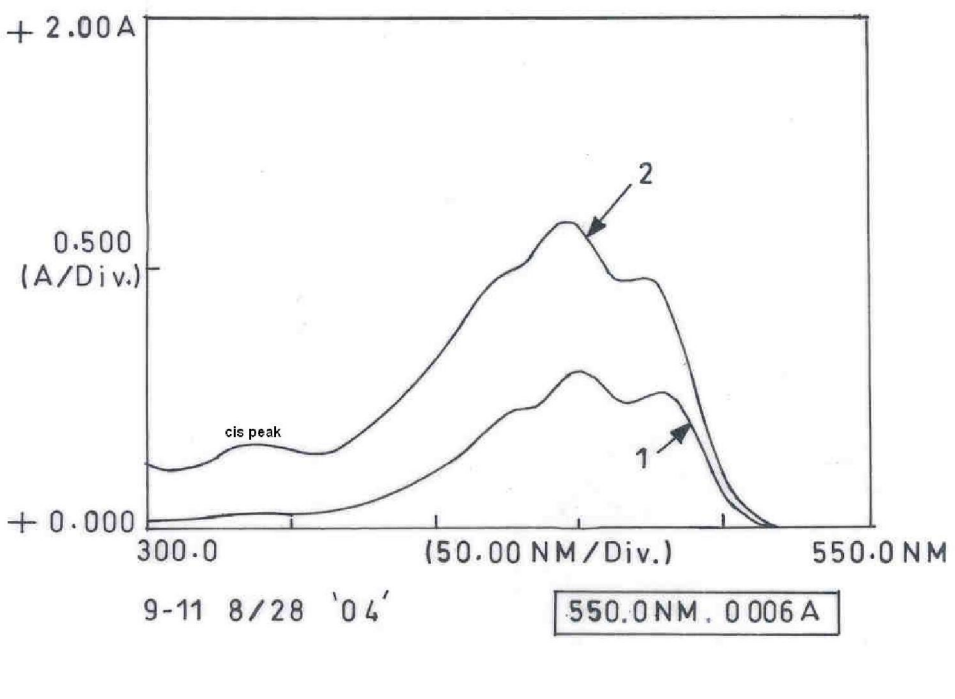


Figure 48. UV visible spectra of β - carotene before (1) and after (2) isomerization, *cis* isomer has shown a peak at 340 nm.

[*Cis* isomer of β- carotene was initially confirmed after subjecting the trans isomer for chemically induced isomerisation after exposing solution of carotene containing 0.01% iodine to florescent light for 30 minutes]

All *trans* β- carotene was eluted at retention time of 9.088 minutes and that of *cis* form was eluted before *trans* at 8.960 min and all *trans* when present along with *cis* isomer was eluted at 9.151 minutes (Figure-49).

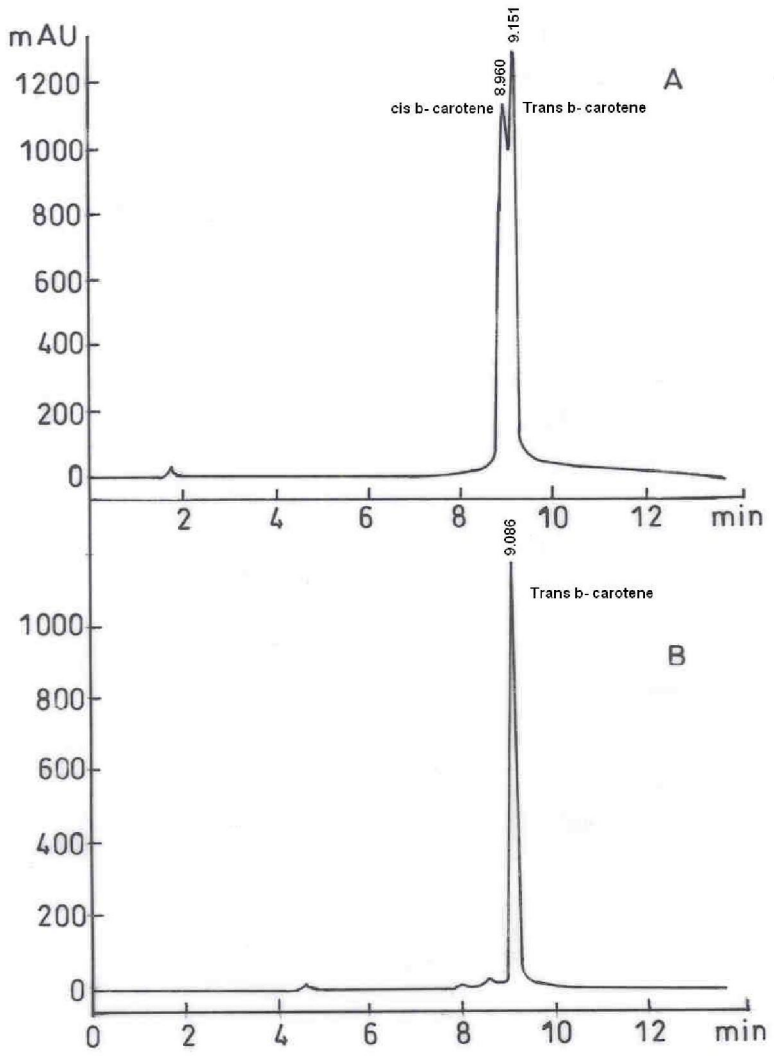


Figure 49. HPLC of β-carotene before and after isomerization; *cis* isomer elutes prior to its *trans* counter part.

[HPLC was done on silica C_{18} column using Methanol: Acetonitrile: Dichloromethane (50:41:9) as mobile phase at a flow rate of 1.0 mL min^{-1}]

6.25 Preparative TLC for separation of carotenoids.

Preparative TLC using n-hexane/acetone/ ethyl acetate (19.5/80/0.5) solvent system carotenoids resolved into 11 spots, which were scraped, re-extracted in acetone and further analyzed by HPLC using gradient solvent system. Some of the spots identified were β-carotene (Rf-0.93), zeaxanthin (Rf-0.55), lutein (Rf-0.23) and chlorophyll appeared as two spots with Rf 0.32 and 0.28 and these were further confirmed as chlorophyll a and b respectively by absorption maxima.

6.26 Separation of carotenoids by column chromatography.

Separation of carotenoids was done on a glass column (300 x 10 mm) using various adsorbants namely, silica gel, magnesium oxide and diatomaceous earth, alumina and silica impregnated with 10.0 % phosphoric acid. Among all the materials tried, silica impregnated with 10.0 % phosphoric acid (overnight) provided better separation. The solvents used for elution were in the order of n-hexane (100%), hexane: acetone (9:1), hexane: acetone (8:2), hexane: acetone (1:1) and acetone (100 %).

Based on the UV- absorption spectroscopy the major carotenoids were identified (Table-25).

Table 25. Different carotenoids identified in *D.bardawil* by UV-Spectrophotometer.

No.	Absorption Maxima	Name	% Content
1	275, 285,296	Phytoene	3.57
2	332,348,367	Phytofluene	5.42
3	425,450,478	β- Carotene	78.57
4	422,444,473	α- Carotene	5.14
5	420,445,475	Lutein	0.86
6	418,440,470	Violaxanthin	1.28
7	417,437,465	Neoxanthin	1.28
8	425,422, 418	Zeaxanthin	1.21
9	422, 436,452	Unidentified	2.67

6.27 Separation of carotenoids by preparative HPLC.

Preparative HPLC was carried out using methanol: acetonitrile: dichloromethane (50:41:9) on silica column. Chromatogram has shown clear separation of chlorophyll and β- carotene and other carotenoids as seen in both green and red cell extracts of *Dunaliella* (Figure- 50). Further, the unidentified carotenoids were separated by column chromatography and identified by UV-visible spectrophotometry.

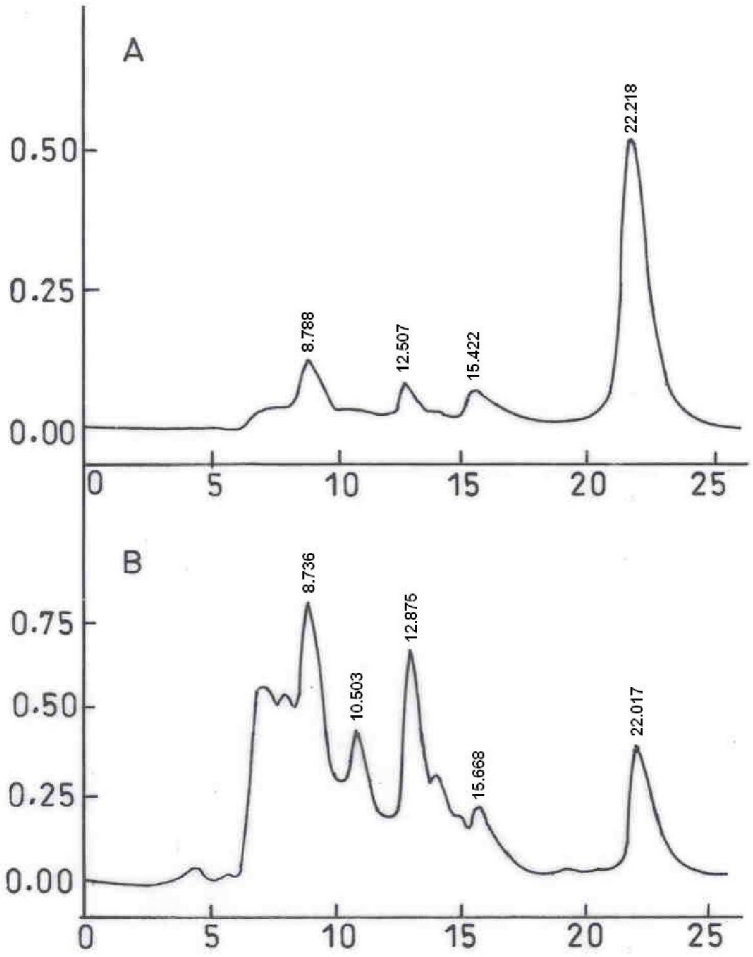


Figure 50. Separation of chlorophyll and carotenoids in preparative HPLC in vegetative (green) and carotenoid accumulated (red) cell extracts of *Dunaliella bardawil*.

[HPLC was done on ODS (Bonda pack) preparative column using Methanol: Acetonitrile: Dichloromethane (50:41:9) as mobile phase at a flow rate of 10.0 mL min^{-1}]

6.28 Estimation of carotenoids by HPLC.

The β-carotene is eluted after 14 min and was identified with standard β-carotene (ICN Chemicals, *trans* β-carotene). A clear separation of carotenoids was noticed when methanol was used as the solvent. Hence methanol was used in further analyses. The chromatogram of *Dunaliella* showed the presence of β-carotene as the major component along with other carotenoids. α-carotene, lutein and lycopene, were confirmed by UV- absorption maxima. The identities of these peaks were confirmed by determination of relative retention times and by

spiking with standard β-carotene. The relative percentage of β-carotene was 78.57 % (Figure-51).

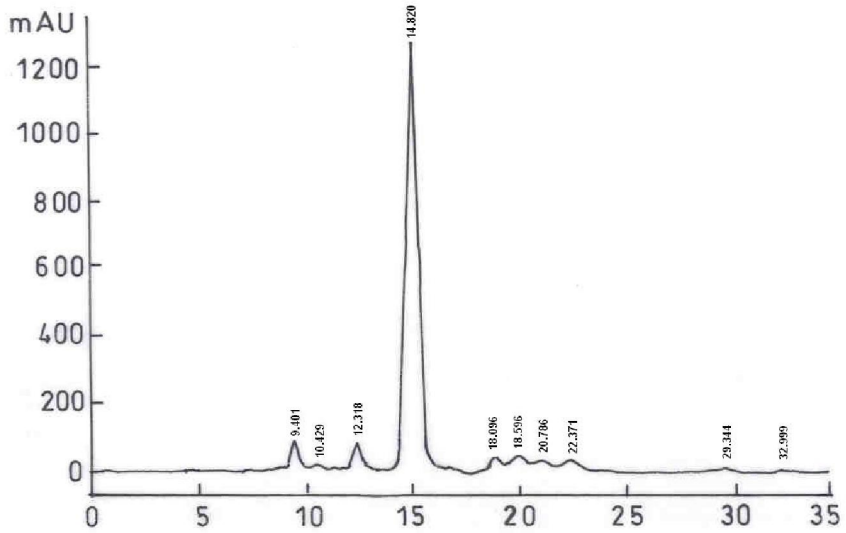


Figure 51. HPLC chromatogram of *Dunaliella* extract in gradient elution system**.**

[HPLC was done on silica C_{18} column (25cm x 4.6nm I.D 5µ particle size) column using Acetonitrile and Chloroform in gradient as mobile phase at a flow rate of 0.7 mL min^{-1}, Rt 9.401-Lutein, 12.318-zeaxanthin, 14.82-β-carotene, 18.096-phytoene]

6.29 MALDI analysis of carotenoids.

MALDI graph clearly showed the presence of β- carotene, lutein, zeaxanthin, neoxanthin in case of *D.salina*. However *D.bardawil* showed the presence of β- carotene, lutein and phytoene which were identified based on the molecular (M^+) peaks (Figure-52 & 53). Mass spectroscopy also confirmed the presence of similar carotenoids.

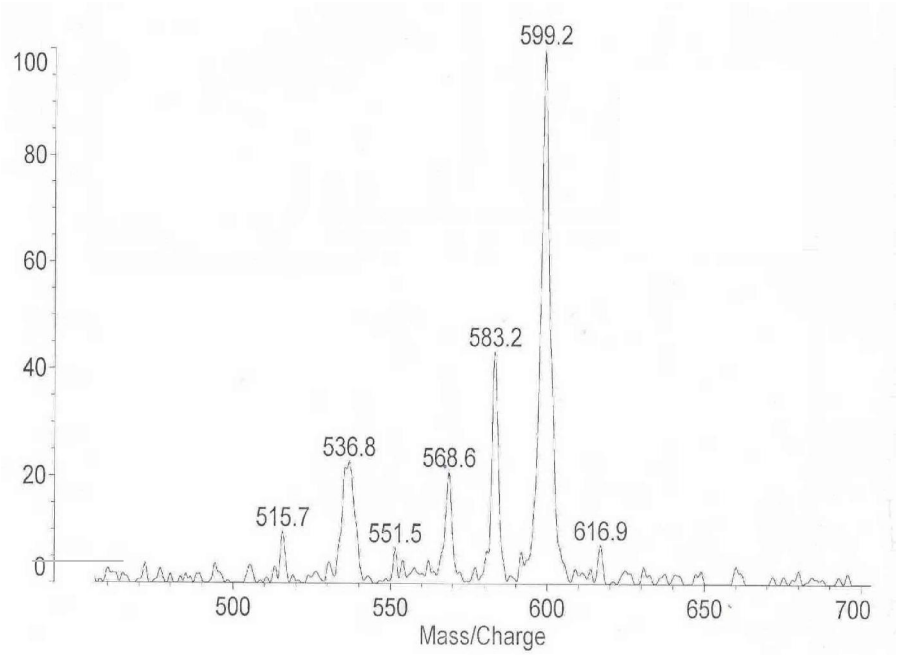


Figure 52. Matrix Assisted Laser Desorption Ionization analysis of carotenoids of *D .salina.*

[MALDI was performed using n-hexane and Isopropyl alcohol mixture (1:1) extract of *D.salina* employing α-cyano 4 hydroxy-cinnamic acid as matrix]

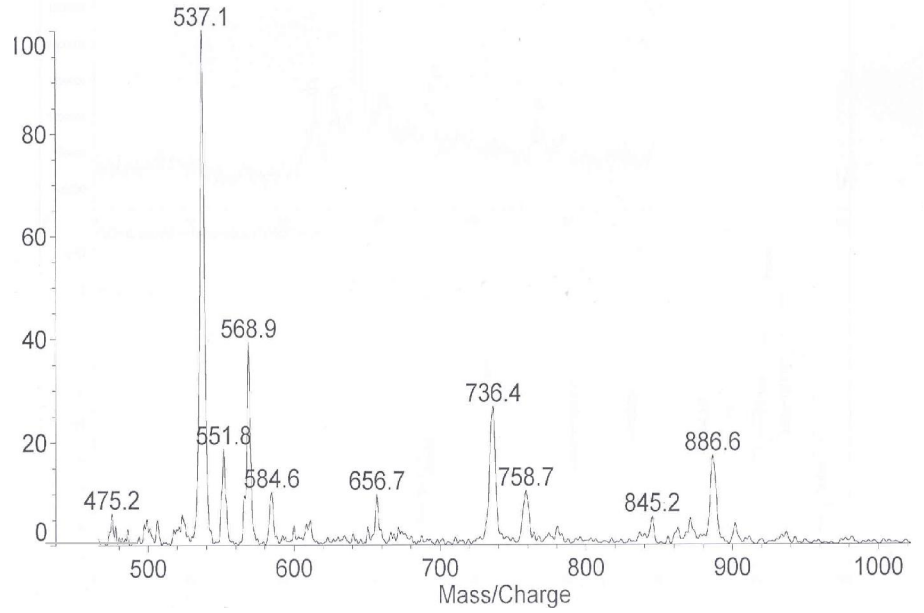


Figure 53. Matrix Assisted Laser Desorption Ionization analysis of carotenoids of *D .bardawil.*

[MALDI was performed using n-hexane and Isopropyl alcohol mixture (1:1) extract of *D.bardawil* employing α-cyano 4 hydroxy-cinnamic acid as matrix]

6.30 Identification of carotenoids by Mass Spectroscopy (MS).

Mass spectrum has shown the molecular peak at 536.544 for standard all *trans* β- carotene (M^+) and also fragmentation pattern in case of ESI-TOF (electro spray ionization) mode, peak at 444.467, 431.356 and 345.243 indicating the fragmentation due to opening of benzene ring and loss of methyl group attached (Figure- 54).

In case of APCI mode molecular peak (M^+) at 537.45 and 538.46 were observed (M^{+1} and M^{+2}). This may not give an idea of isotopes, hence the same was not employed for LCMS (Figure- 55).

Further, among the different fractions analysed for identification of carotenoids (ESI-TOF), acetone extract showed the presence of phytoene and cryptoxanthin apart from β- carotene. Ethyl acetate extract showed the presence of β- carotene as major compound (spectra not shown). However, n-hexane: isopropyl alcohol (1:1) fraction has shown the presence of β-carotene (536.52), lycopene (537.52), phytoene (544.49 and 583.55 corresponding to + K), viloxanthin (599.57) and neoxanthin (639.58, + K). Lutein and zeaxanthin were also seen in the extract but could not be distinguished due to same molecular weight (~568), which have shown a peak at 567.56 and 591 (+Na). Interference of both potassium and sodium ions were seen in mass spectra hence the molecular peaks were considered with addition of respective molecular weight. This extract was further subjected to LCMS in order to confirm the presence of various carotenoids (Figure-55).

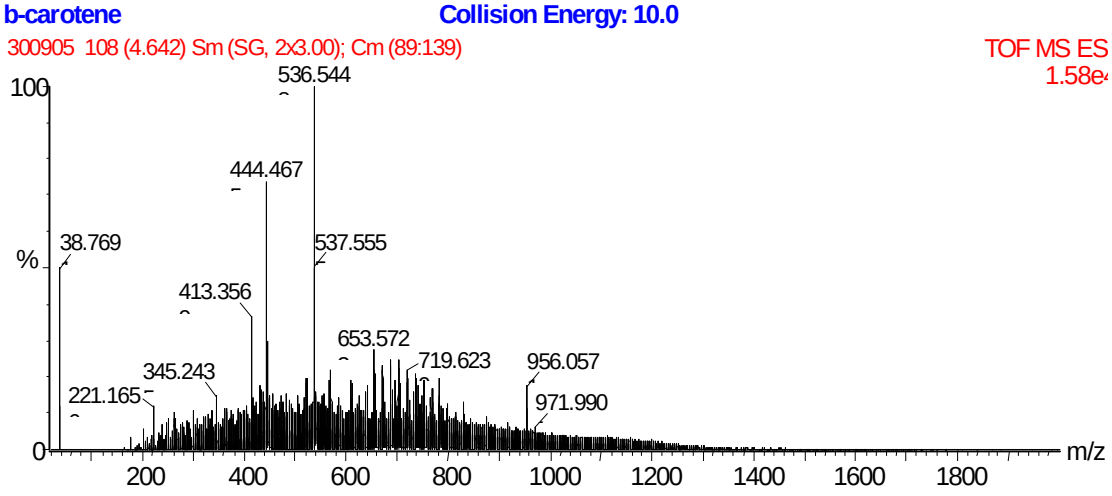


Figure 54. Mass spectra of standard all *trans* β- carotene by-ESI^+.

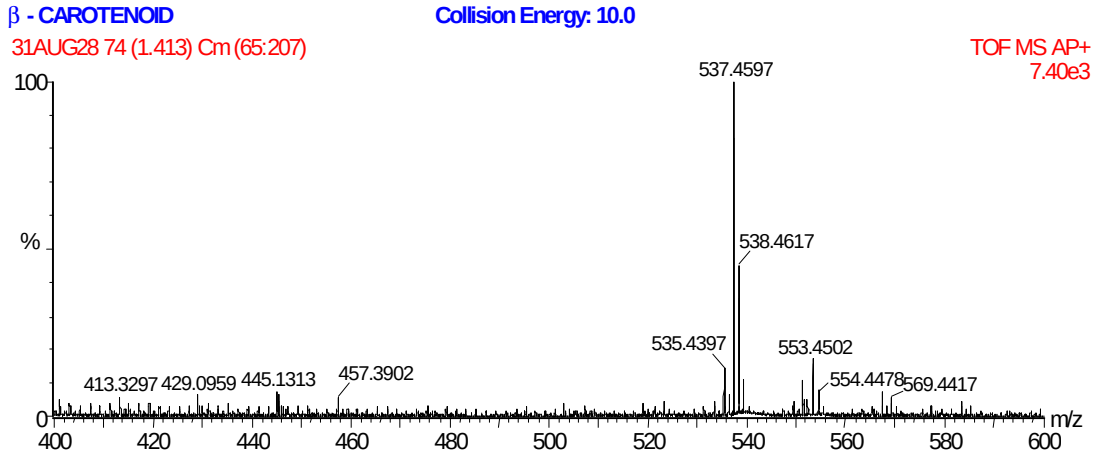


Figure 55. Mass spectra of standard all *trans* β- carotene by APCI mode.

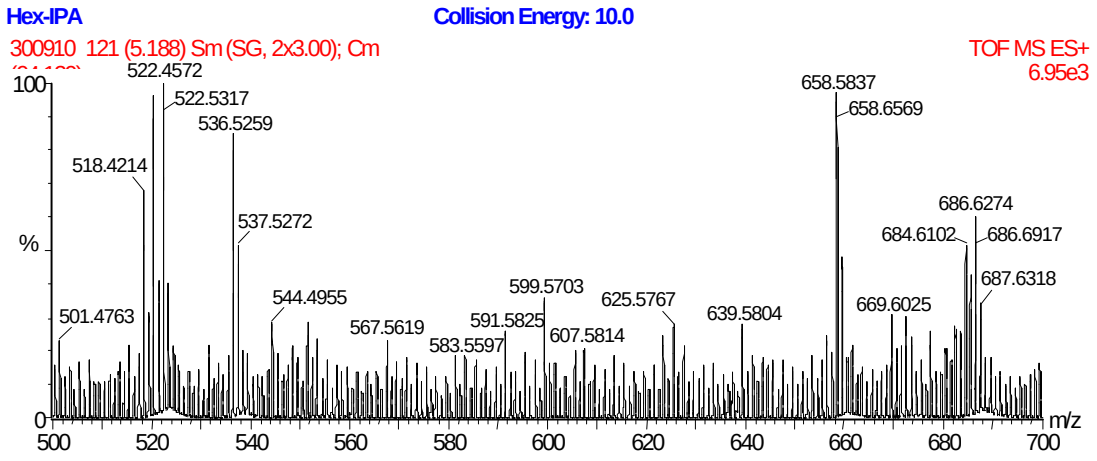


Figure 56: Mass spectra of n-hexane: IPA (1:1) extract of *Dunaliella* using ESI^+ mode.

6.31 Identification of carotenoids by LC-MS.

LCMS of both *D.salina* and *D.bardawil*, n-hexane: IPA (1:1) extracts carried out using C_{18} column with 100 % methanol as mobile phase at 0.2 mL min^{-1}. This system could not resolve the compounds and only β- carotene and lutein were.

In case of LCMS done using methanol: actonitrile: dichrolomethane (70:20:10) as mobile phase on silica C_{18} column at flow rate of 0.2 mL min^{-1} results revealed, revealed the total of nine peaks, the major one being β- carotene constituting more than 60 % of total constituents (Figure-57). First peak eluted at 3.238 was identified as lycopene (mol wt of 536). Other peaks were in the order of, lutein (Rt-4.06), phytoene (Rt-4.125), zeaxanthin (Rt-6.407), β- cryptoxanthin (Rt-9.122), α- carotene (Rt 12.694) and β- carotene (Rt 13.852). These

carotenoids were also confirmed by their fragmentation pattern. LC-MS confirms the results of UV spectral study of carotenoids separated by column chromatography (Figure 58-60).

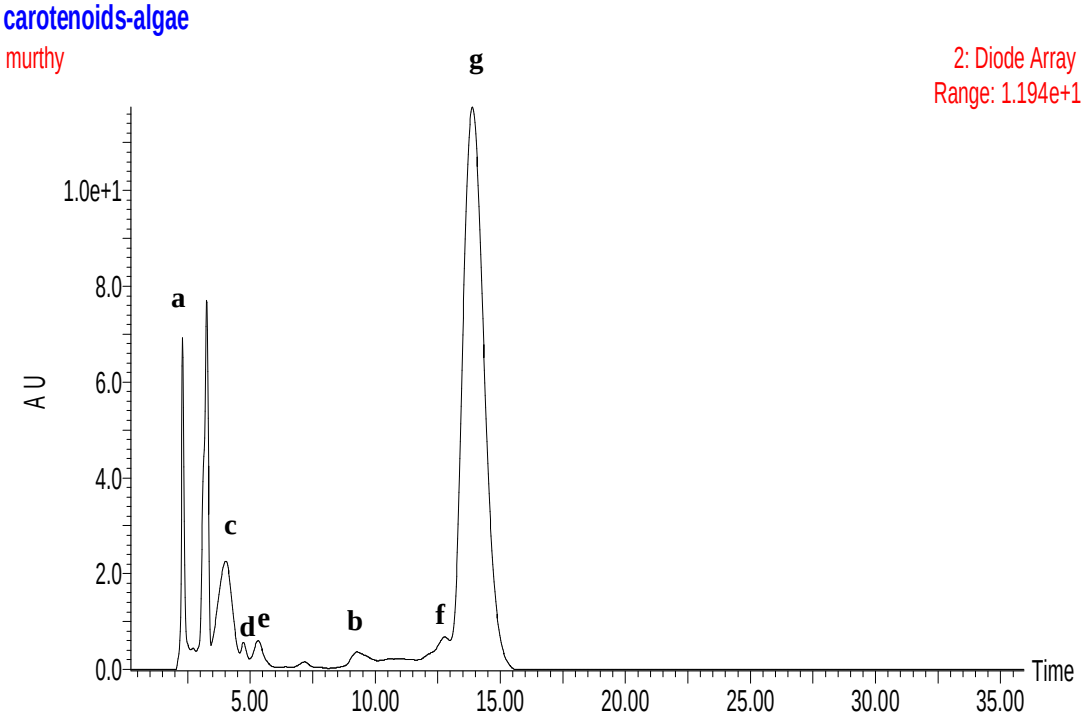


Figure 57. Liquid chromatography of carotenoids of *Dunaliella salina* as separated by methanol: actonitrile: dichrolomethane (70:20:10) on silica C_{18} column at flow rate of 0.2 mL min^{-1}.

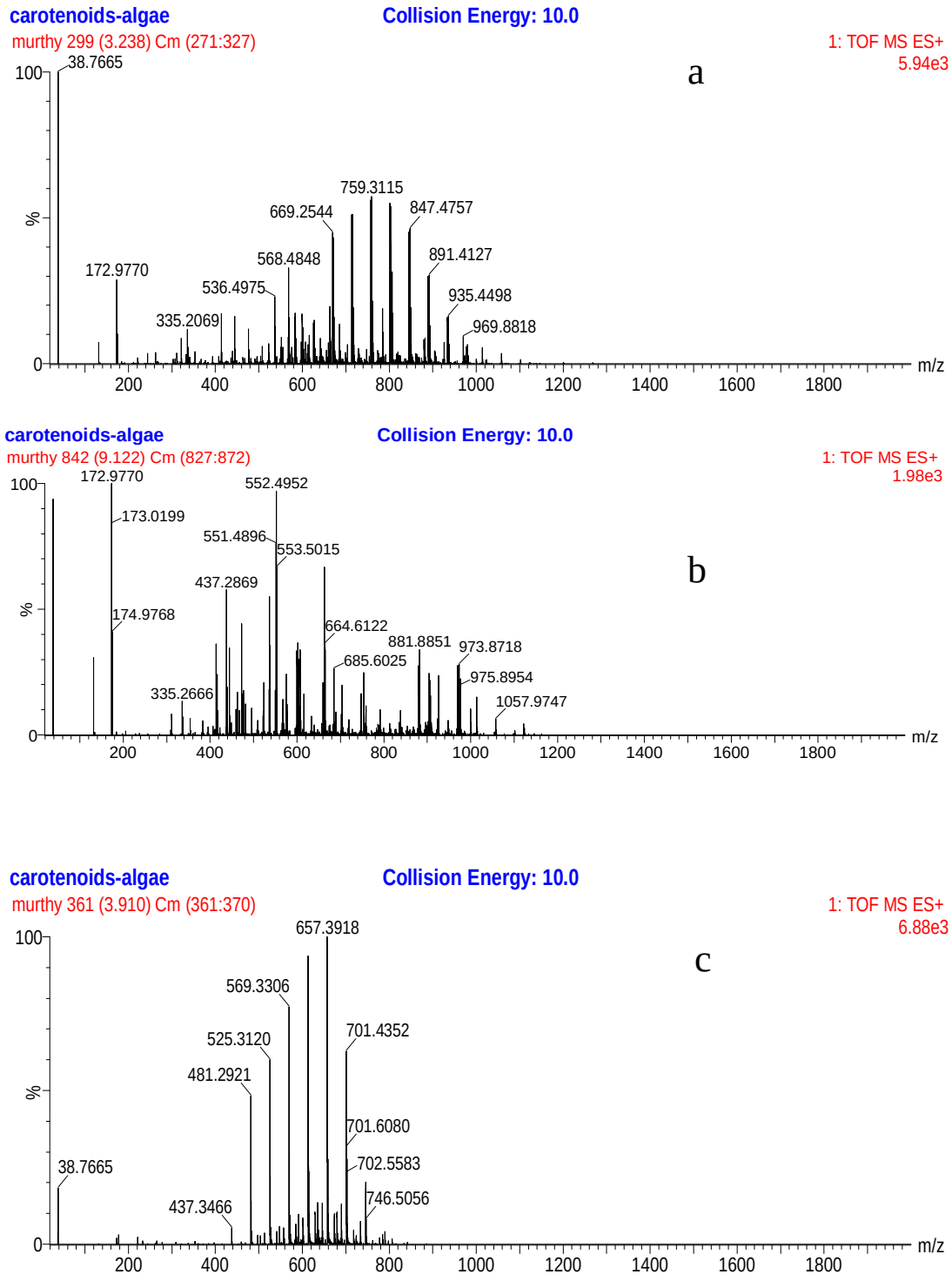


Figure 58. Mass spectra of separated compounds of *Dunaliella* by LCMS, **a**- lycopene, **b**-β-cryptoxanthin and **c**-lutein.

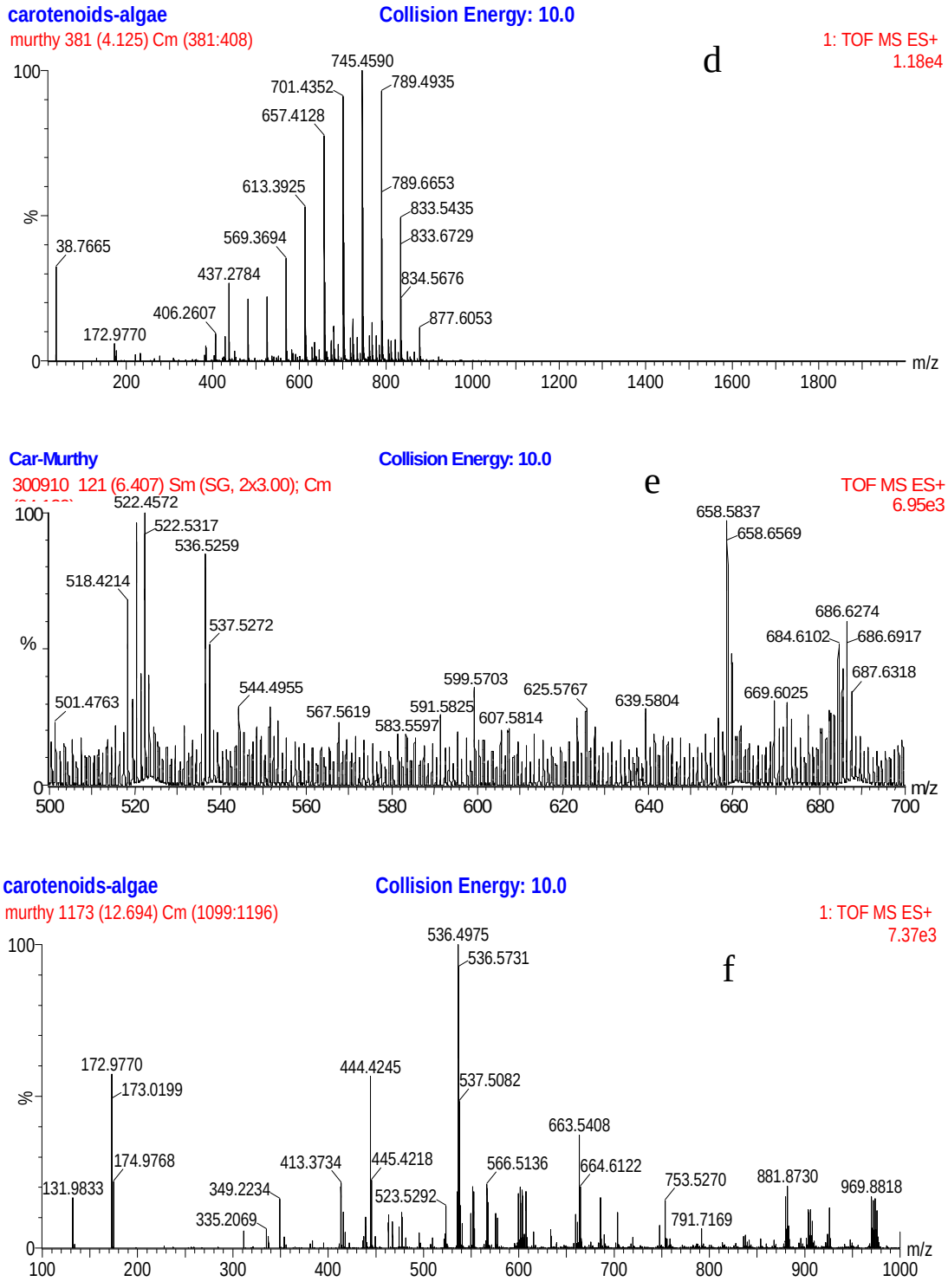


Figure 59. Mass spectra of separated compounds of *Dunaliella* by LCMS, **d**- zeaxanthin, **e**- phytoene and **f**-α- carotene.

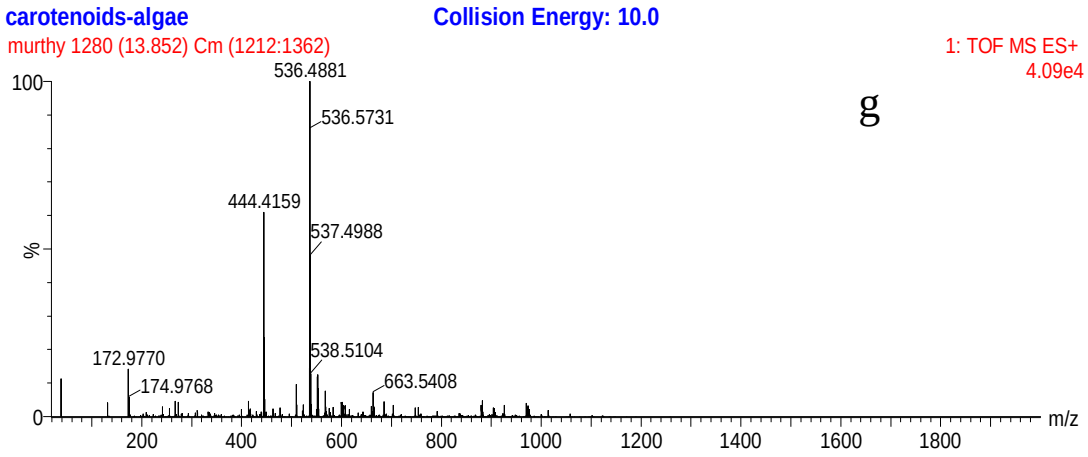


Figure 60. Mass spectra of separated compounds of *Dunaliella* by LCMS, **g** - β- carotene.

6.32 Proximate composition of Dunaliella.

Proximate analysis of *Dunaliella* showed the presence of high protein and ash. Content of ash is possibly due to the presence of salts like sodium chloride and sodium bicarbonate and salts, which can be removed by washing with 0.1% HCl in water. Other constituents of algae include carbohydrates, fat, protein and moisture, however the presence of crude fiber was not seen in both the algal forms (Table-26).

Table 26. Proximate composition of *Dunaliella salina* and *Dunaliella bardawil.*

Components	***D.bardawil***	***D. salina***
	% w/w ± S.D	
Protein	21.51 ± 0.75	19.5 ± 0.26
Fat	07.80 ± 0.38	07.2 ± 0.87
Carbohydrate	26.70 ± 1.11	24.2 ± 1.30
Total carotenoids	02.80 ± 0.30	01.7 ± 0.85
β- Carotene	02.07 ± 0.70	2.45 ± 0.57
Ash	22.80 ± 1.20	20.3 ± 0.87
Moisture	06.70 ± 0.90	05.1 ± 0.15

6.34 Biotic elicitation studies.

The yield of biomass in *D. salina* was maximum after elicitation with *A. paraciticus* followed by yeast and *A.oryzae* at concentration of 2.5 % v/v (equivalent to 0.05 mg dry mycelium mL^{-1} of elicitor). In case of *D. bardawil* the maximum biomass was obtained when treated with *A. paraciticus* (5.0%, equivalent to 0.1 mg mL^{-1} of elicitor), *Rhizopus* (2.5%) and yeast extracts (10 %, equivalent to 0.2 mg mL^{-1} of elicitor) when compared to control (Figure-61). The biomass content was less when treated with *A.ochraceus*, *A. flavus* and *A. oryzae* extracts compared to control indicating the growth suppressant activity.

Among the fungal extracts used *A.ochraceus* (0.1 mg mL^{-1}) exhibited 2.3 and 1.30 folds elicitation in case of *D.salina* and *D.bardawil* respectively. *A. flavus* (0.2 mg mL^{-1}) contributed to 2.8 folds increase in carotenoids of *D.salina* (Figure-62) while, *A. niger* (0.1 mg mL^{-1}) elicited the same by 2 folds in case of *D. salina* (Figure-63). Biomass production was maximum when treated with *A. paraciticus* extracts. However, carotenoids elicitation was not significant. This was followed by *A.oryzae, A.niger* and *A.flavus* in case of *D. salina*. In case of *D. bardawil* similar pattern was observed except that *Rhizopus* at concentration of 0.05 mg mL^{-1} and 0.1 mg mL^{-1} % significantly enhanced the biomass compared to control. Throughout the study of elicitation, it was noticed that the increase in concentration of carotenoids was proportional to the decrease in chlorophyll content, which is a significant observation for large-scale production.

Fungal cell wall extracts are potent elicitors of plant cell cultures, the probable mechanism of elicitation being involvement of key messenger Ca^{2+}, factors affecting the cell membrane integrity, inhibition or activation of intracellular pathways and change in the osmotic pressure acting as stress agent (Alvarez et al., 2000). The extracts of fungi or bacteria contain metal ions like zinc, copper, cobalt, which could act as abiotic elicitors. Apart from these they also contain amino acids, vitamins and minerals. It is the possible effect of these components that is responsible for the elicitation (Ertola et al.,. 1998). In general, carbohydrates are known for elicitation of secondary metabolites either by interfering in biosynthetic pathway or stress (Funk et al., 1987). Other possible mechanisms of the elicitation are receptor binding to the elicitor, changes in the flux across the cell membrane, synthesis of secondary messengers, activation of enzymes responsible for the ROS activation, accumulation of defense related proteins, like chitinase and glucanases, structural change in cell wall, systemic acquired resistance etc. (Radman et al., 2003).

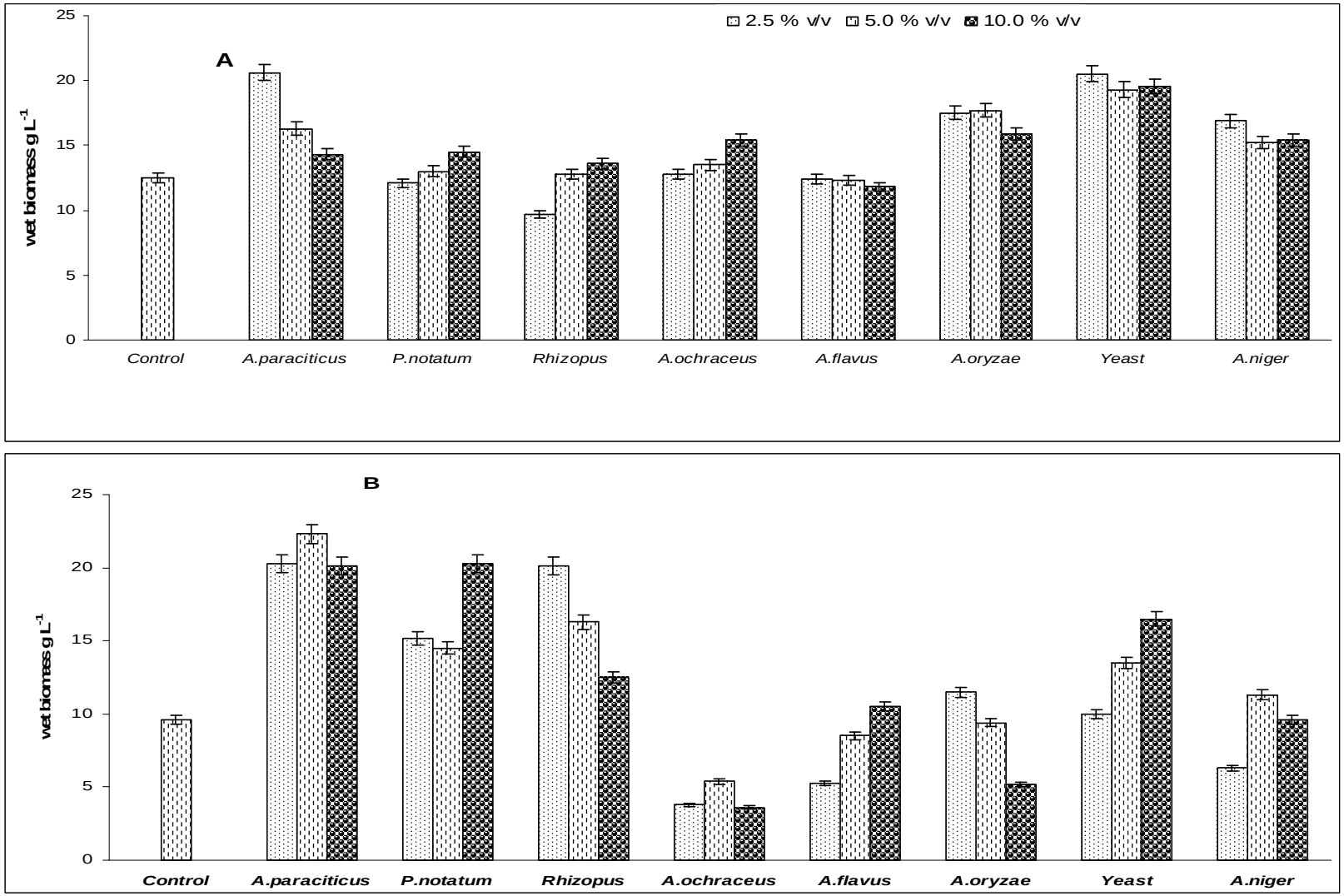


Figure 61. Effect of fungal extracts on production of biomass in *D.salina* (A) and *D.bardawil* (B)[0.05,0.1,0.2 mg dry mass of mycelium L^{-1}]

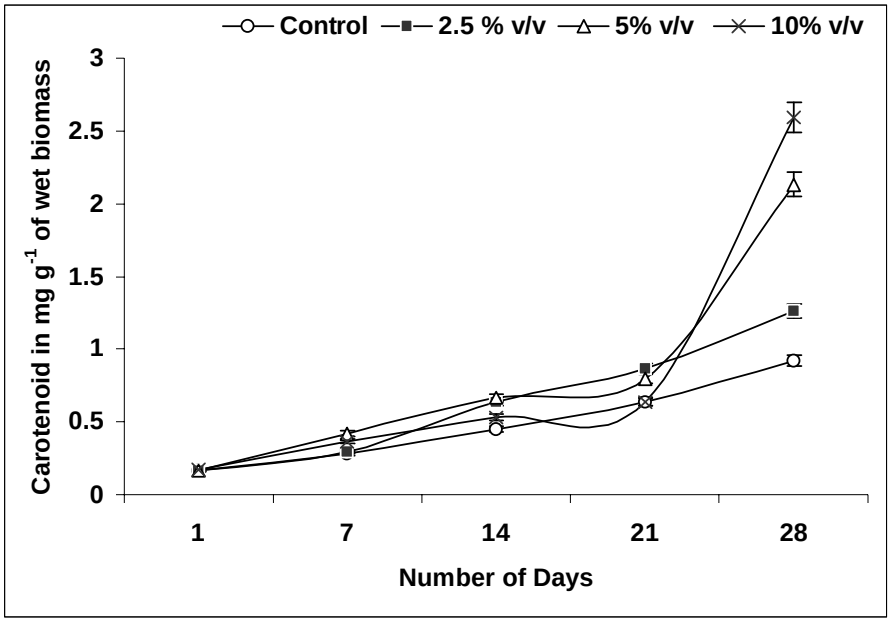


Figure 62. Effect of *A.flavus* on carotenoid contents of *D.salina.*

[*Dunaliella salina* was incubated with water extract of *A.flavus* in different concentration and carotenoids were estimated in wet biomass spectropohotometrically after extracting in n-hexane: Isopropyl alcohol (1:1) concentration corresponds to 0.05,0.1,0.2 mg dry mass of mycelium L^{-1}]

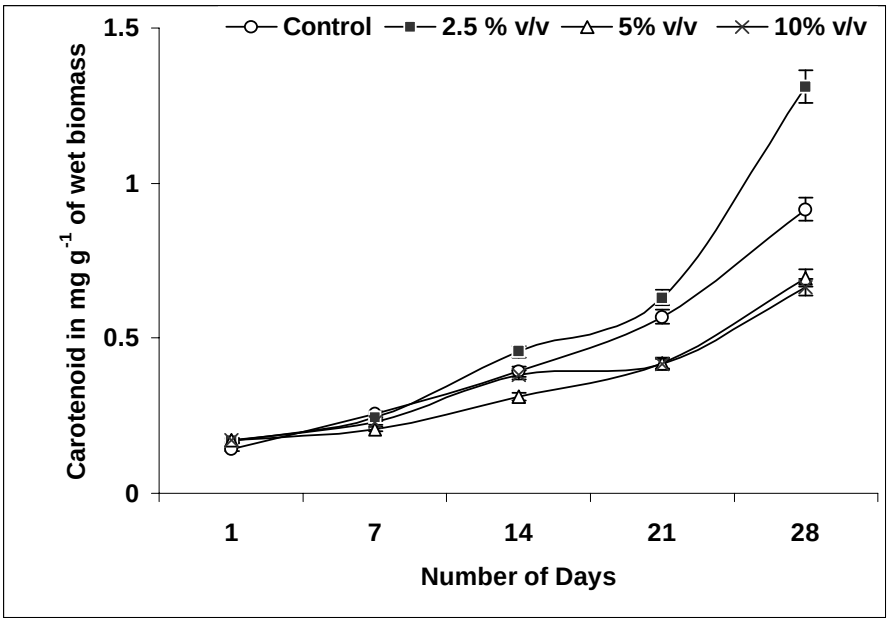


Figure 63. Effect of *A.niger* on carotenoid contents of *D.salina.*

[*Dunaliella salina* was incubated with water extract of *A. niger* in different concentration and carotenoids were estimated in wet biomass spectropohotometrically after extracting in n-hexane: Isopropyl alcohol (1:1), concentration corresponds to 0.05,0.1,0.2 mg dry mass of mycelium L^{-1}]

6.35 Atorvastatin intervention studies on production of carotenoids in Dunaliella.

In general it is well known that chlorophyta family contains both 1, Deoxy-D-xylulose-5 Phosphate (DOXP) and Mevalonic acid pathway (MVA) for the production of Isopentyl pyrophosphate (IPP), which is initial step in biosynthesis of carotenoids. In general DOXP is meant for the steroids production and MVA for carotenoids, if one of the MVA pathway is blocked it can produce carotenoids from DOXP pathway. This is proven in case of other single cell organisms like, *Chlorella, Scenedesmus*. This is the first report supporting involvement of DOXP pathway for carotenoids biosynthesis in halotolerant micro alga *Dunaliella bardawil and D.salina.* Possible mechanism involved in enhancement of carotenoids content is blockade of Mevalonic acid pathway leading to production of IPP via DOXP pathway and resulting in enhancement of carotenoids content in statin treated group of organisms (Schwender et al., 1997).

Growth was not affected when cultures were treated with 5.0 and 10.0 mg L^{-1} atorvastatin, However higher concentration 25.0 mg L^{-1} affected the growth. The content of total carotenoids was increased in case of atorvastatin treated group in both the species (Figure-64). Carotenoid content in 25.0 mg L^{-1} statin treated group was two fold more than control group as seen on the 16th day. Maximum content of carotenoids was observed on 12th day in case of *D. salina.* In case of *D.salina* maximum of 3.5 mg L^{-1} of carotenoids was accumulated at 10 mg L^{-1} of statin concentration on 16th day (Figure-65). In case of *D.bardawil* maximum carotenoid accumulation was observed on 16th day, which was 5.7 mg L^{-1}, when treated with 10 mg L^{-1} of statin (Figure-66).

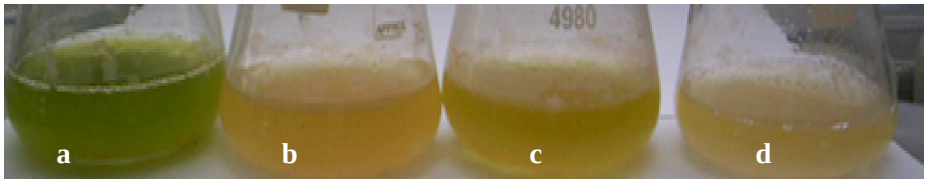


Figure 64. Effect of statin treatment on carotenoids accumulation in *D.bardawil* as on 12th day (a- control, b- 5.0 mg L^{-1}, c-10.0 mg L^{-1} and d- 25.0 mg L^{-1}).

[*D.bardawil* treated with statin at different concentration on 3rd, 6th and 9th day and maintained in modified medium under 2.0Klux light intensity at18 ±2^{0}C]

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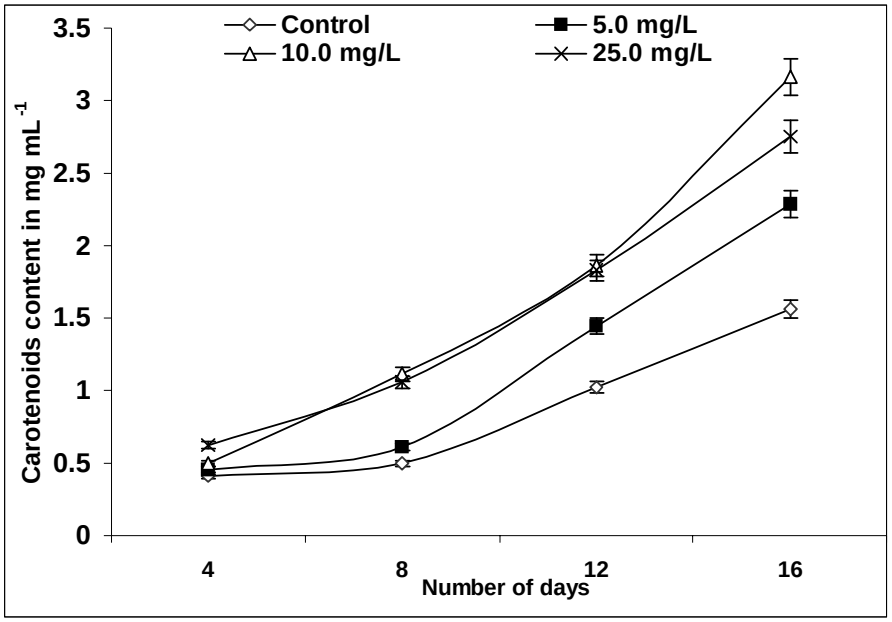


Figure 65. Effect of statin on carotenoids concentration in *D.salina.*

[*D.salina* was treated with statin at different concentration on 3rd, 6th and 9th day and maintained in modified medium under 2.0Klux light intensity at18 $\pm 2^0$C and carotenoids content was recorded]

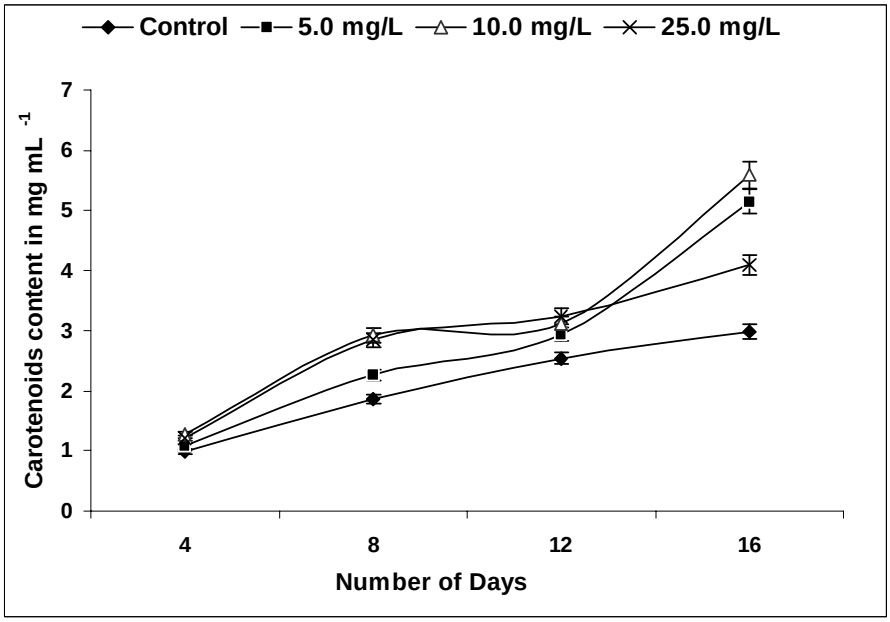


Figure 66. Effect of statin on carotenoids treatment in *D.bardawil*.

[*D.bardawil* was treated with statin at different concentration on 3rd, 6th and 9th day and maintained in modified medium under 2.0Klux light intensity at18 $\pm 2^0$C and carotenoids content was recorded]

6.35.1 Lycopene cyclase analysis.

Ammonium sulphate fractions of both *D. salina* and *D.bardawil* show the presence of significant amount of lycopene cyclase at 43 kDa providing the information supporting the enhancement of carotenoids content as seen in SDS - PAGE (10.0%, Figure-67). Ammonium sulphate fraction (40 %) has shown maximum content of the enzyme, the same was further confirmed by assay using HPLC. Enzyme concentration was quantified in terms of amount of lycopene converted to cyclic β- carotene.

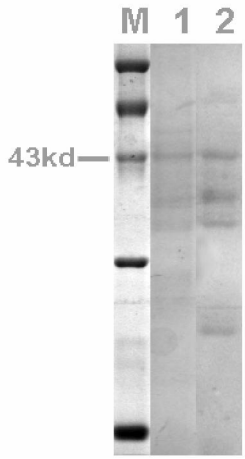


Figure 67. SDS-PAGE of 40 % ammonium sulphate fractions showing the presence of lycopene cyclase in (1) *D.salina* and (2) *D.bardawil*.

[Protein was extracted from both algal forms by gradient precipitation with ammonium sulphate and 40% fraction rich in protein was subjected for SDS and band was confirmend using standard marker]

When the incubated lycopene was subjected of HPLC, lycopene eluted at R_T of 21.08 while β - carotene was eluted at 31.25 min. Concentration of lycopene cyclase was calculated in terms of amount of lycopene converted to β - carotene according to method of Schnurr et al., (1996). The amount of lycopene cyclase in case of control group (without statin treatment) of *D.salina* and *D.bardawil* were significantly lower and the same was magnified to 1.75 and 2.02 in case of 5.0 mg L^{-1} atorvastatin treated group respectively (as calculated by band intensity using Hero lab easy win 3.2 software). Maximum enzyme concentration was observed in case of 40% fraction. This fraction showed 1.534 nM conversion of lycopene to β- carotene in case of *D.bardawil* and the activity was 0.348 nM hr^{-1} as calculated from HPLC results. The amount of lycopene cyclase was 32.61± 0.15 % of total protein in *D.bardawil* and 27.28 ± 0.25 % in case of *D.salina* in 40% fraction. Chromatogram shows lycopene as well as β- carotene in assay system incubated for four hours (Figure-68).

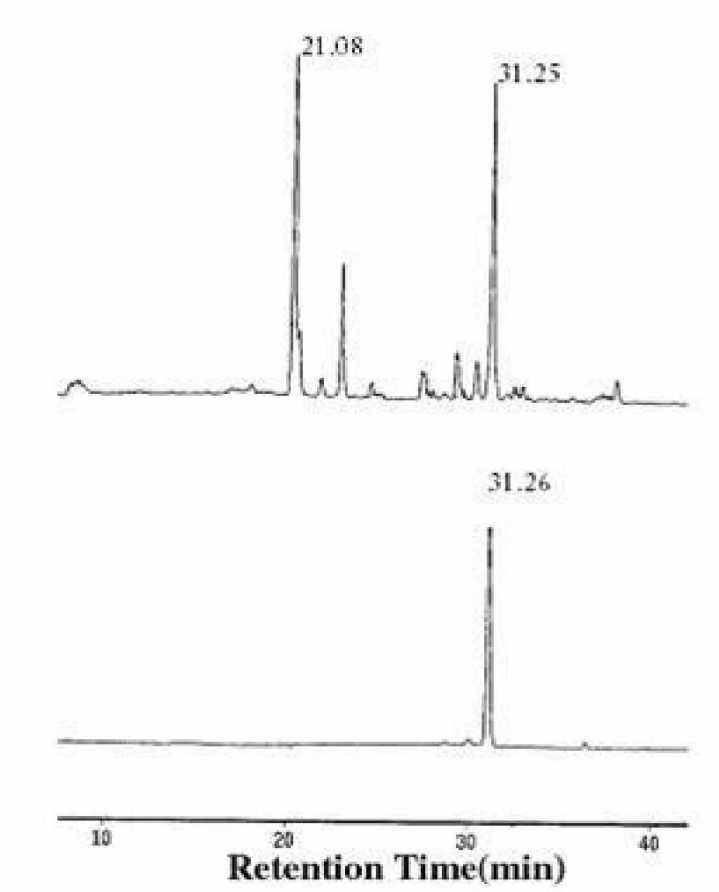


Figure 68. HPLC showing the content of and β- carotene converted in lycopene cyclase assay system.

[β- carotene was incubated with protein fraction prepared using L-α-phosphotidylcholine and NADP samples were removed at intervals by extracting in diethyl ether and petrol (1:9) and reaction mixture was analyzed by HPLC with C_{18} column using acetonitrile: methanol: propanol (85:10:5) with UV detector at 440.0 nm]

6.35.2 Analysis of squalene content.

Squalene content of control group of *D.salina* was 0.53 ± 0.25µg g^{-1} and in case of *D.bardawil* it was 0.62 ± 0.18 µg g^{-1}. The same was found to be nil in case of statin treatment in all the concentrations in both *D.salina* and *D.bardawil*. The GC-MS reveals the presence of squalene at M^+ at 410 (Figure-69).

Figure 69. Mass spectra of squalene showing the presence of squalene at M^+ of 410.0.

[n-hexane fraction of lyophilized algal biomass after treatment with statin was analysed for squalene content by GCMS using ELITE -5 capillary columns (0.5 x 30.0 mtrs) using helium as carrier gas]

In general, *Dunaliella* requires high light intensity (20-25 Klux) along with salt stress to accumulate carotenoids. Treatment of cells with statin induced accumulation of carotenoids even at white fluorescent light of intensity 1.8 - 2.2 Klux. The possible mechanism is blocking the formation of squalene, which diverts the same precursor to carotenoids pathway. Since statin used was in the form of calcium salt, which is water soluble, washing of biomass can remove the content and medium-containing statin can be recycled.

Salient feature:

We have developed a new gradient elution method for separation of all the carotenoids of *Dunaliella* on C_{18} silica column using acetonitrile and chloroform. Carotenoids were also identified using mass spectra and elution pattern were confirmed by LCMS, adopting positive electron ionization spray mode. Proximate analysis of the biomass showed the presence of protein (~20.0%), carbohydrates (~24.0%) as major components and high amount of total ash (both acid soluble and acid insoluble). For the first time an attempt was made to know the possible elicitation of carotenoids by cell extracts of bacteria and fungi. The results have shown 2-2.5 folds of enhancement of carotenoids content as seen in case of *A.flavus.*

An intervention study of statin has revealed that the carotenoids production could be enhanced, possibly due to blockade of melonaldehyde pathway, which is evident from complete blockade of squalene synthesis.

vi. Biological activity of Dunaliella biomass and extracted carotenoids.

Background:

This section deals with the evaluation of biological activity of algal biomass as well as carotenoids, with emphasis on free radical mediated antioxidant and hepatoprotective activity. In order to evaluate these properties biomass and carotenoids obtained from our process was subjected to successive extraction using solvents of varied polarity. The extracts of different solvents were subjected to analysis of radical scavenging ability by various *in vitro* models, like DPPH, iron induced reducing power assay and protection against metal ion induced peroxidation of lipids of egg, human erythrocytes, kidney and brain of albino rats using the protocols mentioned under methods.

Further the evaluation of efficacy of biomass was studied in rat models. Effect of biomass feeding on the gain in body weight, hematological profile and on weight of different organs were studied. A study was also undertaken to know the possible antioxidant activity of biomass fed at 2.5 and 5.0 g kg^{-1}, upon challenging animals with carbon tetrachloride, which is known to generate high amount of free radicals. The same group was examined for possible protection of liver, which is damaged upon administration of carbon tetrachloride as measured by various biochemical markers in serum. Similarly *in vivo* studies on antioxidant and hepatoprotective activity was undertaken by treating the rats with carotenoids of algae at 125 and 250 μg kg^{-1}. These results were compared with control group fed with normal diet rats and animals supplemented with 100 μg kg^{-1} of synthetic all *trans* β- carotene based diet.

6.36 Antioxidant activity of Dunaliella extract.

6.36.1. β*-Carotene linoleic acid model system.*

Dunaliella ethanolic extracts of both vegetative and carotenoids accumulated cells have shown good antioxidant activity at the concentration 2.25 and 4.5 mg (500 and 1000 ppm respectively) in β-CLAMS models when compared to BHA as a standard. Activity exhibited by red cells at 1000 ppm was almost equivalent to 80% of the BHA. However the activity at 250 and 500 concentrations in case of green cells was 70% of that of red cell extracts (Figure-70).

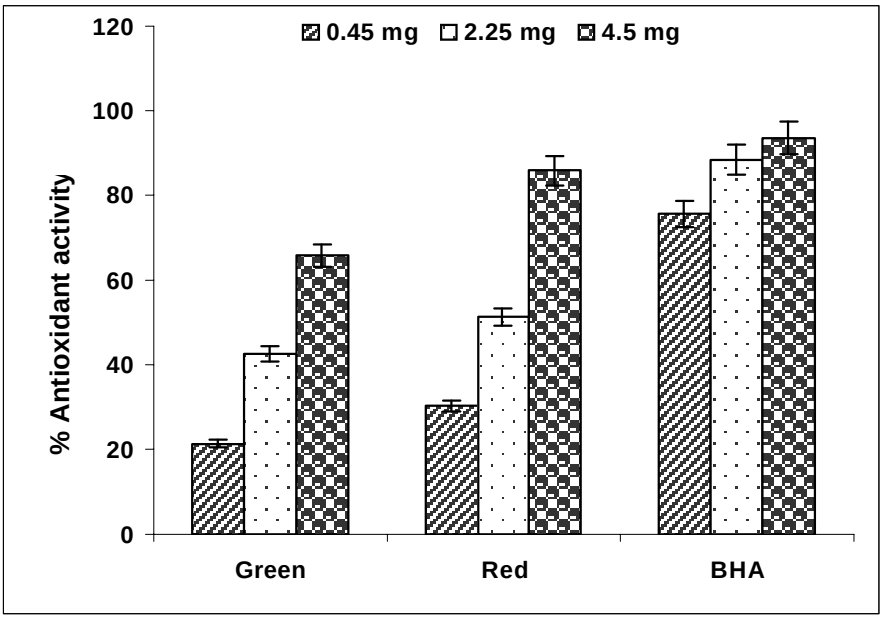


Figure 70. Percentage antioxidant activity of ethanolic extract of *Dunaliella bardawil.*

6.36.2. α,α *Diphenyl* β *- picryl hydrazine model.*

Both the green and red cell extracts have shown significant radical scavenging activity at higher dose compared to ascorbic acid. In case of red cells extract at 500 ppm concentration radical scavenging activity was more than 60 % compared to standard ascorbic acid. Activity of green cells were comparatively lesser (Figure-71).

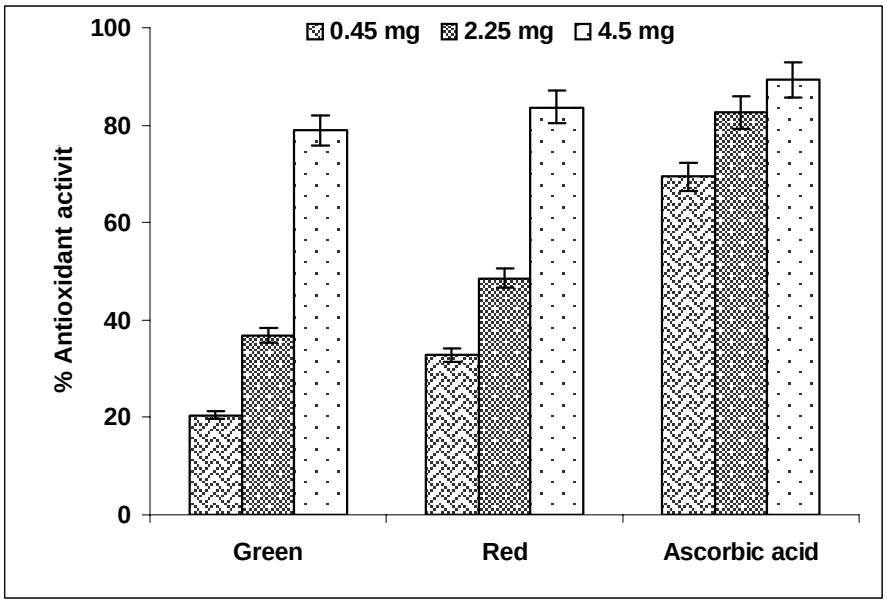


Figure 71. Percentage radical scavenging activity of ethanolic extract of *Dunaliella bardawil*.

6.37 Successive extraction of Dunaliella constituents.

Both D.salina and *D.bardawil* had shown maximum extractability in case of methanol, which was 18.41 and 12.07 % respectively. This was followed by n-hexane, which extracted 8.o% in both the species (Table-27).

Table 27. Percentage yield of total extractables in different solvent in *D.salina* and *D.bardawil*.

Solvents	***D.salina***	***D.bardawil***
n-Hexane	8.21 ± 1.1	8.78 ± 0.88
Chloroform	3.13 ± 0.98	2.93 ± 1.20
Ethyl acetate	2.12 ± 0.56	0.55 ± 0.74
Acetone	0.29 ± 1.02	1.35 ± 0.89
Methanol	18.41 ± 0.59	12.07 ± 0.55
Water	3.04 ± 1.02	4.02 ± 1.08

6.37.1 Radical scavenging assay using α, α- diphenyl-β-picrylhydrazyl (DPPH).

Antioxidant activity as demonstrated by DPPH model revealed highly significant radical scavenging ability in acetone extracts of both *D. salina* and *D. bardawil*, which was 83.92 and 93.21 % respectively compared to ascorbic acid. This was followed by ethyl acetate and water in case of *D.salina* and ethyl acetate and methanol in case of *D.bardawil* (Table-28).

Table 28. Antioxidant activity of *Dunaliella* by DPPH model.

Organism	Extract	Concentration (ppm)	% Scavenging activity
D.salina	n-Hexane	100	22.98 ± 1.05
		250	37.13 ±1.64
	Chloroform	100	40.75 ± 2.06
		250	46.40 ±1.68
	Ethyl acetate	100	50.59 ± 2.65
		250	61.49± 1.35
	Acetone	100	47.74 ± 2.06
		250	83.92 ± 1.69
	Methanol	100	37.74 ± 0.69
		250	55.31 ± 0.36
	Water	100	50.32 ± 1.65
		250	66.9 ± 1.98
D.bardawil	n-Hexane	100	31.72 ± 1.26
		250	57.76 ± 0.86
	Chloroform	100	34.42 ±1.48
		250	43.08 ± 0.86
	Ethyl acetate	100	47.21 ±0.68
		250	59.08 ± 0.88
	Acetone	100	87.36 ±1.06
		250	93.21 ± 0.95
	Methanol	100	51.82 ± 0.24
		250	71.97 ± 1.49
	Water	100	46.28 ± 0.19
		250	69.71 ± 2.37
Standard	Ascorbic acid	100	95.06 ± 0.24
		250	97.32 ± 0.35

6.37.2 Reducing power assay.

Reducing power assay has shown that n-Hexane and water fraction of *D.salina* exhibited the highly significant activity compared to ascorbic acid. Other extractables shown significant activity are Chloroform and methanol extracts of *D.bardawil* (Figure -72). This indicates that the non-polar components of algae are also responsible for the radical scavenging potentials

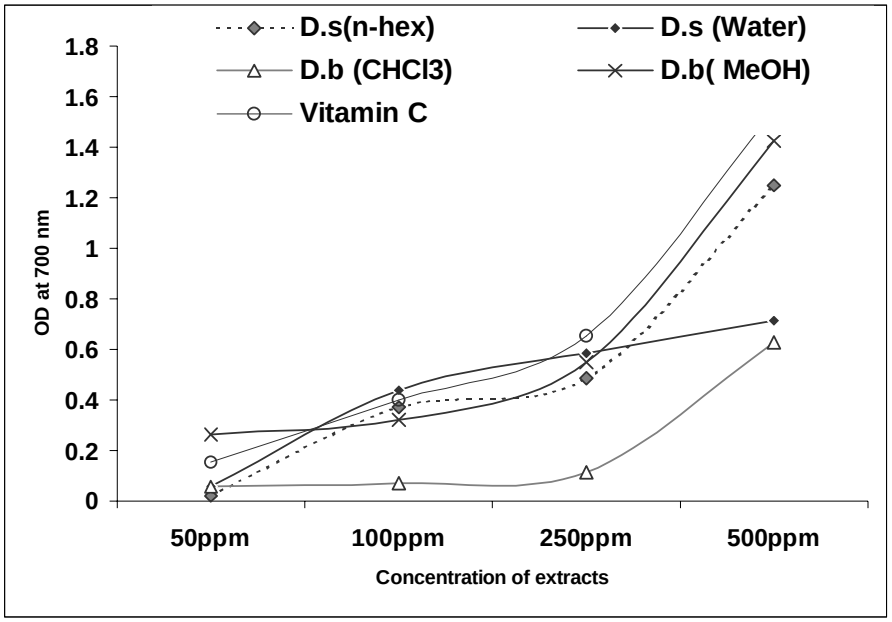


Figure 72. Reducing power assay of various extractables of *Dunaliella.*

[Successive extracts of *Dunaliella* were subjected for reducing power assay, in which effect of extract in reduction of ferric to ferrous was measured as marker of antioxidant ability at different concentration]

6.37.3 LDL peroxidation.

6.37.3.1. Egg lecithin peroxidation assay.

Lecithin peroxidation has been inhibited to maximum extent in ethyl acetate and acetone extract of *D.salina* and the same is maximum in acetone and water extract of *D.bardawil*, which was highly significant compared to standard ascorbic acid. In *D.salina* almost all the extractable have shown significant activity compared to standard. The n-hexane and chloroform have shown comparatively less significant activity indicating *D. salina* contain more of polar compounds responsible to the activity. However in case of *D.bardawil* , activity is more in case of polar fractions i.e., methanol and water (Table - 29).

Table 29. Antilipid peroxidation activity of *Dunaliella* as measured by egg lecithin peroxidation assay.

Organism	Extract	Concentration (ppm)	% Antilipid peroxidation
D.salina	n-Hexane	100	63.43 ± 1.06
		250	71.47 ±0.86
	Chloroform	100	75.44 ± 0.94
		250	83.27 ± 1.64
	Ethyl acetate	100	85.84 ± 0.85
		250	91.74 ± 1.12
	Acetone	100	89.70 ± 0.67
		250	93.13 ± 1.67
	Methanol	100	80.58 ±0.95
		250	83.48 ± 0.67
	Water	100	74.15 ± 1.89
		250	86.59 ±1.59
D.bardawil	n-Hexane	100	20.64 ± 0.49
		250	43.70 ± 1.32
	Chloroform	100	60.96 ±0.98
		250	70.83 ± 1.65
	Ethyl acetate	100	49.16 ± 0.59
		250	74.90 ± 1.67
	Acetone	100	75.89 ± 2.09
		250	87.77 ± 0.73
	Methanol	100	54.20 ± 1.61
		250	76.40 ± 1.49
	Water	100	81.12 ± 0.67
		250	93.02 ± 2.05
Standard	Ascorbic acid	100	95.63 ± 2.4
		250	97.01 ± 1.65

6.37.3.2 Human Erythrocyte Peroxidation assay.

Human erythrocytes have shown slightly different pattern of results. In case of *D. salina* acetone and water has shown prevention of erythrocytes peroxidation equivalent to ascorbic acid and activity was significantly less in case of n-hexane and chloroform. *D.bardawil* minimum activity was seen in case of chloroform, other extracts exhibited relatively significant activity in comparison with standard (Table- 30).

Table 30. Antilipid peroxidation activity of *Dunaliella* as measured by Human Erythrocyte peroxidation assay.

Organism	Extract	Concentration (ppm)	% Antilipid peroxidation
D.salina	n-Hexane	100	44.50 ± 0.94
		250	67.34 ± 1.36
	Chloroform	100	43.75 ± 0.95
		250	69.02 ± 1.92
	Ethyl acetate	100	72.37 ± 2.19
		250	75.30 ± 1.11
	Acetone	100	91.21 ± 0.87
		250	93.02 ± 0.61
	Methanol	100	51.43 ± 0.37
		250	72.51 ±0.09
	Water	100	86.04 ± 2.08
		250	88.00 ± 1.55
D.bardawil	n-Hexane	100	67.62 ± 1.56
		250	82.00 ± 0.96
	Chloroform	100	23.10 ± 0.29
		250	63.15 ± 0.14
	Ethyl acetate	100	89.53 ± 0.92
		250	91.21 ± 0.07
	Acetone	100	87.44 ± 1.38
		250	91.35 ± 1.22
	Methanol	100	76.70 ± 0.67
		250	91.63 ± 1.68
	Water	100	85.35 ± 1.57
		250	87.58 ± 0.99
Standard	Ascorbic acid	100	92.36 ± 0.49
		250	94.65 ± 0.61

6.37.3.3 Rat kidney lipid peroxidation.

In case of *Dunaliella* maximum activity against lipid peroxidation was seen in case of acetone extracts of both *salina* and *bardawil,* which was 90.20 and 89.15 %. Over all activity were lesser compared to human erythrocytes peroxidation in both the algal forms. Acetone extract has shown maximum activity in both the algal forms (Table -31).

There was no proportional enhancement of activity as concentration enhanced from 100 to 250 ppm.

Table 31. Antilipid peroxidation activity of *Dunaliella* as measured by rat kidney lipid peroxidation assay.

Organism	Extract	Concentration (ppm)	% Antilipid peroxidation of rat kidney lipids
D.salina	n-Hexane	100	81.54 ± 1.12
		250	84.63 ± 0.69
	Chloroform	100	83.95 ± 0.64
		250	85.98 ± 2.63
	Ethyl acetate	100	88.32 ± 0.76
		250	88.85 ± 2.96
	Acetone	100	89.90 ± 0.64
		250	90.20 ± 1.44
	Methanol	100	85.61 ± 1.05
		250	86.06 ± 0.95
	Water	100	84.17 ± 0.54
		250	89.30 ± 1.52
D.bardawil	n-Hexane	100	68.20 ± 0.63
		250	82.67 ± 1.05
	Chloroform	100	76.26 ± 1.92
		250	82.30 ± 0.75
	Ethyl acetate	100	85.98 ± 0.61
		250	88.47 ± 1.05
	Acetone	100	88.77 ± 0.49
		250	89.15 ± 0.78
	Methanol	100	84.70 ± 1.09
		250	85.08 ± 2.09
	Water	100	82.60 ± 1.06
		250	87.26 ± 1.35
Standard	Ascorbic acid	100	95.77 ± 0.98
		250	96.53 ± 1.06

6.37.3.4 Rat brain lipid peroxidation.

Maximum activity against rat kidney lipid peroxidation was seen in case of acetone extracts of both *salina* and *bardawil*, which was 90.20 and 89.15 % respectively in 250 ppm concentration. Overall activity was significantly high in case of kidney compared to brain of rats. Activity was in almost same range of 80-85 % in all the extracts in *D.salina* and *D.bardawil*. Results of the study indicates *Dunaliella* is best for prevention of peroxidation of lipids in kidney as it is prone for extensive peroxidation induced by drugs and their metabolites and heavy metals (Table -32).

Table 32. Antilipid peroxidation activity of *Dunaliella* as measured by rat brain lipid peroxidation assay.

Organism	Extract	Concentration (ppm)	% Antilipid peroxidation of rat brain lipids
D.salina	n-Hexane	100	24.81 ± 0.36
		250	29.43 ± 1.02
	Chloroform	100	59.55 ± 0.65
		250	61.43 ± 0.98
	Ethyl acetate	100	23.87 ± 1.11
		250	49.22 ± 0.68
	Acetone	100	63.01 ± 0.96
		250	73.96 ± 0.64
	Methanol	100	38.17 ± 0.34
		250	56.01 ± 0.36
	Water	100	37.52 ± 1.63
		250	65.04 ± 0.69
D.bardawil	n-Hexane	100	11.52 ± 1.23
		250	40.84 ± 0.69
	Chloroform	100	10.36 ± 0.33
		250	18.74 ± 0.56
	Ethyl acetate	100	40.56 ± 0.96
		250	56.04 ± 1.09
	Acetone	100	63.67 ± 0.87
		250	65.26 ± 0.67
	Methanol	100	52.33 ± 0.92
		250	56.74 ± 0.73
	Water	100	60.92 ± 1.09
		250	62.95 ± 1.38
Standard	Ascorbic acid	100	83.61 ± 0.69
		250	88.56 ± 1.32

6.38 Estimation of nutritional quality of Dunaliella salina cells.

Based on the results of the above studies *Dunaliella salina* were further subjected for evaluation of nutritional activity using albino rats. Treatment of *Dunaliella salina* biomass at the 2.5 g kg $^{-1}$ and 5.0 g kg $^{-1}$ has not shown any death or symptoms of toxicity. Animals of algal treatment group were active similar to that of normal diet fed ones. Over all growth as measured by gain in body weight (once a week) shows that in general male gained higher body weight compared to females in all the groups. Gain in body weight was maximum of 39.09 % in case of males treated with 2.5 g kg $^{-1}$ algae, followed by 20.0 % in case of females of same treatment. 5.0 g kg $^{-1}$ *Dulnaliella* has shown relatively lesser gain in body weight compared to normal animals (Table-33).

Table 33. Effect of *Dunaliella salina* treatment on the body weight (g) of experimental rats.

Treatment	**Sex of animal**	**Body weight initially**	**Body weight after 7 days**	**Body weight after 14 days**
Normal diet	Male	127.66 ± 2.51	151.0 ± 3.60	163.33 ± 7.63
	Female	129.66 ± 6.10	158.33 ± 2.88	173.33 ± 2.88
2.5 g kg^{-1} of *Dunaliella* cells	Male	136.66 ±15.27	172.0 ± 20.30	190.0 ± 15.00
	Female	130.0 ± 17.32	143.32 ± 15.27	156.66 ± 12.58
5.0 g kg^{-1} of *Dunaliella* cells	Male	126.0 ± 6.0	146.6 ± 2.88	158.0 ± 3.75
	Female	130.33 ± 5.50	146.33 ± 4.04	153.5 ± 5.0

Effect of algal treatment on the absolute and relative weight of vital organs is summarized in Table-34. Decrease in the weight of liver, lungs and testes were observed in both the dose of algae compared to normal animals. Relative weight of kidney and thymus were significantly high in both 2.5 g kg^{-1} and 5.0 g kg^{-1} *Dunaliella* treated group. Significant decrease in the relative weight of testes was observed in both the doses, however relative liver and spleen weight were less at 2.5 g kg^{-1}.

Table 34. Effect of *Dunaliella salina* treatment on the vital organs weight of experimental rats.

Organs	**Average weight in gm ± SD**		
	Normal	**2.5 g kg^{-1} of *Dunaliella* cells**	**5.0 g kg^{-1} of *Dunaliella* cells**
Average body weight	168.33 ± 9.5	173.33 ± 6.5	155.75 ± 5.6
Absolute (g)			
Liver	6.8	6.75	6.60
Heart	0.70	0.70	0.65
Kidney	1.25	1.3	1.35
Lungs	1.05	1.0	0.95
Thymus	0.25	0.30	0.30
Testes	2.30	2.20	2.10
Spleen	0.55	0.45	0.50
Relative (g 100 g $^{-1}$b.w)			
Liver	4.04	3.89	4.23
Heart	0.40	0.4	0.41

Kidney	0.74	0.75	0.86
Lungs	0.62	0.57	0.60
Thymus	0.14	0.17	0.19
Testes	1.36	1.26	1.34
Spleen	0.32	0.25	0.32

6.38.1 Effect of Dunaliella treatment on hematological profile of experimental animals.

Treatment of algal biomass has not shown any adverse effect on the hematological profile of the experimental animals. Hemoglobin content of both 2.5 g kg^{-1} and 5.0 g kg^{-1} *Dunaliella* treated animals were lowered, which was more in lower dose. White blood cells were in the normal range in all the group of animals. Its count decreased in case of 2.5 g kg^{-1} and it was not much altered in higher dose compared to normal group of animals. Serum protein content was significantly lower in both the concentrations of *Dunaliella* treated compared to the values of normal animals (Table-35).

Table 35. Effect of *Dunaliella salina* treatment on the hematological profiles of experimental rats.

Hematology profile	**Normal diet**	**2.5 g kg^{-1} of *Dunaliella* cells**	**5.0 g kg^{-1} of *Dunaliella* cells**
% Hemoglobin	8.78	7.73	8.54
WBC	7380	6686	7400
RBC ($x10^6$)	6.31	6.32	6.60
Mean corpuscular hemoglobin (MCH)	26.3	28.8	25.4
Mean corpuscular hemoglobin concentration (MCHC)	26.6	23.4	23.6
Mean corpuscular volume (MCV	36.4	38.0	37.2
Total Protein	6.3	6.2	6.15

6.39 Estimation of antioxidant activity of whole cells in vivo.

Measurement of hepatic enzymes as biochemical markers for antioxidant activity in toxin challenged rats fed with *Dunaliella* at 2.5 g kg^{-1} and 5.0 g kg^{-1} dose shows that algae can serve as potential antioxidant. Treatments of rats with toxin at 2.0 g kg^{-1} body weight significantly reduced the levels of catalase, peroxidase and SOD by 45.99, 54.56 & 55.97 % respectively. On the other hand, lipid peroxidation increased by 1.8 folds as compared to normal due to the CCl_4 treatment. However, pre-treatment of rats with 5.0 g kg^{-1} of algae

preserves catalase, peroxidase and SOD activities, which are comparable with control values of the enzyme. Restoration of catalase was 11.35 and 93.61 in animals pretreated with 5.0 g kg $^{-1}$ and 2.5 g kg $^{-1}$ of biomass. Higher dose of algae has shown restoration of 221.67 % (peroxidase) and 132.43 (SOD) and that of lower dose had shown 200.0 % and 83.91% of peroxidase and SOD respectively. Protection shown by synthetic β- carotene (100 μg kg^{-1}) was comparatively lower (Table-36).

Table 36. Effect of *Dunaliella salina* whole cells treatment on the antioxidant markers of experimental rats (hepatic enzymes).

Treatment	Catalase	Peroxidase	SOD	Anti lipid Peroxidase
Normal	597.98 ± 5.98	8.43 ± 5.6	16.81 ± 3.6	23.12 ± 2.8
Control	322.96 ± 8.36	3.83 ± 1.6	7.4 ± 1.8	41.48 ± 2.0
2.5 g kg^{-1} of *Dunaliella* cells	625.3 ± 3.6	11.50 ± 1.1	16.01 ± 1.3	14.52 ± 2.3
5.0 g kg^{-1} of *Dunaliella* cells	682.6 ± 4.5	12.32 ± 0.9	17.2 ± 1.12	16.8 ± 1.54
Synthetic β- carotene (100 μg kg^{-1})	604.63 ± 10.8	10.21 ± 1.4	15.79 ± 2.6	17.75 ± 1.6

6.40 Hepatoprotective activity of whole cells in vivo.

Dunaliella salina at 2.5 g kg^{-1} and 5.0 g kg^{-1} b.w caused decrease in SGPT by 12.63, 33.84 %, SGOT by 38.30, 57.80 % respectively. However there was not significant decrease in case of synthetic β- carotene fed rats, which was found to be 2.02 and 24.32 % of SGPT and SGOT respectively. Similarly the alkaline phosphatase was higher in case of CCl_4 toxin treated rats, where as it was nearly half in case of 2.5, 5.0 g kg^{-1} biomass treated groups. Synthetic β- carotene treated group animals have also shown decrease in the SALP, which was not significant. The total protein content decreased by 35.31 % in case of control compared to normal animals and it was found to be enhanced by 28.71% and 33.82 % respectively in case of 2.5, 5.0 g kg $^{-1}$ biomass treated group of animals. The serum albumin content also supported the above results, Serum albumin was higher in case of control group in contrast to algae treated ones (Table -37).

Histopatology studies also support the above studies, normal animals showed the intact hepatic architecture with normal portal vein and portal duct structure, in-group treated with carbon tetrachloride with normal diet, the same was destructed with fatty infiltration and. The extent of destruction was significantly more in case of male (d) compared to female (Figure-73). Feeding of algal biomass at 2.5 g kg^{-1} b.w. and carbon tetrachloride has shown significantly lesser extent of fatty infiltration, interestingly 5.0 g kg^{-1} showed complete protection (H). Results were also significant compared to synthetic all *trans* β- carotene fed at 100 μg kg^{-1} b.w. as seen in Figure-73-J.

Table 37. Effect of *Dunaliella salina* whole cells treatment on the hepatoprotective markers of experimental rats (serum enzymes).

Treatment	**SGPT**	**SGOT**	**SALP**	**Serum protein**	**Serum albumin**
Normal	83.61 ± 6.92	147.78 ± 6.35	52.63 ± 1.33	6.06 ± 0.66	3.83 ± 0.86
CCl_4	92.71 ±3.56	191.16 ± 5.42	84.16 ± 4.4	3.92 ± 0.62	2.46 ± 1.56
2.5 g kg^{-1} of *Dunaliella* cells	81.0 ± 3.87	117.93 ± 6.24	44.73 ± 5.60	7.8 ± 0.68	3.36 ± 2.61
5.0 g kg^{-1} of *Dunaliella* cells	61.33 ± 5.61	80.66 ± 9.60	52.91 ± 4.73	8.11 ± 0.59	3.21 ± 1.99
Synthetic β- carotene (100 μg kg^{-1})	90.83 ± 9.56	144.66 ± 5.9	48.21 ± 5.41	5.59 ± 0.77	3.48 ± 2.57

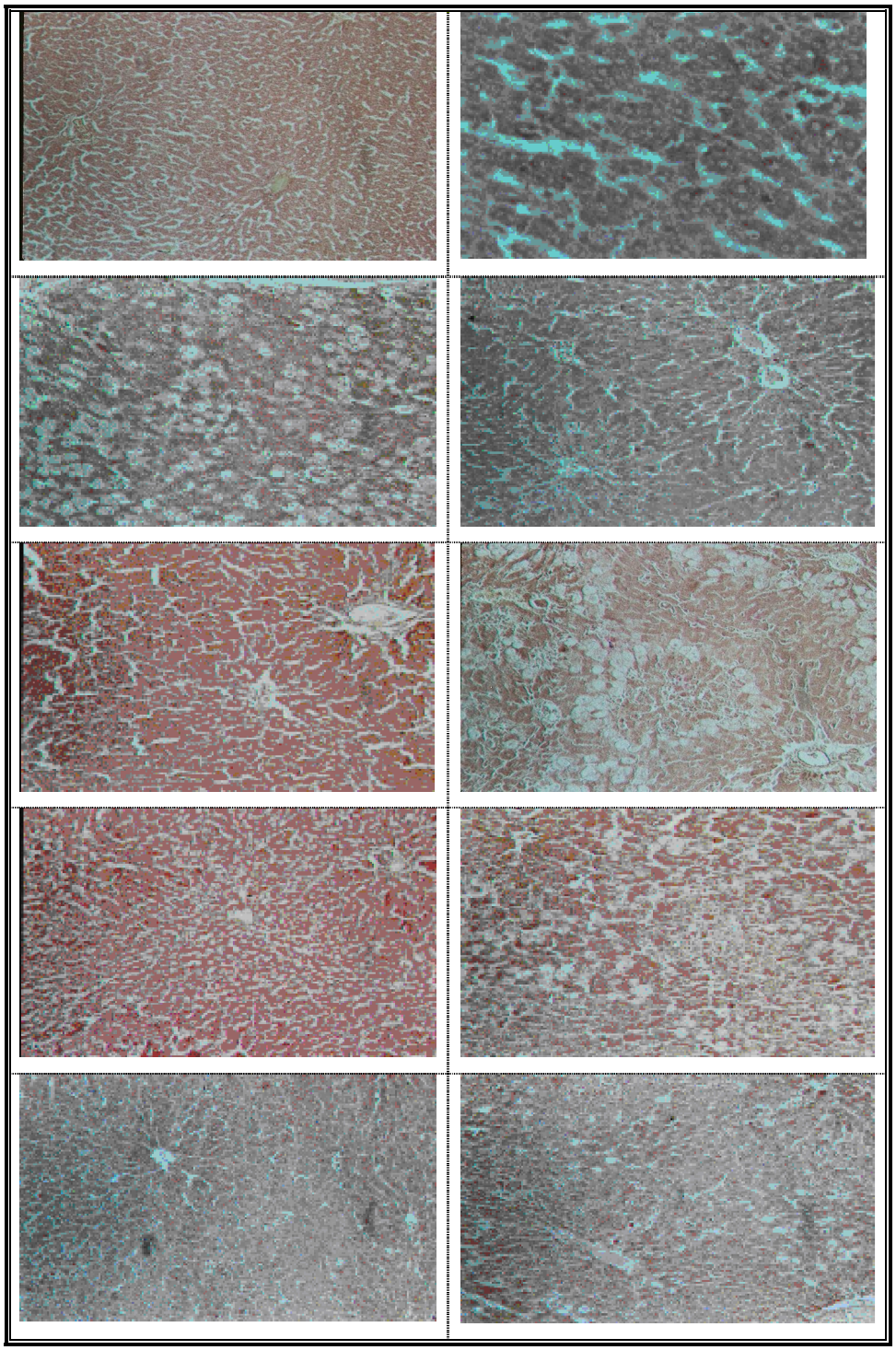


Figure 73. Photomicrographs of liver tissue of rats of different groups examined for hepatoprotective effect of whole cells of *Dunaleilla salina*. Hematoxylin and Eosin (H&E) x100 (except Bx400x).

[A: and B: Control, Normal Histoarchetecture C: CCl_4 treated male rats showing fatty infiltration, D: CCl_4 treated female partial cell altered, E: Rats fed with 2.5 g kg^{-1} *Dunaliella* biomass- Normal picture, F: Rats fed with 2.5 g kg^{-1} *Dunaliella* biomass and CCl_4- moderate fatty infiltration, G: Rats fed with 5.0 g kg^{-1} biomass- Normal picture, H: Rats fed with 5.0 g kg^{-1} biomass and CCl_4- less fatty infiltration (compared to F) I: Rats fed with 100µ g kg^{-1} syn β- carotene - Normal picture, J: Rats fed with 100µ g kg^{-1} syn β- carotene and CCl_4- fatty infiltration observed].

6.41 Estimation of antioxidant activity of carotenoids in vivo.

Among the groups the one which was treated with *Dunaliella* carotenoids at the concentration of 250µ kg^{-1} has shown maximum activity i.e. protection when compared to control (untreated with carotenoids) and synthetic carotenoid treated group. Treatment of toxin to *Dunaliella* carotenoids and synthetic β-carotene have shown protection, which was estimated in terms of content of Hepatic enzymes namely, Catalase, Peroxidase, Super oxide Desmutase and Anti lipid peroxidase. Treatments of rats with toxin at 2.0 g kg^{-1} body weight significantly reduced the levels of catalase, peroxidase and SOD by 45.97, 54.56 and 59.97 respectively. On the other hand, lipid peroxidation increased by 1.8 folds as compared to normal due to the CCl_4 treatment. However, pre-treatment of rats with 250µg of carotenoids preserved catalase, peroxidase and SOD activities, which are comparable with control values of the enzyme. Restoration of catalase was 100.1, 152.51 and 87.21 % when compared to toxin treated groups, respectively in case of 250µg, 125µg of *Dunaliella* carotenoids and 250µg synthetic β-carotene treated group. The similar trend was seen in case of peroxidase and SOD enzymes (Table-38).

This shows the protection provided by feeding *Dunaliella* extract to these rats by maintaining the levels of these enzymes even after treatment of toxin. The lipid peroxidation was restored by 7.18 folds in case of 250µg *Dunaliella* carotenoid treated group, and the same was restored by 3.6 folds in case of 125µg *Dunaliella* carotenoid treated group.

Table 38. Effect of *Dunaliella salina* carotenoids treatment on the antioxidant markers of experimental rats (hepatic enzymes).

Groups	Units of Catalase	Units of Peroxidase	Units of SOD	% Anti lipid peroxidase
Normal	597 ± 5.98	8.43 ± 5.6	16.81 ± 3.6	23.12 ± 2.8
Control (CCl_4 treated)	322.96 ± 8.36	3.83 ± 3.6	7.4 ± 2.8	41.48 ± 2.0
Dunaliella carotenoids (125 µg kg^{-1})	647.83 ± 5.90	13.44 ± 5.8	17.8 ± 3.2	11.51 ± 1.4
Dunaliella carotenoids (250 µg kg^{-1})	815.52 ± 8.95	16.86 ± 3.1	20.52 ± 1.2	5.77 ± 0.98
Synthetic β- carotene (100 µg kg^{-1})	604.63 ± 10.8	10.21 ± 4.0	15.79 ± 2.6	17.75 ±1.6

6.41 Estimation of hepatoprotective activity of carotenoids in vivo.

Carotenoids of *Dunaliella* have shown significant hepatoprotective activity as measure by serum enzymes. As explained afore the content of SGPT, SGOT and SALP were enhanced

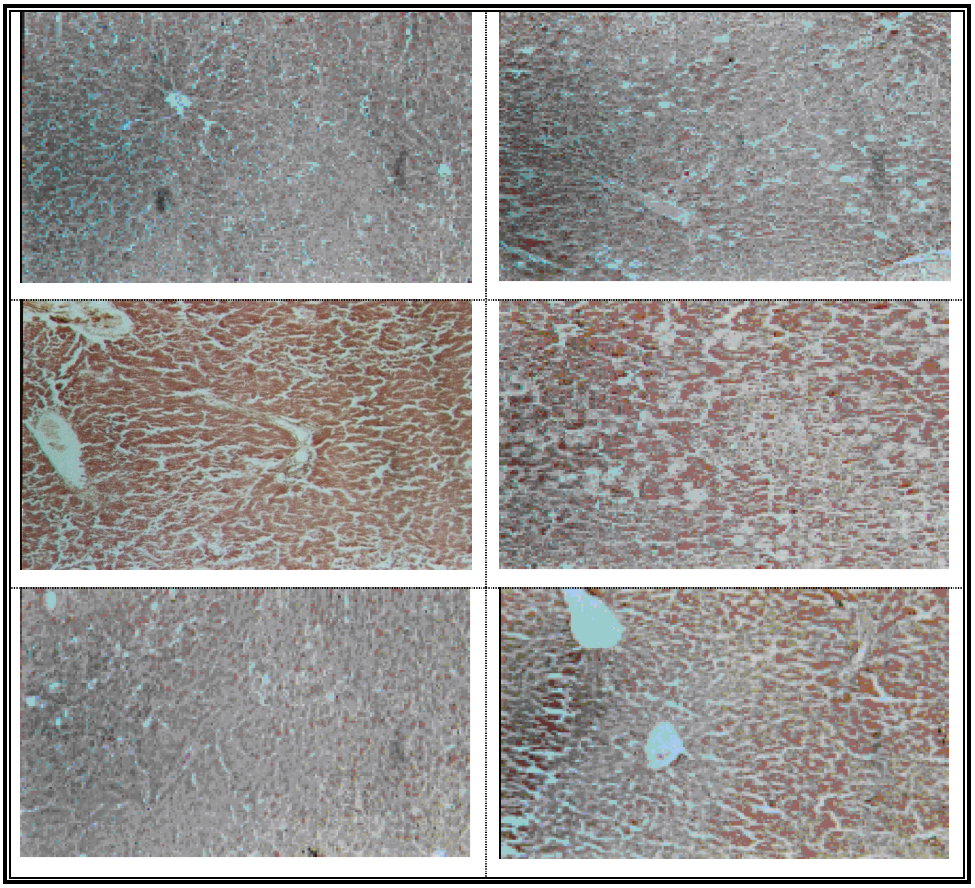
indication the significant damage caused by carbon tetrachloride. Animals treated with 125 μg kg^{-1} carotenoids of *Dunaliella* have shown to decline the raised concentrations of SGPT by 51.17 %, SGOT by 57.07% and SALP by 54.47 %. Where as 250 μgkg^{-1} has restored 55.19 (SGPT), 61.22 (SGOT) and 58.87 % (SALP) and the same were not significantly in animals treated with synthetic β- carotene (100 μg kg^{-1}). Carotenoids of algae fed to the experimental animals also showed to enhance content of serum protein, which is generally lower in case of toxin treated group. Content of serum albumin, which was significantly declined by toxin, showed restoration in both the doses of algal carotenoids (Table-39).

Histopathology has also shown supportive results, significant protection has been provided by both 125 μg kg^{-1} and 250 μg kg^{-1} *Dunaliella* carotenoids compared to animals fed with synthetic β- carotene (Figure-74). There was less fatty infiltration and destruction of hepatocytes seen in case of algal carotenoids treated group of animals.

Table 39. Effect of *Dunaliella salina* carotenoids treatment on the hepatoprotective markers of experimental rats (serum enzymes).

Treatment	**SGPT**	**SGOT**	**SALP**	**Serum proteins**	**Serum albumin**
Normal	83.61 ± 6.92	107.83 ± 6.35	52.63 ± 1.33	6.06 ± 0.69	3.83 ± 0.86
CCl_4	92.71 ±3.56	191.16 ± 5.42	84.46 ± 4.4	3.92 ± 1.32	2.46 ± 1.56
Dunaliella carotenoids (125 μg kg^{-1})	62.83 ± 3.87	73.62 ± 6.24	38.45 ± 5.60	6.96 ± 6.32	4.59 ± 2.61
Dunaliella carotenoids (250 μg kg^{-1})	57.65 ± 5.61	66.51 ± 9.60	34.73 ± 4.73	5.32 ± 2.84	3.97 ± 1.99
Synthetic β- carotene (100 μg kg^{-1})	90.83 ± 9.56	144.66 ± 5.9	48.21 ± 5.41	5.59 ± 0.77	3.48 ± 2.57

Figure 74. Photomicrographs of liver tissue of rats of different groups examined for hepatoprotective effect of carotenoids of *Dunaleilla salina*.
Hematoxylin and Eosin (H&E) x100



[a: Rats fed with 100µ g kg^{-1} synthetic β- carotene - Normal picture, b: Rats fed with 100µ g kg^{-1} synthetic β-carotene and CCl_4- fatty infiltration observed c: Rats fed with 125 µg kg^{-1} carotenoids of *Dunaliella*- normal picture, d: Rats fed with 125 µg kg^{-1} carotenoids of *Dunaliella* and CCl_4- minimal fatty infiltration, e: Rats fed with 250 µg kg^{-} carotenoids of *Dunaliella*- normal picture, d: Rats fed with 250 µg kg^{-1} carotenoids of *Dunaliella* and CCl_4-almost normal].

Salient feature:

The above result reveals that the carotenoids are the major constituents responsible for the radical scavenging ability. The activity of *in vitro* studies shows that the carotenoids rich fractions i.e., acetone and methanol have shown the maximum activity significantly compared to reference standards. We also found the presence of ascorbic acid as one of the ingredient as contributing for the radical scavenging ability of water extract. Carotenoids were also shown significance prevention of lipid peroxidation as seen in case of egg lecithin, human erythrocytes and brain and kidney of rats measured in terms of thiobarbituric acid reactive species (TBARS).

Feeding of *Dunaliella salina* biomass has shown no adverse effects or toxicity on animals upon feeding for 14 days. The net effect on hematological and organ weight was not much varied compared to normal diet fed animals.

For the first time we have estimated the antioxidant and hepatoprotective activity of the carotenoids of the algae and biomass against carbon tetrachloride induced radical toxicity as measured by various biochemical marker enzymes and witnessed by histological studies on liver.

7.0 Discussion

7.1 Selection of the research problem

Vitamin A deficiency (VAD) is the leading cause of preventable blindness in children and raises the risk of disease and death from severe infections. In pregnant women VAD causes night blindness and may increase the risk of maternal mortality. This is a public health problem in 118 countries, especially in Africa and South-East Asia including India. According to 2003 survey of WHO, nearly 100 and 140 million children are deficient in vitamin A. An estimated 2,50,000 to 5,00,000 vitamin A-deficient children become blind every year, half of them dying within 12 months of losing their sight and nearly 6,00,000 women die from childbirth-related causes each year, the vast majority of them from complications, which could be reduced through better nutrition, including provision of vitamin A (www.who.int/nut/vad.htm). In order to meet the demand of vitamin-A, apart from the administration of retinol palmitate, supplementation of the carotenoids in general and β- carotene in particular is the widely used. In this connection carotenoids are gaining more and more attention in the direction of searching newer sources and renewable production systems. Biotechnology as a tool to enrich carotenoids production is receiving attention as evident from the interest generated in golden rice. Micro algae are also rich sources of a range of carotenoids. Some of the well-known micro algal forms, which are important sources of carotenoids are *Dunaliella, Spirulina* and *Haematococcus*. Of them *Dunaliella* is known to accumulate high quantity of carotenoids in response to various physical stress and is used as commercial source of β-carotene in certain parts of the world.

Some of the industries involved in the production of carotenoids from *Dunaliella* are,

- Betatene Ltd, Cheltenham, Vie. 3192, Australia, a division of Cognis Ltd, Australia.
- Cyanotech Corp., Kailua-Kona, HI 96740, USA. .
- Inner Mongolia Biological Eng. Co., Inner Mongolia, 750333, P. R. China.
- Nature Beta Technologies (NBT) Ltd, Eilat 88106, Israel, a subsidiary of Nikken Sohonsha Co. Gifu, Japan.
- Tianjin Lantai Biotechnology, Ine. Nankai, Tianjin, in collaboration with the Salt Scientific Research Institute of Light Industry Ministry, P. R. China.
- Western Biotechnology Ltd. Bayswater, WA 6053, Australia, a subsidiary of Cognis Ltd, Australia.

- Aqua Carotene Ltd, Subiaco, W A 6008, Australia, and Small plants are also located in Chile, Mexico, Cuba, Iran, Taiwan, and Japan.

Utilization of *Dunaliella* is not much popular in rest of the world due to unavailability of the information regarding the cultivation in public domain as most are in the form of patents with restricted availability. In spite of this, carotenoids from *Dunaliella* are being used for various therapeutic and prophylactic applications. There are very limited scientific information available on the production system in large scale as applicable to industrial utility and on the safety of the whole algal cells (except for a paper from Mokady et al., 1989). Algal cultivation has some advantages over other plant production systems as they are fast growing and can be cultivated utilizing solar energy and carbon dioxide in the environment as a source of carbon as seen in case of blue green alga *Spirulina,* cultivated for the protein and nutritional supplement. However for industrial cultivation, effective utilization of natural resources for cultivation and utilization of minimum energy are the aspects to be addressed to make the technology economically and commercially viable.

In light of this understanding and available scientific information in literature regarding the organism, the present work was aimed in identifying the strain of *Dunaliella* (local, as *Dunaliella* is found in the salt pans of seashore), which can produce maximum content of quality carotenoids, Development of suitable methodology for maintenance, production of algal biomass enriched with carotenoids using effective economical protocols. Work was also planned to develop simpler and economic protocols for harvesting the cultivated cells, downstream processing for effective extraction of carotenoids from cell and storage of the biomass and extracts without degradation as carotenoids are sensitive to light, oxygen and to temperature to some extent. Attempts were also planned to identify the carotenoids produced from algae and to evaluate the safety of *Dunaliella* biomass and also to evaluate biological efficiency of biomass and carotenoids of the algae in comparison with synthetic counterpart all *trans* β- carotene in terms of hepatoptotective and antioxidant ability.

The above work plan was carried out with following broad objectives in order to contribute a technology for public utility to fight against major national health issue of VAD, which is of more relevance to developing nations.

- Establishment of culture of *Dunaliella* and production of biomass in pilot scale.
- Downstream processing of carotenoids for application in food and pharmaceuticals.

@ Analysis of various carotenoids and xanthophylls of *Dunaliella.*

@ Studies on elicitation of carotenoids in *Dunaliella.*

@ Studies on biological activity of carotenoids and whole cells of *Dunaliella*.

@ Complete technology information for the cultivation and utilization of the algae as potent source of carotenoids.

7. 2 Establishment of culture of *Dunaliella* and production of biomass in pilot scale.

Number of studies has been done to understand the environmental effect on the growth and carotenoids production in *Dunaliella* by various researchers (van Auken and McNulty 1973; Massyuk 1968; Vonshak 1986; Ben Amotz and Avron 1989; Avron and Ben Amotz 1992; Lears et al., 1990; Jahnke 1999). Though most of the reported studies give information on cultivation of *Dunaliella,* there is little information on the medium required for cultivation of the vegetative cells as well as the enhancement of carotenoids in biomass. The present study made an effort to find out the media requirements and factors to obtain such biomass for production of carotenoids from micro alga *Dunaliella*.

Various mechanisms have been postulated to explain the acclimatization of *Dunaliella* to a varied range of salinity and the ability of these algae to survive in concentration of glycerol (Borowitzka and Brown 1974). A linear relation has been observed between the concentration of salt and glycerol production. Absence of a rigid polysaccharide cell wall permits a rapid adjustment of the intracellular osmotic pressure by fluxes of water through cytoplasmic membrane (Ben Amotz et al., 1982). The level of a plasma membrane *Mr* 60,000 protein, p60, was found to increase with rising external salinities. Results have shown that p60 is a structurally novel carbonic anhydrase transcriptionally regulated by CO_2 availability and exhibiting halophilic-like characteristics (Fisher et al., 1996). It is observed that environmental stress conditions like, high salt (NaCl), extreme temperature and pH or nutrient deficiency resulted in enhanced β- carotene to chlorophyll ratio and an inverse relation to cell growth rate as seen in case of *D. bardawil* explained in section 6.11to 6.14. The light intensity provided during the cell division is the prime factor responsible for increase in β- carotene to chlorophyll ratio; longer the doubling time higher is the ratio and similar results were observed in our study as seen in results section 6.10. Figures- 22 and 23 shows the growth pattern and Figure-24 shows the culture accumulated high carotenoids upon exposing to high intensity light. It is proposed that the β- carotene accumulates in cells as a protective agent against the high light induced damage. *D. bardawil* is able to accumulate relatively higher content of

carotenoids and the accumulation is also proportional to chlorophyll content indicating the photosynthesis ability (Ben Amotz and Avron 1983). Other membrane protein, which is expressed during stress condition, is of 150 kD, identified as a unique transferrin-like molecule, containing three lobe forming units along with COOH terminal extension including an acidic amino acid cluster (Sadka et al., 1991). This transferrin like structure, generally found in animals were identified in *Dunaliella* for the first time, which is essential for uptake of iron (Fisher et al., 1997). A study conducted using norflurazone to block phytoene formation has revealed that high light intensity along with nitrate, phosphate and sulphate stress enhance the carotenoids accumulation. Enhanced light intensity is known to increase oxygen level in the form of free radicals. The light induced free radical generation leads to plastoquinone mediated carotenoids production, which inturn quench these radicals (Salguero et al., 2003).

In order to understand the required environmental conditions and media constituents for growth, a number of experiments were initially planned in laboratory conditions of 18 ± 2^0C temperature, light intensity of 2.0 Klux. Most of these were done in Erlenmeyer flask of 250, 500 and 1000 mL capacity.

7.2.a Studies on the growth requirements of the algae at laboratory scale.

Both the cultures i.e., *Dunaliella salina* and *D. bardawil* were maintained in AS-100 medium (Table- 13) in agar slants as well as liquid culture. Initially growth was observed to be very slow, which took almost 25-30 days for complete growth as measured by optical density at 560.0 nm. Comparison of growth of algae in AS-100 as well as Ben Amotz medium (Table-15) yielded similar growth pattern (Figure-20). It was found that *D.bardawil* grows at faster rate compared to *D.salina* in these media.

Studies on various parameters influencing the growth such as, Initial pH of the cultivation medium, concentration of sodium chloride as the algae is halotolerant, essential nutrients including, carbon source, sulphate, nitrate, phosphate and micronutrients were undertaken. Results of these studies indicates that *Dunaliella* can grow (multiply) well at initial pH of 7.0-7.5 (Figure-13), initial content of sodium chloride in the concentration of 1.5M (Figure-14), light intensity of 2-4 Klux (Figure-15). Further the results indicates that , exposing the algae to high light intensity which are grown for 14-20 days under low light can enhance the accumulation of carotenoids to greater extent (Figure-16). Results of the studies on source of carbon indicates that the external gaseous CO_2 is not much helpful for the growth, instead it can utilize the carbon

from inorganic salt, sodium bicarbonate, efficiently when used at concentration of 4.0 gL^{-1} (Figure-18). In case of micronutrient a stock of boron, cobalt, manganese, molybdenum, iron supplemented as stock at concentration of 10.0 mL L^{-1} (containing H_3BO_3- 0.5mM, $CoCl_2$ $6H_2O$-1.0mM, $MnCl_2$ $4H_2O$- 7mM, $ZnCl_2$ -1.0mM, $FeCl_3$ - 1.5mM and $(NH_4)_6$ MO_7O_2 $4H_2O$ -1.0mM.) was found to be ideal, whereas that zinc concentration of 0.21 µM in the form of chloride salt is found to be ideal for growth (Figure-19). Based on the results of these experiments, a new medium containing ideal composition of chemicals for optimum production of biomass was developed denoted as '*modified medium*' (Table-14). The composition of the modified medium for growth and carotenoids production was ideal under the local environmental conditions and also for the strains used in the study. Composition of the '*modified medium*' has been submitted for protection as Indian patent (Murthy et al., 2004).

Dunaliella was grown in media reported in the literature as well as modified medium and results have shown that both growth and carotenoids accumulation were significantly higher in case of modified medium (Figure-20 and 21).

7.2.b Studies on the factors dictating the carotenoids accumulation.

Upon standardizing the medium and methodology for optimum multiplication of the algal cells, various methods of stress induction were tried for carotenoids accumulation (based on the literature available) viz., high light, salt (sodium chloride) and total nutrient stress (Ben Amotz and Avron 1983). Exposure of culture to high light (25-30 Klux) initially showed a decline in the growth rate (Figure-22), however it is known to enhance the carotenoids content significantly almost 2- 2.5 folds more compared to white light of 2.0 Klux intensity (Figure-23). Among various conditions tried, salt stress which could be achieved by diluting the whole culture (1:2) and exposing to high light (25-30 Klux) has proven to be a highly effective mode of carotenogenesis. Apart from this subjecting the grown cells to sulphate, nitrate and phosphate stress has shown that only nitrate stress can beneficial (Figure-25) in accumulation of carotenoids, which is in concurrence with earlier reports (Ben Amortz et al., 1983). Studies on culture height, which play vital role in availability of ideal light intensity for induction of stress for carotenoids accumulation has revealed that the culture height of 5.0-7.0 cm is ideal for carotenoids accumulation (Table – 17a and 17b). An attempt was made to utilize commercial fertilizer as source of nitrogen, phosphorous and potassium (Figure-30). Results were initially encouraging but later it was found that upon recycling it could not

produce the quantity and quality of the biomass as that of modified medium. Therefore it was not used for further trials as utilization of spent medium is of great significance in commercial scale production. After standardizing the methodology for the cell multiplication as well as carotenoids accumulation at small scale of 20-25 L culture volume, it was further taken for scale up studies.

7.2.c Studies on scale up of *Dunaliella* culture.

Scale up of algal culture is a challenging process as both micro and macro algal forms are highly potent source of numerous potential nutritional compounds. It is necessary to cultivate them apart from their natural habitat for commercial exploitation. Problems associated with natural habitat are slow growth due to fierce competition for existence with other marine forms, for nutrients, accidental contamination with toxins or heavy metals, cross contamination with toxic forms of same species etc., In order to avoid these things the best way of production is cultivation of these algal forms in artificial systems. An ideal cultivation technique should be economical as the production per unit volume is very low (biomass will be in the range of 0.25-1.0 gL^{-1}) and viable to utility. Process can be made economical by utilizing natural resources like, seawater, solar energy and natural resources. There are reports which have shown cultivation of *Dunaliella* using closed tubular bioreactor with a significant increase in the content of 9- *cis* β- carotene, lutein and other carotenoids (Garcia -Gonzalez et al., 2005). Present investigation was aimed at making the scale up of cultures economically viable. Hence some of the reported media chemicals like, TRIS (buffer), vitamin B_{12} and EDTA were avoided and were replaced by economic alternates without compromising the quality and productivity. An attempt was also made to utilize agricultural fertilizer as source of nutrients, but the same was not useful in utilization of media for recycling.

Four major method have been used for large-scale cultivation of *Dunaliella* viz, using no mixing and minimal control of environment. This can yield less than 0.1 g β-carotene m^{-3} and requires larger area. Second one by using biotechnology for control of all the factors affecting growth and chemistry of cells. The ponds were oblong or raceway type with slow revolving paddle wheels for mixing, up to 3000-m^2 surface was employed for this methodology, yield up to 200.0 mg β-carotene m^{-2} day^{-1}. Third one is in by mode consisting of ponds of about 50,000 m^2 each with partial control and no mixing. The fourth one is cultivation employing cultivation in closed photo-bioreactors, like narrow,

very long, plastic tubes, plastic bags, trays and more. This has not gone beyond laboratory scale as they are not economically viable for *Dunaliella* (Ben Amotz 2003).

Scale up studies of these cultures were done using modified medium by growing axenic cultures in 500 mL - 1.0 L flasks, followed by semi opened carboys of 5-10 L capacity and slowly transferring these cultures to 3 and 5 L plastic carboy of wide mouth and then to 20-25 L rectangular glass tanks (Figure-24). Initially cultures could not survive in this open-air condition due to contamination of protozoa and diatoms. This was suppressed by enhancing the salt concentration by 2.0% w/v of sodium chloride and pH was maintained by using sodium bicarbonate (1.0 gL^{-1}). These cultures, which could survive even after contamination, were transferred into circular cement tanks with agitation and grown for 15-16 days under shade, after which they were exposed to direct sunlight after dilution (Figure-26). These cultures could accumulate carotenoids in 2-3 days i.e., after exposure to 10-12 hours of direct sunlight (25-30 Klux). Cultures were grown in pilot scale raceway ponds of 500 L and 2000 L capacity under low light with the help of shade nets (50% cutoff light i.e., 12-15 Klux) for 14 ± 2 days, with culture height of 50-60 cm and further transferred to higher capacity raceway ponds with dilution to a 15-20 cm layer which provides optimum carotenogenesis (growth pattern in Figure-28 and 29, photographs of culture is as shown in Figure-27). Occasional agitation for 2-3 minutes twice a day was provided using motor driven paddle wheel at 8-10 rpm. This condition was repeated several times to confirm the productivity under similar culture conditions using fresh and recycled medium in different climatic environments, which yielded 1.2-1.5 gL^{-1} of wet biomass. Different experiments were conducted to know the concentration of media chemicals to be replenished in order to utilize the spent medium i.e., to recycle the medium. Chemical composition of sodium chloride (2.0 %w/w), bicarbonate ($1/3^{rd}$ of original), sulphate ($1/3^{rd}$ of original), and nitrate ($1/5^{th}$ of original) along with adjustment of the pH to 7.0 ± 0.25 was suitable to achieve optimum growth in recycled medium.

7.2. d Studies on harvesting of *Dunaliella bardawil* cells from culture and studies on drying.

As *Dunaliella* is a small, motile algal form, it is difficult to separate the cells effectively from culture without loss of carotenoids and has remained a challenge for many industries. Upon optimization of cultivation protocol, various harvesting methods were examined for harvesting like, online and batch centrifugation, flocculation using various

flocculants like, alum (Figure-34), chitosan (Figure-33), ferric chloride (Figure-32) and alteration in pH (Figure-31; Figure-36). Both batch and online centrifugation of cultures proved to be an expensive process as both require huge electricity (Table-18). Hence it was planned that flocculation of these cells will reduce the culture volume almost 8-9 times and further centrifugation can be employed for effective harvesting. Among the techniques examined flocculation using alum (0.7-0.8 g L^{-1}) followed by centrifugation was found to be an effective method of harvesting the biomass (Poelman et al., 1997). Due to loss during open-air centrifugation, online centrifugation was adopted after flocculation. Micro algae carry negative charges, which prevents the aggregation of the cells in suspension. The surface charges can be neutralized or reduced by addition of flocculants, such as multivalent cations and cationic polymers. The same is observed when alum or chitosan are used to flocculate the *Dunaliella* biomass. *D. tertiolecta* has been reported to be harvested by enhancing the pH of the culture broth by addition of NaOH. This has shown successful flocculation between pH ranges of 8.6-10.5, flocculating more than 90% of the cells. The only problem associated with the technique is that the cells will loose certain amount of protein, which are leached into the medium (Horiuchi et al., 2003). Multivalent metal salts which have been tried for flocculation of micro algae, (*Scenedesmus* and *Chlorella*) are ferric chloride, aluminium sulphate and ferric sulphate and polyferric sulphate (Grima et al., 2003). Some other cell harvesting methods adopted for micro algae are pressure filter (belt press, suction filter), vacuum filter (drum filter, belt filter, pre-coat vacuum filter). Yet another method to harvest the cells is electrolytic flocculation where electric current is applied instead of flocculants. Application of 0.3 k W h^{-1} m^3 could harvest more than 95.0 % of cells as seen in case of *Closterium* sp (Poelman et al., 1997).

Potash alum is known to bring down negative charges on the surface of cell wall of algae and tends to aggregate the cells into larger masses, which settle down due to density. This flocculated biomass was subjected for filtration to collect the intact biomass. Biomass was subjected to various drying methods for storage and extraction of carotenoids. The drying methods adopted include, shade, sun, oven, spray (both under oxygen and nitrogen) and IR drying and all the methods have shown a loss of carotenoids in the range of 15-55 % (Table-19). However, IR drying which exhibited minimal loss has also shown to enhance the *trans/cis* ratio of β - carotene (7-8 times). Hence this method can be employed in such process, which requires β - carotene with

enhanced isomeric form (Table-19), there was no effect on the nutritional quantity in case of spray drying except for the loss of carotenoids (Table-20).

The present study has resulted in eco-friendly technology for production of highly valuable carotenoids from cultured microalgae. As the medium is recyclable and production involved utilization of natural resources like solar energy and sea water and can be taken up as zero energy utilization process. The technology is highly suitable to equators with ideal weather conditions of light intensity and temperature necessary for the process. However it can also be grown in low light with some modifications of parameters like, like culture depth and intensity. Hence this can serve as best system for production of carotenoids world wide.

7.3 Downstream processing of carotenoids for application in food and pharmaceuticals and studies on stability.

Most appropriate and economical downstream processing methodology is of paramount value in an industrial process as it accounts for the main cost of the product. The methodology to be employed for the process also decides the choice of down stream technique. As explained earlier drying of the biomass will lead to loss of carotenoids invariably. For the therapeutic supplementation of carotenoids at the rate of 10-20 mg carotenoids day^{-1}, direct oil extraction can be employed for extraction as oils which take up the carotenoids up to 1000-1,200 ppm. Thus 1.0 mL of oil containing 10 mg carotenoids is feasible for administration. For the commercial utilization of carotenoids a concentrate of 5-30% w/v dispersion in olive oil is generally prepared, which have advantage of stability and transportation. This concentrate is used for formulations on food and pharmaceuticals after adequate dilution. Production of such concentrate is possible only by extraction in permitted organic solvents and subsequent dispersion in oil.

Different methods have been tried to extract carotenoids from algal biomass, including direct extraction into edible oil, extraction in organic solvents and continuous extraction using various organic solvents, like octane, decane, pentane, hexane and tetradecane with biphasic systems, without compromising the viability of cells (Hejazi et al., 2002). An ideal extraction procedure should employ a solvent, which can extract maximum content while being sufficiently volatile so that it can be easily removed without causing significant degradation of the compounds. Utilization of oil for extraction has limitations, as it cannot take up more than 1.0 mg carotenoid mL^{-1} of oil due to limitation in

solubility. Therefore in order to make a concentrate of carotenoids it was found that the best method was to use a solvent for extraction and resuspend in oil. Present process utilizes ethyl acetate which is capable of extracting maximum content of carotenoids and can be easily removed so as to suspend the extract in oil, which acts as a stable carrier matrix.

It was also found that direct extraction of wet biomass was highly suitable. Extraction was tried using various combinations of solvents. Among them, ethyl acetate and n-hexane: isopropyl alcohol (1:1) showed maximum extractability (Table-21). An attempt was also made to extract pigments using various edible oils from low moisture biomass (< 5.0%). Palm and olive oils showed maximum extractability, which may be due to the chemical features of these oils, which implies that the chemical nature and natural antioxidant contents in these oils is ideal for stability. Solvent extracted carotenoids were suspended in various edible oils like, coconut oil, gingili (Sesame), palm, olive, groundnut, rice bran, mustard and sunflower oil and subjected for stability studies under different conditions of temperature, stabilizer and light (Figure-36 to 43). Analysis of degradation rate constant day^{-1} (R^2) shows that storage in palm oil and olive oil at 4^0C along with BHA provides maximum stability as seen after nine months of storage (Table-23), which can be attributed to the physicochemical properties of these oils.

7.4 Analysis of various carotenoids and xanthophylls of *Dunaliella*.

7.4.a Qualitative analysis of carotenoids of *Dunaliella*.

Carotenoids of *Dunaliella* were quantified in terms of total carotenoids by measuring optical density at 450 nm and found to yield 2.25 ± 0.25 % (w/w) of total carotenoids in terms of dry weight. This was also identified by comparison of spectral pattern of the carotenoids with authenticated samples (Figure-45). These carotenoids were subjected to identification using TLC adopting various solvents on silica gel coated plate. Among the different solvents employed, n-hexane/acetone/ ethyl acetate (19.5/80/0.5) has shown high-resolution in separation of pigments. Based on the TLC separation it was found that *Dunaliella* contains total of eleven pigments including chlorophyll a and b (Figure-46 and 47). Spectral method was also used to find out the *cis* isomer of β-carotene, which apart from helping in quantification of total carotenoids also help in detection of adulteration of various natural β- carotene with synthetic one as synthetic form contain only *trans* isomeric form (Figure-48). This isomeric form was also confirmed by isocratic HPLC method (Figure- 49). The pigments were separated on silica and alumina

column using various solvents and were identified based on their absorption maxima as, phytoene, phytofluene, α- carotene, β- carotene, lutein, violaxanthin, neoxanthin and zeaxanthin (Table-24). This observation was further strengthened by means of gradient HPLC analysis developed by us using acetonitrile and chloroform as mobile phase (Figure-51).

7.4.b Quantitative analysis of carotenoids of *Dunaliella bardawil.*

Number of HPLC methods have been reported to identify the carotenoids from various biological materials, using C_{18} silica column employing various solvents including methanol, acetonitrile, dichloromethane, methylene chloride, hexane in gradient and isocratic elution (Khachik et al., 1986). Another method using C_{18} column has been reported using reverse phase chromatography in acetonitrile, methanol and dichloromethane in gradient elution pattern (Darko et al., 2000). Utilization of methanol-acetonitrile and chloroform (47:47:06) in C_{18} column to separate *cis* and *trans* isomers of β- carotene has been reported by Schwartz and Killam (1985). Most of the methods are applicable for either carotenoids containing hydrocarbon carotenoids or oxygenated carotenoids (xanthophylls). It is difficult to have a single HPLC method for samples containing both of these. Some of the HPLC techniques reported by various researchers are as shown in Table- 40. After a number of attempts to have a suitable solvent system which can resolve all the pigments of algae, a gradient solvent consisting of acetonitrile and chloroform has been developed and this gave better resolution of all the algal pigments. HPLC confirmed the presence of *cis* and *trans* β- carotene along with other carotenoids identified by TLC. It was also found that 100 % methanol can also resolve the pigments and the same was employed for LC-MS.

Different methods of liquid chromatography-mass spectrometry have been reported by van Beerman (1997). He used C_{30} column and gradient elution of methyl-*tert*- butyl ether in methanol containing 1.0 mM ammonium acetate. Five LCMS techniques have been tried, including moving belt, particle beam, continuous flow fast atom bambardment (FAB), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Among these, ESI and APCI are most suitable for carotenoids. APCI produces molecular ions along with protonated and deprotonated ones depending upon the mobile phase condition.

Table 40. Comparison of various methods employed for estimation of carotenoids by liquid chromatography.

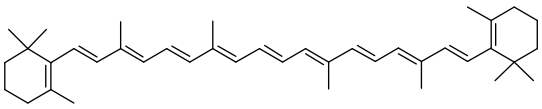
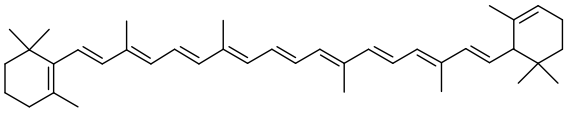
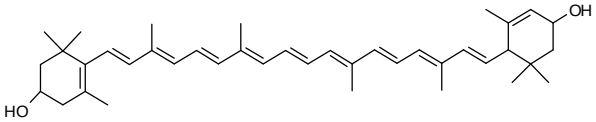
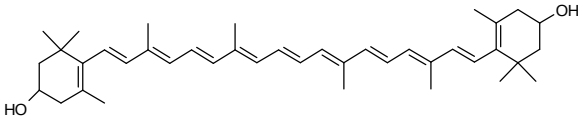
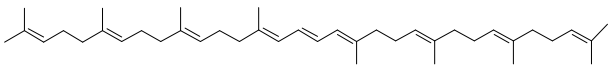
Method	**Column**	**Mobile phase**	**Carotenoids detected**	**Reference**
Isocratic	C_{18}	CH_3CN, $CHCl_3$, and CH_3OH	α- carotene, β- carotene, β-cryptoxanthin, lycopene	Thurnham et al., 1988
Isocratic	ODS	CH_3CN, THF, and CH_3OH	α- carotene, β- carotene, β-cryptoxanthin, lycopene	Hess et al., 1991
Isocratic	ODS	CH_3CN, THF and CH_3OH	α- carotene, β- carotene, β-cryptoxanthin, lycopene, lutein	Steinberg 1989
Gradient	ODSI	CH_3CN, CH_3OH and H_2O	α- carotene, β- carotene, β-cryptoxanthin, lycopene, lutein/zeaxanthin	Zaman et al., 1993
Gradient	S5-ODS2	CH_3CN, CH_3OH, $CHCl_3$ and NH_4AC	α- carotene, β- carotene, β-cryptoxanthin, lutein/ zeaxanthin, *trans*-lycopene, *cis*-lycopene	Su et al., 1999
Gradient	C_{18}	C_6H_{14},CH_2Cl, Dioxane	lycopene	Froescheis et al., 2000
Gradient APIC-MS	C_{30}-silica	C_2H_4O, H_2O	Lutein/ zeaxanthin, α- carotene, β- carotene, lycopene	Dachtler et al., 2001
Gradient*	C_{18}	CH_3CN and $CHCl_3$	α- carotene, β- carotene, phytoene, β- cryptoxanthin, lutein/ zeaxanthin, lycopene, lycopene	Murthy et al,. 2005
Isocratic* (ESI-MS)	C_{18}	CH_3OH, CH_3CN and $CHCl_3$	α- carotene, β- carotene, phytoene, β- cryptoxanthin, lutein/ zeaxanthin, lycopene	Present report

* Indicates ourstudies.

ESI will show fragmentation along with molecular peak, which is essential to identify carotenoids of same molecular weight. MALDI, MS and LCMS analysis revealed the presence of two more carotenoids, lycopene and β- cryproxanthin.

β- carotene, lutein, zeaxanthin, phytoene and neoxanthin were identified by MALDI as observed by their characteristic molecular peaks (Figure -52 and 53). It also revealed the association of lutein with C_{11}, neoxanthin with $C_{12:0}$ and $C_{20:\ 3}$ and zeaxanthin with $C_{20:4}$ fatty acids. Mass spectra confirmed the presence of β- carotene (m/z, 536/537 (M^{+} and M^{+1}); 444.0 (M^{-92}), 429 (M^{-107})), α- carotene (536 (M^{+}); 523 (M^{-13}); (444.0 (M^{-92}); 413 (M^{-123})), lutein (569 (M^{+1}), 525 (M^{-44}), 437 (M^{-132})), zeaxanthin (568 (M^{+}), 537 (M^{-31}), 406 (M^{-162}), lycopene (536 (M^{+}); 444 (M^{-92})), phytoene (544 (M^{+}), 536 (M^{-8}), 522 (M^{-22}), 518 (M^{-26})) and β-cryptoxanthin (552 (M^{+}), 437 (M^{-115})). The same was further confirmed by LC MS (Figure-58-60). Structure and compositions of these are summarized in Table -41.

Table.41. Structural formulae and composition of major carotenoids of *Dunaliella*.

Carotenoids (Common and chemical names)	**Chemical structure**	**% Content (Approximate)**
β- carotene (β,β-carotene)		78.5
α- carotene [(6'R)- β,ε- carotene]		5.14
Lutein ((3R,3'R,6'R)-β,ε carotene-3,3'-diol)		0.86
Zeaxanthin ((3R,3'R)- β,β carotene-3,3'-diol)		1.21
Phytoene (15-*cis*-7, 8,11,12, 7', 8', 11', 12'- octahydro -ψ,ψ- carotene)		3.75

For the fist time we have reported details of all the carotenoids present in the algae , which can extend its utility for a wider range compared to sources containing β- carotene alone. Analysis of various carotenoids indicates that *Dunaliella* is not only rich in β-carotene, which is major projected bioactive molecule, it also contain optical isomer *cis* form of β- carotene and α-carotene essential for better biological activity. It has also shown the presence of oxygenated carotenoids namely xanthophylls like, lutein and zeaxanthin, which are of great significant in enhancement of bioavailability of β-carotene, in eye health and other health benefits.

7.4. c Analysis of proximate composition of *Dunaliella salina* and *Dunaliella bardawil.*

Analysis of proximate composition of algae shows the presence of 20 ± 2.0 % protein in both the algal forms. Apart from this, fat (7.5 ± 0.4%) and carbohydrate (25.0 ± 2.0 %) are the major constituents. Ash content was more in both the species, which may be due to the presence of high sodium chloride and bicarbonate in biomass (Table-26).

7.5 Studies on elicitation of carotenoids in *Dunaliella.*

Plants have developed a number of defense mechanisms to defend themselves against microbial attack. This includes synthesis of secondary metabolites. Some times this leads to immediate production of reactive oxygen species (Davis et al., 1993). Yeast extract was used as an elicitor in *Daucus carota* hairy root cell cultures, where three and a half fold enhancement of peroxidase levels was observed (Zenk 1991). Fungal cell wall extracts are potent elicitors in plant cell cultures (Hamerski et al., 1990). The cell wall extract of *Phytoptera nicotinae*, a potent pathogen, cell wall was investigated for the production of phytoalexins in cultures of *Nicotiana tabacum* (Olelofse and Dubery 1996).An enhanced production of capsidiol and debneyol and production of two unknown phytoalexins were observed in Capsicum (Threlfall and Whitehead 1988). Carbohydrates have also shown this ability as seen in case of oligogalacturonides employed as an elicitor in tobacco, which acts by enhancing Ca^{+} uptake and significant enhancement in the extracellular pH compared to control (Binet et al., 1998).

Some of the abiotic elicitors used for the purpose are, benzo (1,2,3)-thiadiazole-7 carbothiolic acid S-methyl ester (BTH) was used from the cell walls of *Phytophthora sojae* is used as conditioner in elicitation of systemic acquired resistance in parsley (*Petroselium crispum*, Siegrist et al., 1998; Pitta-Alvarez et al., 2000). Some of the possible elicitation mechanisms in plants include, binding of elicitor to a plasma membrane receptor (Cheong and Hahn 1991), changes in ion influx across membrane

(Mathieu et al., 1991), synthesis of secondary messengers (Mahady et al., 1998), activation of NADPH oxidase responsible for ROS and cytosol acidification (Lebrun-Garcia et al., 1999), cytoskeleton reorganization (Kobayashi et al., 1995), production of ROS (Apostol et al., 1989), accumulation of defense related proteins such as chitinase and glucanase (Benhamou 1996), structural changes in cell wall and synthesis of secondary messengers like, salicylic acid and jasmonic acid (Radman et al., 2003).

Biotic elicitors like, fungal cell extracts viz., *A.ochraceus* (5.0 %), *A. flavus* (10.0%) and *A. niger* (5.0%) significantly elicit both biomass and carotenoids content of the two algae *D.salina* and *D.bardawil* (Figure-61). Both *A. flavus* and *A. niger* extracts have shown the enhancement of carotenoids content in *Dunaliella salina* (Figure-62 and 63). However its utility in large scale production system was not studied. Mechanism of elicitation in algae is also a subject matter of future studies.

For the first time, an attempt has been made to study the effect of statin which acts by interfering with biosynthetic pathway and results give clear evidence about the blockade of squalene biosynthesis by statin, which in turn increases the content of lycopene cyclase and ultimately results in enhanced synthesis of carotenoids (Figure-65 and 66). Results of this study can be utilized for enhancing the carotenoids content even at low light intensity as blocking of squalene will enhance the carotenoids content at low light also (Figure-64). Statin was used at very low concentration and the same is not extracted along with the carotenoids (as it is insoluble solvents used for extraction of carotenoids), its utility to enhance the carotenoids in large scale production is a question?

This study reveals that elicitors which are generally employed in minute quantities to get more of valuable product can be of great significance in production of carotenoids. Fungal extracts which elicits carotenoids production either by triggering biosynthesis will be of great significance.

7.6 Studies on biological activity of carotenoids and whole cells of *Dunaliella salina.*

Due to high demand and health conscious life style there is urge for newer potential sources of these compounds. Apart form land plants, Marine forms are also of promising potential sources. Algal forms are also reliable source of bioactive molecules and nutrients. Among them, microalgae *Spirulina, Chlorella*, *Dunaliella* and *Hematococcus* are some of commonly utilized ones and Eucheuma, *Enteromorpha*, *Prophyra* are commonly used macro algal forms. These algae are also a source of Nutraceutical like, Vitamin, Protein, Structured lipids and essential amino acids.

Apart from the nutritional effect as pro vitamin A, carotenoids are known to posses various pharmacological activity, which are antioxidant mediated, which act by following mechanism. Carotenoids are integral parts of membranes but xanthophylls are variable in their position. Therefore carotenoids are effective antioxidants if radicals are generated inside the membrane. Apart from this carotenoids have a remarkable effect in the immune response and intracellular communication (Britton 1995: Charluex 1996). Many of the carotenoids including β-carotene shows an efficient induction of GAP junction communication induction and free radical scavenging are two independent mechanisms, which act independently to prevent cancer (Stahl et al., 1997). Carotenoids are associated with LDL, where as xanthophylls are distributed equally between high and low density lipoproteins. Carotenoids upon biotransformation convert to retinol, retinoic acid and other products. *Dunaliella* is known to contain both *cis* and *trans* isomeric forms of β- carotene, which is known to have better bioavailability and bio efficacy (Yeum and Russel 2002).

7.6. a Studies on in vitro antioxidant activity.

Studies on the antioxidant activity of both ethanol extracts by β-Carotene linoleic acid model system (Figure-70), DPPH (Figure-71) have shown that they have significant antioxidant activity, which is mainly due to carotenoids, which acts by readily donating required electrons as evident from the color of the extract.

Further subjecting the successive extracts (Table-27) to different radical scavenging and total antioxidant activity by DPPH (Table-28), reducing power assay (Figure-72) shows that apart from carotenoids there are some other ingredients responsible for the radical scavenging activity, which may be attributed to vitamin-C and other biological amino acids. This result was further supported by the ability of successive extracts in prevention of various lipid peroxidations as measured by egg lecithin (Table- 39), human erythrocytes (Table- 30), brain and kidney of albino rats (Table- 31 and 32).

7.6.bStudies on nutritional activity of Dunaliella salina in vivo.

Dunaliella can be a good source of nutrition as it contains high quality proteins with most of the essential amino acids, which can be easily digested. It is a rich source of vitamin A precursor and minerals. Available in natural habitat and easy for cultivation, it contains some of the nutraceutical components like, antioxidants, natural pigments, preservatives and essential fatty acids (gamma linoleic acid, eicosapentaenoic acid). It is also a source of chlorophyll, which helps in cell regeneration. Hence the present study

was aimed at examining the nutritional benefits of the algae in terms of body weight and hematological profile along with its effect on weight of various vital organs.

There are few studies that have examined the effect of β- carotene supplementation *in vivo* (Paetan et al., 1997; Astorgs et al., 1994; Albanes, 1999). Lin et al. (1998) reported that 6.16 μ Moles of β- carotene day^{-1} is sufficient for maximum protection of LDL in women. Seddon et al. (1994) reported the beneficial effects of β - carotene from diet in prevention of age related macular degeneration. It is proved that plasma concentration of carotenoids has influence on cytochrome P450 and other key enzymes required for metabolism in the body (Gradelet et al., 1996).

Nutritional and safety (sub acute) studies showed no visible toxicity in rats when fed at a dose of 2.5 and 5.0 g kg^{-1} for 15 days. However, there was a significant gain in average body weight in case of male rats, which is possibly due to protein content of algae. Effect of algae on weight of various organs revealed no abnormal changes indicating the non-toxic effects of the algae (Table-33). Hematological profile supported the above results with not much variation in the contents of hemoglobin, RBC, WBC and other factors (Table-34).

7.6.c Studies on antioxidant activity of *Dunaliella salina* biomass in vivo.

Carbon tetrachloride has been extensively studied as a liver toxicant. Its metabolites such as trichloromethyl radical ($CCl_3^{\bullet}$) and trichloromethyl peroxyl radical ($CCl_3O_2^{\bullet}$) are involved in the pathogenesis of liver (Gil et al., 2000) and kidney damage. The massive generation of free radicals by CCl_4 induced liver damage with a sharp increase of lipid peroxidation. When free radical generation is massive, the cytotoxic effect does not remain localised but propagated intracellularly, resulting in interaction of these radicals with phospholipids, thus enhancing peroxidation process and destroying organ structure. In biological system, carotenoids are expected to exert most of their antioxidant effect in lipid rich environment because of their lipophilic nature (Oshima et al., 1993). Lycopene, even though has a high reaction rate constant, is least stable under light wherein 50% degradation is seen in 3 hours. The same is almost 20 hours in case of β-carotene (Christopersen 1991).

Carbon tetrachloride induces fatty liver and cell necrosis and plays a significant role in inducing triacylglycerol accumulation, depletion of GSH, increased lipid peroxidation, membrane damage, depression of protein synthesis and loss of enzyme activity (Ahmed et al., 2002; Castro et al., 1974). This is mainly due to metabolites of carbon

tetrachloride, which provoke a sharp increase in the lipid peroxidation in liver and release of markers into plasma (Recknagel et al., 1980). Being cytoplasmic in location, the damage marker enzymes like GOT, GPT and ALP are released in serum (Chenoweth and Hake 1962; Yasuda et al., 1980). The carotenoids exerts their protective activity by impairment of CCl_4-mediated lipid peroxidation, either by decrease in production of free radical derivatives or by the antioxidant activity or both.

Dunaliella biomass fed (2.5 and 5.0 g kg^{-1}) animals have shown significant protection against carbon tetrachloride induced free radicals compared to normal diet treated and toxin administered control group. Results shows that the activity is attributed mainly to carotenoids present and also may be due to ascorbic acid present in small quantities. Biomass apart from defending animals against toxin administered, has a benefit in terms of supplementing protein, which generally undergoes destruction under the oxidative stress (Table-37). Thus *Dunaliella* can be a nutraceutical with liver protective function. Such studies need to be extended to human volunteers for wide application.

7.6.d Studies on antioxidant activity of *Dunaliella salina* carotenoids in vivo.

Algal carotenoids fed animals (125 and 250 μ g kg^{-1} b.w.) have shown highly significant antioxidant activity in terms of restoration of hepatic enzymes after challenging animals with the toxin (carbon tetrachloride). The lipid peroxidation was restored by 6 folds with the later dose and the same was restored by 3 - folds in case of *Dunaliella* carotenoid treated group at the dose of 125μg kg^{-1} b.w.(Table-38).

The free radicals reduce the activity of chief liver detoxification enzymes (catalase, SOD and peroxidase), mainly due to enzyme inactivation during the catalytic cycle. Under oxidative stress, carotenoids of *Dunaliella* containing both *cis* and *trans* isomers along with other carotenoids and xanthophylls act as potent free radical scavengers, reducing the levels of hydrogen peroxide and superoxide anion and consequently lipid peroxidation and enzyme inactivation leading to restoration of the enzyme activity. Bioavilability of *trans* β- carotene is 3 times more than that of *cis*. While the same is found to be more with *cis* isomer of lycopene (Castenmiller and West 1998; Stahl and Sies1992), this may also point towards the possible *de novo* synthesis of these enzymes induced by carotenoids of *Dunaliella*.

Tan and Chu (1991) fed carotenoid from palm to rats and measured hepatic cytochrome P_{450}-mediated metabolism of benzo (*a*) pyrene. β-Carotene was found to be more potent than α-tocopherol. A study in humans revealed that maintenance of a carotenoid-free diet

for two weeks followed by feeding with β-carotenoids demonstrated a decrease in the serum lipid peroxide value, indicating *in vivo* antioxidant effects in humans (Mobarhan et al., 1990). The bioavailabilities of other carotenoids like lutein, zeaxanthin, lycopene, and α-carotene has an effect on each other, which is favorable for biological responses. β-carotene supplementation increases concentrations of α-carotene in a dose-dependent fashion (Rebouche and Seim 1998). It was also found that carotenoids in oil are absorbed and metabolized faster compared to other forms. The World Health Organization has concluded that 6 μg of β-carotene in a meal provides 1μg of retinal, whereas the International Union for Pure and Applied Chemistry concluded from two studies that 3.33 μg will provide 1 μg of retinal (IUPAC 1959). Because treatment with 100 μg of carotenoids will provide 16-30 μg of retinol, the activity exhibited in our study is the result of retinol (vitamin A).

7.6. d Studies on hepatoprotective activity of *Dunaliella salina* carotenoids and biomass in vivo.

Similar results were observed in terms of hepatoprotective ability, which was marked by restoration of serum biochemical markers, like SGPT, SGOT and alkaline phosphatase along with serum protein and albumin content. Histopathology also shows extensive damage (fatty infiltration and necrosis) in animals treated with toxin alone and the same was prevented significantly in algal biomass and algal carotenoids treated group (Figure-74).

As there are very few studies on safety of *Dunaliella*, one of the study reported by Mokady et al., 1989 has revealed that the biomass feeding to albino rats has not shown any symptoms of toxicity as seen till three generations and recent publication by Kuroiwa et al., (2006) has shown that no-observed-adverse-effect levels (NOAELs) were estimated to be 1.25% (696 $mg^{-1}kg^{-1}day^{-1}$) for male rats and 5% (2879 $mg^{-1}kg^{-1}day^{-1}$) for female rats. In light of these reports a to study was taken up to know the effect of feeding biomass as well as carotenoids of *Dunaliella* and to evaluate their effect on nutrition, antioxidant ability as well as hepatoprotective activity in experimental rats. Results indicates that the biomass fed at level of 2.5 and 5.0 g kg^{-1} is found to be safe as there were no symptoms of any kind of toxicity. Results have also shown both biomass as well as carotenoids have shown highly significant protection against carbon tetrachloride induced radical damages as evident form vital enzymes level both in blood as well as liver and histopathology as also supported the same. Hence the algae and carotenoids can

be employed in various formulations used as therapeutics for ailments cirrhosis of liver induced by alcohol or drugs, free radical induced stress conditions. It will not be long before the algae and algal carotenoids, with these potential activity can find their place as ingredient in treatment of conditions like, alzheimer's, cancer, diabetics and coronary heart disease, which are the major health problems of present world.

Results of the present study have revealed that the cultivation of *Dunaliella* was successful in pilot scale at two stages namely vegetative for cell multiplication and carotenoids accumulation under light, salt and nitrate stress. Most of the experiments on cultivation and chemical analysis was done both in *D.salina* as well as *D.bardawil*, however pilot scale studies were done using *D.bardawil*, indigenous strain. Biological activity was studied in both the strains by *in vitro* models and *D.salina* in case of *in vivo* system. Further harvesting process was done flocculating the cells followed by centrifugation. Extractions of the carotenoids were done by permitted solvents and the extracted carotenoids were identified by various instrumentation techniques and also subjected to stability studies under different storage conditions in edible oils. It was observed that palm and olive oils could serve as best carriers for carotenoids. Both algal biomass as well as carotenoids were subjected for *in vivo* antioxidant and hepatoprotective activity and found to be superior compared to synthetic all *trans* β-carotene.

Findings of the present research project will be of relevance in solving problems associated with carotenoids and vitamin-A deficiency. Results also have implications in the scientific understanding of the *Dunaliella* for further consideration in order to exploit the same for value added products related to carotenoids, as an extension of present work. The biomass and carotenoids extract both find application in formulations requiring supply of carotenoids for nutritional or therapeutic needs.

8. 0 Summary and conclusion.

There is a considerable interest, demand and search world wide for novel and sustainable source of carotenoids in general and β- carotene in particular. This is mainly due to its functional attributes viz., precursor for vitamin-A, antioxidant potentials and also as natural color. With current consumer awareness regarding the benefits of naturally derived carotenes over synthetic ones, extensive research is going on worldwide in utilizing of biotechnology as a tool for such compounds. Micro algae repertoire of various pigments, have received the attention of food technologist. The green micro alga *Dunaliella*, which was discovered in early 1800, was initially identified and used as a source of glycerol and with advance in research it was found that it is also a potential source of carotenoids. It was found to accumulate upto 5-8 % weight of carotenoids under different stress conditions. Carotenoids are tetraterpenes containing carbon and hydrogen and few of them contain oxygen. Even though β- carotene produced by synthetic route is economical (US $ 200-300 kg^{-1}), it is losing the customer attention as it is reported to be cancerous on long term usage instead of anti cancerous agent (Woutersen et al., 1999) and also less efficient as provitamin-A due to monoisomeric form compared to naturally derived ones. Production of β- carotene by other biotechnological routes will be too expensive, as they require large-scale fermenters with sophisticated devices. Micro algae can serve as economically viable and feasible technology, which can be easily commercialized. The present cost for natural β- carotene being US $ 2000-2500 kg^{-1}, employing some of the natural resources and simpler techniques cost can be made still economical as it will be highly helpful for meeting the needs especially in developing countries.

The aim of present investigation was to study the method for maintenance of cultures of *Dunaliella*, factors responsible for growth as well as carotenoids production. Culture conditions, for improved algal cells growth and high carotenoids production with respect to media composition, nutrient requirements, illumination conditions, temperature and pH of the media were studied. Enhancement of carotenoids production was carried out adopting various stress conditions like, salt, nutrients, light etc., Further scaleup studies were done to grow algae in outdoor conditions in raceway ponds upto 2000 L. Efforts were also made for downstream processing of *Dunaliella*, analysis of carotenoids qualitatively and quantitatively and studies on biological efficacy of algal carotenoids in comparison with synthetic β- carotene.

Initially cultures were maintained in reported medium as stock cultures and used for further studies. AS-100 medium, was employed for maintenance of culture and to study the effect of various culture conditions on growth of algal cells. Some of the parameters studied were range of pH, concentrations of sodium chloride, carbon, nitrate, phosphate , sulfate and vital micronutrient zinc. Results have shown the requirement of medium constituents necessary for optimum cell growth. Based on these results, composition was modified and a medium was developed denoted as *'modified medium'*. This was able to produce cell density of >10.0 million cells mL^{-1}, which is significant compared to international benchmark.

Further the grown cells of *Dunaliella* were subjected for carotenoids accumulation through stress induction in terms of salt, nitrate and light. High light stress could induce the carotenoids accumulation upto 2.5 % w/w in terms of dry weight. Upon standardizing the conditions for growth and carotenoids accumulation in small scale, scaleup studies were done to grow the culture in semi-open using carboys, followed by open rectangular tanks. Further it was grown in circular cement tanks and raceway ponds, cell count of ~12.0 x 10^6 cells mL^{-1} was achieved in carboys and ~ 10.0 ± 0.5 x 10^6 cells mL^{-1} was achieved in both circular and raceway ponds.

Efforts were made to find out the suitable method for harvesting of cells by various methods viz., batch, online centrifuge and flocculation using various flocculants. Results have shown that flocculation using alum followed by centrifugation will be efficient and less energy consuming rather than direct centrifugation. Harvesting efficiency of more than 95% cells could be achieved by this method. Harvested biomass were subjected for drying using different methods. Significant loss of carotenoids was encountered in all the methods and IR drying has shown to enhance the *cis* to *trans* ratio of β- carotene. Different food grade solvents were used to extract the carotenoids from cells and n-hexane: Isopropyl alcohol (1:1) and ethyl acetate were found to be efficient solvents. Studies on utilization of oil matrix for enhancing the shelf life has shown that palm and olive oils have best shelf-life enhancing ability when stored with BHA (stabilizer) or in low temperature.

Analysis of the carotenoids of *Dunaliella* using various techniques has shown that β-carotene, lutein, zeaxanthin, phytoene and neoxanthin are major carotenoids along with α- carotene. MALDI analysis has shown that lutein is associated with C_{11}, neoxanthin with $C_{12:0}$ and $C_{20:3}$ and zeaxanthin with $C_{20:4}$ fatty acids. *Dunaliella* has shown the

presence of 20 ± 2.0 % protein in both the species studied. Apart from this, fat (7.5 ± 0.4%) and carbohydrate (25.0 ± 2.0 %) are the major constituents.

Dunaliella salina whole cell biomass is shown to be safe on feeding to rats and has potential antioxidant and hepatoprotective abilities. Carotenoids of *Dunaliella* have also shown the significant antioxidant and hepatoprotective ability in carbon tetrachloride challenged albino rats. The whole research work is represented schematically in Figure-75 and Representation of steps of process developed shown in Figure-76.

The results of the present investigation based on the objectives mentioned under introduction section, reveals that the marine micro algae *Dunaliella* can be cultivated in both marine and fresh water using suitable media composition with sodium chloride supplimentation. The algae need to be cultivated in two stages: vegetative stage and carotenogenesis stage under stress conditions. Results also revealed that the alga is rich in β- carotene constituting ~80% of total carotenoids present (3.0 ± 0.5 % w/w on dry weight basis). It is also found that algae contain other carotenoids and xanthophylls, which are significant for biological activity. Present work also elucidates idea of enhancing the carotenoids content under low light using biotic (fungal extracts) and abiotic (statin) elicitors. Safety studies revealed non-toxic effect of *Dunaliella salina* when fed at a dose upto 5.0 g kg^{-1} b.w. Studies on antioxidant and hepatoprotective activity against CCl_4 challenged animals reveals that both algal biomass as well as carotenoids of algae are significant protectants of vital organs like liver compared to the synthetic all *trans* β- carotene against oxidative damage.

Salient features of the thesis.

- Development of eco-friendly and less energy consuming technology for production of *Dunaliella* for carotenoids .
- Identification of various problems and finding solutions for industrial utility of the process.
- Detailed characterization and quantification of carotenoids present in micro algae *Dunaliella*.
- Evaluation of radical scavenging and hepatoprotective ability of both whole *Dunaliella salina* whole cells and carotenoids as applicable for utilizing algae for nutritional, prophylactic and therapeutic applications.

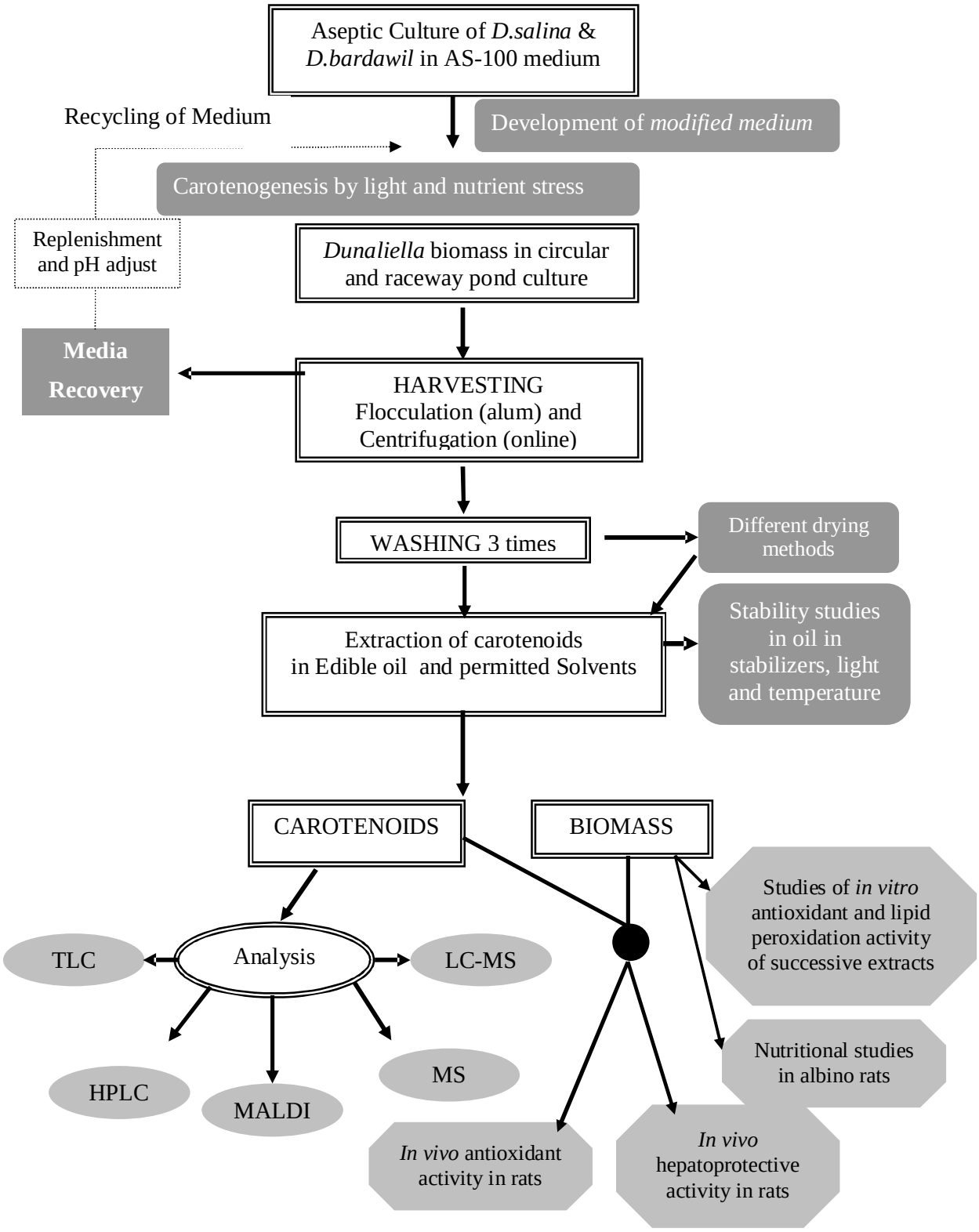


Figure 75. Schematic representation of the various facets of research work presented in thesis.

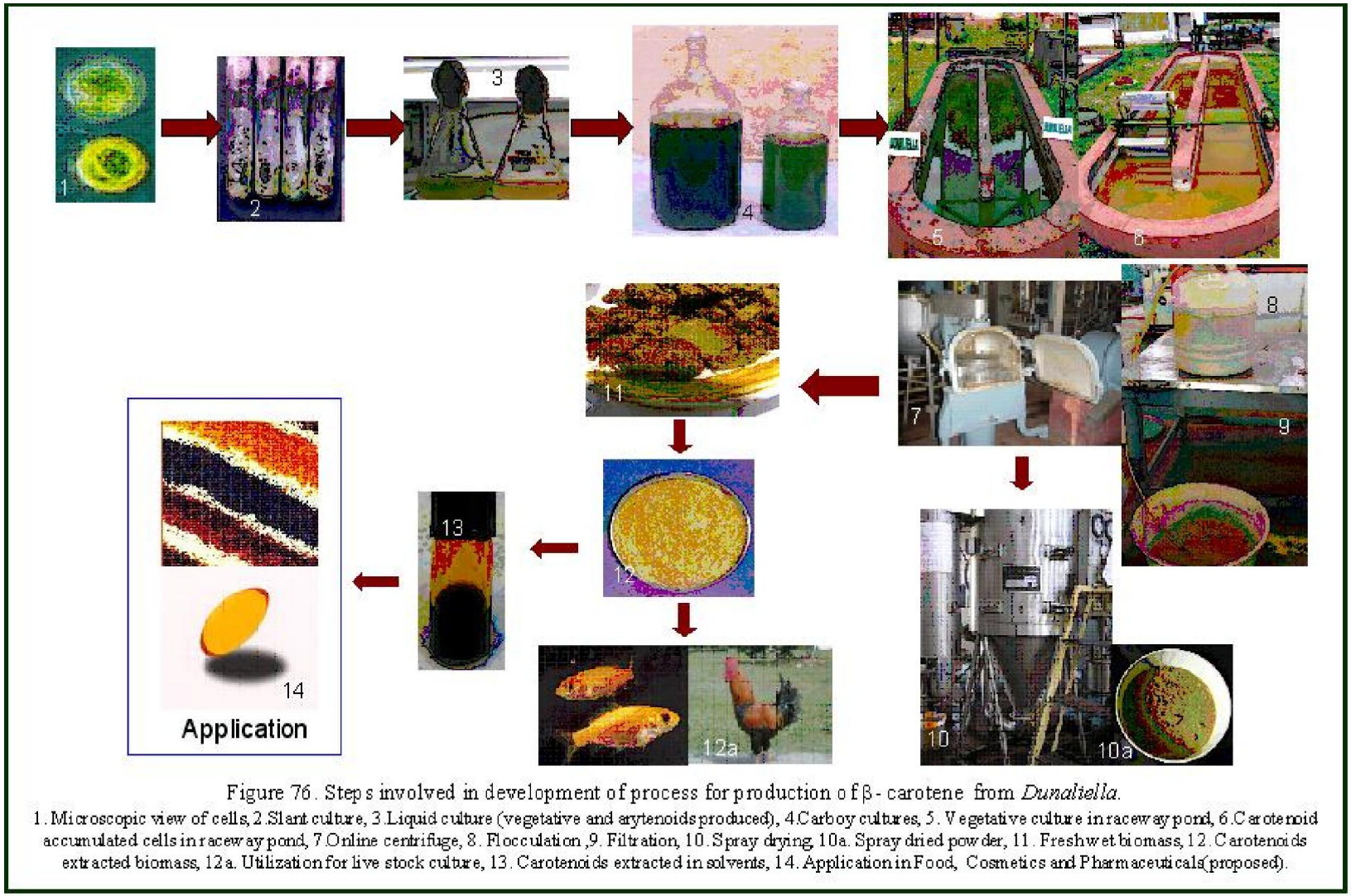


Figure 76. Steps involved in development of process for production of β- carotene from *Dunaliella*.

1. Microscopic view of cells, 2. Slant culture, 3. Liquid culture (vegetative and arytenoids produced), 4. Carboy cultures, 5. Vegetative culture in raceway pond, 6. Carotenoid accumulated cells in raceway pond, 7. Online centrifuge, 8. Flocculation, 9. Filtration, 10. Spray drying, 10a. Spray dried powder, 11. Fresh wet biomass, 12. Carotenoids extracted biomass, 12a. Utilization for live stock culture, 13. Carotenoids extracted in solvents, 14. Application in Food, Cosmetics and Pharmaceuticals (proposed).

9.0 Publications and presentations

Research articles

- « Chidambara Murthy, K.N., Rajesha, J., Vanitha, A., Sowmya, P.R., Mahadeva Swamy, M., Ravishankar, G.A., 2005. *In vivo* antioxidant activity of carotenoids from *Dunaliella salina* –a green microalga. Life Sciences 76(12) 1381-1390.
- « Chidambara Murthy, K.N., Rajesha, J., Vanitha, A., Mahadeva Swamy, M., Ravishankar, G.A., 2005. Hepatoprotective activity of carotenoids from micro algae *Spirulina platensis* & *Dunaliella salina.* Journal of Medicinal Food. 8(4), 523-527.
- « Chidambara Murthy, K.N., Rajesha, J., Vanitha, A., Sowmya, P.R., Mahadeva Swamy, M., Ravishankar, G.A., 2005. Hepatoprotective activity of *Dunaliella salina* - a marine micro alga. Hepetology Research. 33,313-319.
- « Chidambara Murthy, K.N., Namitha, K.K., Ravishankar, G.A., Nutraceutical potentials of commonly utilized micro and macro algal forms. Submitted after revision to Food composition and Analysis.
- « Chidambara Murthy, K.N., Namitha, K.K., Ravishankar, G.A., Antioxidant potentials of commonly utilized micro and macro algal forms. Submitted after revision to Food composition and Analysis.
- « Chidambara Murthy, K.N., Dayananda, C., Ravishankar, G.A., Evidence for squalene biosynthesis blockage to enhance carotenoid production by atarvostatin in micro algae *Dunaliella* species. Communicated to Journal of Applied Phycology.
- « Chidambara Murthy, K.N., Vanitha, A., Narayan, M.S., Ravishankar, G.A., Charecterization of fatty acids associated with carotenoids in *Dunaliella* by MALDI. Communicated to Phytochemical analysis.
- « Vanitha, K.N. Chidambara Murthy, R. Sarada, M.S.Narayan, P. R. Sowmya and G.A. Ravishankar "Fatty acid profile of Micro algae *Dunaliella salina*. Communicated Food composition and Analysis.

Review papers

- « Laurent, D., Galaup, P., Yaron, A., Arad, M.S., Blanc, P., Chidambara Murthy, K.N., Ravishankar, G.A. 2005. Microorganisms and algae as source of

pigments for food use: a scientific oddity or an industrial reality? Trends in Food Science and Technology, 16, 389–406.

« Chidambara Murthy, K.N., Ravishankar, G.A., Algae: Under exploited source of pharmaceuticals". Communicated to Marine Drugs.

Book chapters

« Chidambara Murthy, K.N., Ravishankar, G.A., Carotenoids from *Dunaliella* -A journey from test-tube to customer. In: *Recent advances on applied aspects of Indian marine algae with reference to global scenario*. CSMCRI, Gujarat, India.

« Chidambara Murthy, K.N., Ravishankar, G.A., Micro algae – a potential source of bioactive compounds. In: proceedings of National symposium on Microalgal biotechnology, Bharthidasan University, Tiruchirappali, India.

Patents

« Chidambara Murthy, K.N., Mahadeva Swamy, M., Sarada, R., Ravishankar, G.A., 2004. Seawater based medium for the production of *Spirulina*. NF-281/04 (Indian patent).

« Chidambara Murthy, K.N., Mahadeva Swamy, M., Ravishankar, G.A., 2004. A modified medium for producing the *Dunaliella* species for production of β- carotene. 585/DEL/2004 (Indian patent).

Presentations in symposium and conferences

Posters

« Chidambara Murthy, K.N., Vanitha, A., Mahadeva Swamy, M., Sarada, R., Ravishankar, G.A., Antioxidant activity of *Dunaliella salina* extracts in Indian Convention of Food Science and Technologists -2002 at CFTRI, Mysore, India. December 2002.

« Chidambara Murthy, K.N., Sowmya, P.R., Vanitha, A., Mahadeva Swamy, M., Sarada, R., Ravishankar, G.A., Flocculation- a simple technique to harvest microalgae *Dunaliella salina*. National symposium on Microalgal biotechnology, Conducted by Bharthidasan University, Tiruchirapally, India. February 2004.

« Chidambara Murthy, K.N., Sowmya, P.R., Vanitha, A., Mahadeva Swamy, M., Ravishankar, G.A., Nutritional properties of microalgae *Dunaliella*

salina. National symposium on protein (Solae), Mysore, India. March 2004.

« Chidambara Murthy, K.N., Ravishankar, G.A., Biologically efficient carotenoids from micro algae *Dunaliella* presented in 14th International symposium on carotenoids. Edinburgh UK, July 2005.

« Vanitha, A, Chidambara Murthy, K.N., Mahadeva Swamy, M., Sarada, R., Ravishankar, G.A., Isolation Characterization and purification of carotenoids from *Dunaliella salina*. International Food Conventions-2003, Mysore, India. December 2003.

Oral presentations

« Micro algae- a potential source of bioactive compounds In: National symposium on Microalgal Biotechnology, Conducted by Bharthidasan University, Tiruchirapally. February 2004.

« Production of pharmacologically efficient carotenoids from micro algae at Phytopharma- 2005, held at Aurangabad, India during 4-6th February 2005.

« Micro algae a potential source of carotenoids at GRD college of science, in Symposium on Nutraceuticals and Biotechnology, Coimbatore, India, September 2005.

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