Biotechnological production of microalgal carotenoids with reference to astaxanthin and evaluation of its biological activity

A thesis

submitted to the Department of Biotechnology of

University of Mysore

in fulfillment of the requirement for the degree of

Doctor of Philosophy

by

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Declaration

I hereby declare that this thesis entitled **"Biotechnological production of microalgal carotenoids with reference to astaxanthin and evaluation of its biological activity"** submitted to the University of Mysore, Mysore, for the award of the degree of Doctor of Philosophy in Biotechnology, is the result of research work carried out by me in the Plant Cell Biotechnology Department, Central Food Technological Research Institute, Mysore, India, under the guidance of Dr. R. Sarada, during the period July 2004 - September 2007.

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

Date: 08.10.2007
Place: Mysore (Sandesh Kamath B.)

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Certificate

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Abstract

Microalgal biotechnology has gained importance due to its potential to produce bioactive compounds. Green alga *Haematococcus pluvialis*, being a potent source for ketocarotenoid astaxanthin, has been an attractive species for commercial exploitation. The present work focused on production of astaxanthin from *H. pluvialis* and evaluation of its biological activity. Modified medium was developed for autotrophic cultivation of *H. pluvialis* in open and closed system. *Haematococcus* was grown in different prototype bioreactors under optimized culture condition. The high biomass yield in closed tubular bioreactors suggested that maintenance of the constant carbon dioxide level in the airspace is essential for effective gas-liquid mass transfer. Maximum biomass yield of 0.89 g/L with a growth rate of 0.13 d^{-1} and astaxanthin content of 1.8% (w/w) was obtained in closed tubular bioreactor. *H. pluvialis* culture of 60 L prototype raceway tank, after 9 to 12 days growth period and exposed to sunlight and salinity stress for 5 days, produced a biomass yield of 0.5 g/L and astaxanthin content of 1.4 % (w/w). Digital image processing based method was developed for estimation of carotenoid content in *H. pluvialis* cells, a good correlation of R^2=0.967 was observed between carotenoid content as estimated by analytical method.

H. pluvialis mutants were isolated using chemical and physical mutagen treatment and were characterized for growth, astaxanthin production, photosynthetic property and carotenoid gene expression. Mutants obtained with 1-methyl 3-nitro 1-nitrosoguanidine (NTG) have shown significant enhancement in total carotenoid and astaxanthin content (23-59% w/w) in comparison with parent culture. The mutant obtained by UV irradiation showed highest lycopene cyclase activity (458 nmole of β-carotene formed/mg of protein/hr) followed by NTG mutant (315 nmole of β-carotene formed/mg of protein/hr) when compared to that of parent strain (105 nmole of β-carotene formed/mg of protein/hr). Expression analysis of carotenoid biosynthetic genes in the mutants exhibited increase in transcript levels compared to wild type.

Astaxanthin esters and free astaxanthin from *H. pluvialis* were evaluated for their biological activity. Results indicated that free astaxanthin from *H. pluvialis* has 4.4 fold higher free radical scavenging activity (IC_{50} value of 8.1μg/ml) when compared to that of astaxanthin esters. Free astaxanthin also showed maximum reducing power of 59.6U/g equivalents to that of tannic acid (48.5 U/g). The above data showing better antioxidant activity of free astaxanthin is substantiated by comparing with the activity of standard astaxanthin. Free astaxanthin exhibited 5 fold higher soybean lipoxygenase inhibitory activity (IC_{50} ~3.4 μg/ml) when compared to total carotenoid fraction. Further, astaxanthin esters effectively inhibited the gastric proton potassium ATPase enzyme that is involved in the acid secretion during gastric conditions. Free astaxanthin was potent inhibitor of gastric H^+ K^+ ATPase with IC_{50} -6.2μg/ml than astaxanthin esters (IC_{50} – 18.2 μg/ml). Results of *in vivo* studies revealed that astaxanthin esters at 500μg/kg b.w., protected ulcerous condition in rats by ~67% equivalent to that of known antiulcer drug- omeprazole which offered ~72% protection at 20 mg/kg b.w. Attractive skin colouration in ornamental fish was achieved by feeding astaxanthin rich *H. pluvialis* biomass. Poultry birds fed with astaxanthin rich *H. pluvialis* showed an increase in yolk colour intensity as indicated by Roche Yolk colour fan (Yolk colour score-11.00) and improved egg quality as per FAO standards (Haugh unit score -76 and USDA grade AA). A maximum of 44μg of carotenoid content per gram of yolk was observed in experimental birds, which is 2-3 fold higher compared to control (15μg/g of egg yolk). The findings of this study have substantiated biological activity of astaxanthin such as antioxidant, pigmentation efficiency and established its antiulcer properties. It has also provided insight on autotrophic cultivation of *Haematococcus pluvialis* for production of astaxanthin.

Dedicated to Bhagavathswaroopi

Ajja, Anama, Annu & amma

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Sandesh Kamath B.

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List of Abbreviations

μ	Growth rate
°C	Degree Centigrade
μg	Microgram
μM	Micromolar
Abs	Absorbance
AMD	Age-related macular degeneration
ANN	Artificial neural network
b.w.	body weight
BAP	6-benzyl aminopurine
BBM	Bold's basal medium
BHA	Butylated hydroxy Anisole
BHT	Butylated hydroxy toluene
BKT	β-carotene ketolase
CCD	Charged couple device
Chl	Chlorophyll
CHY	β-carotene hydroxylase
d	day
DAP	Diammonium Phosphate
DCPIP	2, 6-dichlorophenol indophenol
DIP	Digital image processing
DPPH	1,1-Diphenyl 2-picryl hydrazyl
EMS	Ethyl Methane Sulphonate
Fv	Variable fluorescence
GA_3	Gibberellic acid
GOGAT	2-oxoglutarate amido transferase
GPx	Glutathione peroxidase
GS	Glutamine synthetase
H^+K^+ATPase	Proton-Potassium ATPase
HPLC	High performance liquid chromatography
Klux	Kilolux
LCY	Lycopene cyclase
LDPE	Low-density polyethylene
M	Molar
MDA	Malondialdehyde
min	Minutes
MSX	Methionine sulfoximine
NBT	Nitroblue tetrazolium

NTG	1-methyl 3-nitro 1-nitrosoguanidine
PDS	Phytoene desaturase
PSY	Phytoene synthase
R^2	R-squared value (coefficient of determination)
Rf	Retention factor/resolution front
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TLC	Thin-layer chromatography
UV	Ultra-violet
v/v	Volume per volume
w/w	Weight per weight

Chapter 1
Introduction and Reveiw of Literature

Introduction

Colours are deliberately added to food to enhance the appeal. However, concerns regarding the adverse effects of synthetic food colours have led the researchers to explore newer sources of natural colours. Naturally occurring colourants not only impart attractive colouration to food but also have nutraceutical benefits. One group of natural colourants which has wide occurrence in nature is carotenoids. The role of carotenoids in human and animal health is widely recognized. Among the sources of carotenoid, microalgal forms are being explored as rich source of carotenoids. In last few decades, microalgal biotechnology has made significant progress for the production of biomass, mainly as a source of protein. Some species of microalgae have been commercially produced for carotenoids like β-carotene, astaxanthin, lutein etc.

The scientific knowledge of the beneficial role of carotenoids for prevention of specific diseases is rapidly gathering. Ketocarotenoid astaxanthin has gained importance in pharmaceutical, nutraceutical and pigmentation applications. Currently synthetic astaxanthin is the chief ingredient in the aquaculture feed which imparts the attractive red colour to salmon. *Haematococcus pluvialis* – a green alga is one of the natural sources known for its ability to accumulate high amount of astaxanthin (2-3% w/w on dry weight basis). With this background, *H. pluvialis* was selected as a suitable source for production of astaxanthin for the present investigation. Information on technological aspects of astaxanthin production in Indian conditions is scanty. The potential of *H. pluvialis* to produce astaxanthin as nutraceutical and as food colourant has not been fully exploited. Due to growing demand of natural astaxanthin, cultivation of *H. pluvialis* in economically viable system was envisaged. In addition, studies on the biological activity of *H. pluvialis* derived astaxanthin and constituents were carried out to elucidate its role in human health.

1.0. Carotenoids

Carotenoids are recognized worldwide for their unique biological characteristics. They are a group of molecules which can be found in most life forms and are responsible for diverse functions, ranging from their original evolutionary role as photosynthetic or light-quenching pigments to antioxidants, precursors of vitamin A, or pigments involved in the visual attraction of animals such as flower pollinators (Johnson and Schroeder, 1995). Carotenoids have been studied for many years because of their diverse roles in biological system. Britton (1995) has stated that carotenoids are not just "another group of natural pigments", they are substances with special and remarkable properties that form the basis of their many varied functions and actions in living organisms. The unparalleled health benefits derived from them has led the mankind in search of newer and potential sources of carotenoids.

The name 'carotene' was suggested by Wachenroder in 1831 for the hydrocarbon pigment he had crystallized from carrot roots. Berzelius named the yellow pigments from autumn leaves as 'xanthophylls'. Many pigments of this class were separated by Tswett, who called the whole group 'carotenoids' (Olson and Krinsky, 1995)

Till date more than 600 carotenoids have been identified, but only ~60 of them are detected in the human diet and ~20 of them in human blood and tissues. β-Carotene, ∝-carotene, lycopene, lutein and β-cryptoxanthin are the five most prominent carotenoids found in the human body (During and Harrison, 2004). In the human diet, plant food sources are the major contributors of carotenoids: carrots, squash, and dark-green leafy vegetables for β-carotene, carrots for ∝-carotene, tomatoes and watermelon for lycopene, kale, peas, spinach, and broccoli for lutein, and sweet red peppers, oranges and papaya for β-cryptoxanthin.

1.1. Chemistry of carotenoids

The common chemical feature of the carotenoid is a linear polyisoprenoid structure, a long conjugated chain of double bond and a near bilateral symmetry around the central double bond (Britton, 1995). Different carotenoids are derived essentially by modifications in the base structure by cyclization (i.e. formation of β- or ε-ionone rings) of the end groups and by introduction of oxygen groups giving them their characteristic colors and antioxidant properties (Rao and Rao, 2007).

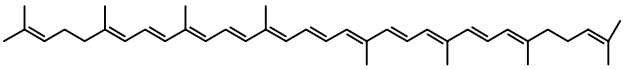
Carotenoids are synthesized *de novo* in bacteria, algae, fungi and higher plants (Goodwin, 1980). Majority are C_{40}-carotenoids and few bacterial carotenoids with 30, 45, or 50 carbon atoms. In bacterial carotenoids, hydroxy groups at the ionone ring may be glycosylated or carry a glycoside fatty acid ester moiety. Furthermore, carotenoids with aromatic rings or acyclic structures with different polyene chains and typically 1-methoxy groups can be found. Typical fungal carotenoids possess 4-keto groups, may be monocyclic, or possess 13 conjugated double bonds. 3-Hydroxy ∝- and β- as well as 5,6-epoxy β-carotene derivatives are abundant in chloroplast of some algal groups and green plants. Structures of major carotenoids are shown in the Figure 1.1.

Some reports also mention that the carotenoids, which possess hydroxy and/or carbonyl substitution on one or both of the molecule's end-groups, as xanthophylls, e.g. astaxanthin, canthaxanthin, lutein, and zeaxanthin. The polyene chain and the other structural features influence the chemical properties (e.g., redox properties) of the carotenoids as well as their location and orientation within lipid bilayers in biological environments (El-Agamey et al, 2004).

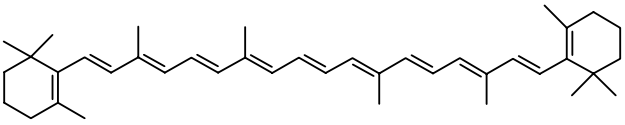
Carotenoids are known to exist in different geometric forms; *cis* and *trans*-isomers. These isomers may be interconverted by light, thermal energy or chemical reaction; for example cooking of vegetable promotes isomerization of carotenoids from the *trans* to the *cis* form. β-carotene, with nine double bonds in its polyene chain that are free to assume *cis/trans* configurations, can theoretically form 272 isomers whereas its asymmetric isomer, ∝-carotene, can form 512. According to Olson and Krinsky (1995), synthetic β-carotene is almost entirely in the *trans*-isomeric form. The total possible number of compounds in the class, including all possible isomers, easily exceeds 200,000. Isomer specific biological functions clearly exist for carotenoids (Rock, 1997).

1.2. Carotenoids as natural food colours

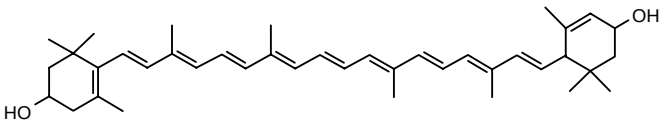
The consumer appeal to the food or food product depends on its colour. On a global scale, the size of the food colour market is estimated to be $940m (www.nutraingredients-usa.com) of which 27% ($ 250m) is market share of natural colours (Figure 1.2).



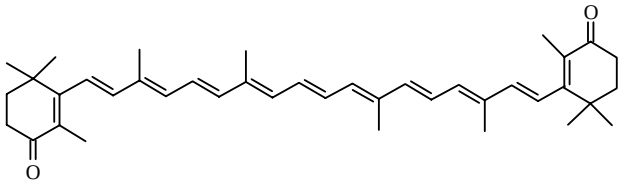
Lycopene



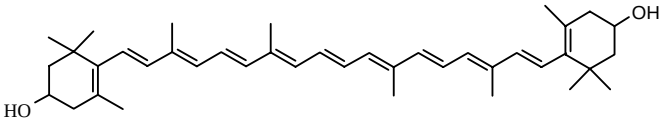
β-carotene



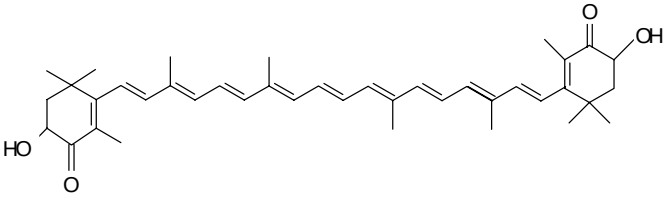
Lutein



Canthaxanthin



Zeaxanthin



Astaxanthin

Figure 1.1. Structures of some major carotenoids.

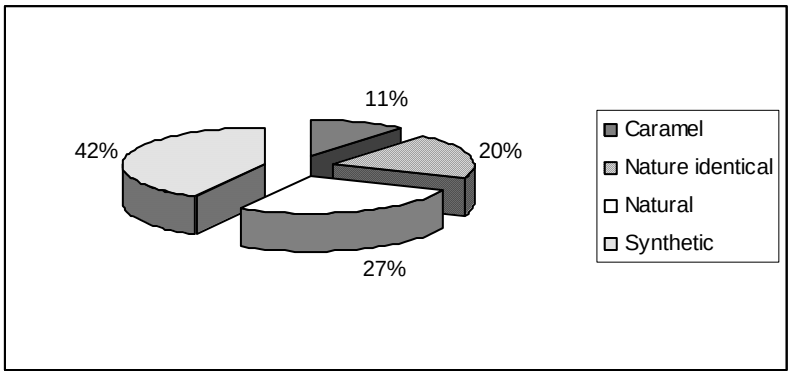


Figure 1.2. Percentage market share of food colours (Downham and Collins, 2000)

Genotoxicity and carcinogenicity of synthetic food colours, mainly azo dyes, has been documented by Combes and Haveland-Smith (1982). A number of azo compounds are mutagenic in assays if chemical reduction or microsomal activation, or both, are induced (Chung and Cerniglia, 1992). In animal model, the DNA damage induced by azo dyes has been reported by Tsuda et al (2001). Because of the adverse effect of synthetic food colours, the current research is being focused on the natural food colours.

Carotenoids are one of the main groups of natural colour substances, the rest being anthocyanins, porphyrins and chlorophylls. Carotenoids are responsible for many of the brilliant red, orange and yellow colour of edible fruits and vegetables. Carrot extract and red palm oil – rich in carotenoids have been widely used as colouring agents mainly to colour fats and margarine. Water-soluble forms of carotenoids are suitable for colouring of sugar confectioneries like candies, toppings, icings, fruit gums, fruit drops etc. An aqueous dispersion of carotenoids in large amounts of dextrin or sugars can be applied to colour breakfast cereals and dried infant food preparations (Pattnaik et al, 1997).

The possible role of carotenoids and their metabolites in disease prevention is far from fully understood, because the bioavailabilities of carotenoids are complicated by multiple factors that affect their absorption, breakdown, transport, and storage (Yeum and Russel, 2002).

1.3. Carotenoids in health and nutrition

Deeply pigmented vegetables and fruits are the major dietary sources of carotenoid. Yellow –orange vegetables and fruits provide most of the β-carotene and ∝-carotene, orange fruits provide ∝- cryptoxanthin, dark-green vegetables provide lutein and tomato and tomato products lycopene (Rao and Rao, 2007). Smaller amounts can be obtained through egg yolk, ocean fish and carotenoids added as colourants to food during processing (Rock, 1997).

The current interest in carotenoid is due to the proposed role of dietary carotenoid in man with respect to disease prevention. The potential functions of β-carotene and other carotenoid on human health have been reviewed by Mayne (1996). Several reactive oxygen species induce degenerative diseases such as cancer, diabetes, cardiovascular diseases etc (Ariga, 2004). The consumption of β-carotene rich foods have been associated consistently with a decreased risk of cardiovascular disease (Kardinaal et al, 1993; Gaziano, 1994). The ability of carotenoid to quench singlet molecular oxygen is well known (Conn et al, 1991; Edge et al, 1997). Dietary carotenoids react with a wide range of radicals such as $CCl_3O_2^{\bullet}$, $RSO_2^{\bullet}$, $NO_2^{\bullet}$, and various arylperoxyl radicals via electron transfer producing the radical cation of the carotenoid (Mortensen et al, 2001).

The epidemiologic literature on intake of lycopene and its relationship with occurrence of cancer has been reviewed by Giovannucci (1999). Cancer chemopreventive effect of lycopene has been reported in mouse lung (Kim et al, 1997) rat urinary bladder (Okajima et al, 1998) and rat colon cancer models (Narisawa et al, 1998). Prevention of carcinogenesis has been reported in rat aberrant colon crypt formation (Narisawa et al, 1996) and the rat hepatic preneoplasia model (Astorg et al, 1997). Organ specific chemoprotective effects of lycopene exerting protective effect on lung and prostate has been established in animal models (Cohen, 2002).

Carotenoids modulate the basic mechanisms of cell proliferation, growth factor signaling, gap junctional intercellular communication, and produce changes in the expression of many proteins participating in the processes. The changes in the expression of multiple proteins suggest that the initial effect of carotenoids involves modulation of transcription, resulting from direct interaction of the carotenoid molecules or their

derivatives with ligand-activated nuclear receptors, or from indirect modification of transcriptional activity of non-liganded transcription factors (Sharoni et al, 2004).

Antioxidant potentials of canthaxanthin in *in vitro* models and in liposomes against oxidation by peroxyl radicals have been reported (Packer, 1993; Woodall et al, 1997). Its antioxidant potency is also shown in membrane model system by Palloza and Krinsky (1992). Inhibition of aflatoxin B1-induced liver preneoplastic foci and DNA damage in rats by canthaxanthin has been demonstrated by Gradelet et al (1998).

Lutein and zeaxanthin consumed in the diet are deposited upto 5 fold higher content in the macular region of the retina as compared to the peripheral retina (Handelman et al, 1988). Zeaxanthin is preferentially accumulated in the foveal region, whereas lutein is abundant in the perifoveal region. These carotenoids, because of their antioxidant properties, provide protection against the adverse effects of photochemical reactions (Snodderly, 1995). Growing number of evidences indicate that oxidative damage plays a role in aetiopathogenesis of age-related macular degeneration (AMD). The possibility that the absorption characteristics and antioxidant properties of macular pigments (lutein and zeaxanthin) confer protection against AMD has been postulated (Landrum et al, 1997). It has been hypothesized that dietary supplementation with lutein and/ or zeaxanthin might protect the retina and/or delay the progression of AMD (Moeller et al, 2000).

In several epidemiologic studies, the role of carotenoids in the prevention of breast cancer recurrence has been suggested by observation that higher levels of carotenoid intakes at diagnosis are associated with greater likelihood of survival (Rohan et al, 1993). Carotenoids appear to modulate redox-sensitive transcription factors such as NF-κB that are involved in the upregulation of IL-6 and other proinflammatory cytokines. Thus carotenoids offer protection against sarcopenia or loss of muscle strength in older adults (Semba et al, 2007).

The study by Zheng et al (1993) strongly suggest that β-carotene from fruits and vegetables is atleast one of the agents responsible for inhibition of mouth and throat cancer. Block et al (1992) have reported that dietary intake of fruits and vegetables is inversely associated with esophageal, gastric and colorectal cancer risk. Carotenoids have been used successfully to treat certain photosensitive diseases. Mathews-Roth (1993) has

demonstrated that the majority of patients with the genetic disease erythropoietic protoporphyria benefit from high-dose supplementation of β-carotene and /or canthaxanthin. All these reports strongly support the beneficial health effects derived from carotenoids and thus exploration of newer and unconventional sources of carotenoids is necessitated.

1.4. Microalgae

The biodiversity of microalgae is enormous and represents an almost untapped resource. It has been estimated that between 200,000 and several million species exist (Norton et al, 1996). Despite being potential producers of a wide spectrum of natural substances of vital human need, microalgae have so far been a rather under explored source in the development of biotechnology (Goyal and Goyal, 1998). In recent years, microalgal biotechnology has gained attention due to advancements in production technology. The microalgal biomass market has a size of about 5,000 t/year of dry matter and generates a turnover of ca. U.S. $ 1.25×10^9/year (Pulz and Gross, 2004).

1.5. Microalgae as a source of food and nutraceutical

Many species of microalgae such as *Spirulina, Chlorella, Scenedesmus* have been used as food for years and is still being used in several countries like China, Fiji, Ecnader, Monogolea (Prasad and Gupta, 2007). Various microalgae have been considered as unconventional source of protein and the microalgae are also source of essential amino acids. Carbohydrates in microalgae are in the form of starch, glucose or other polysaccharides and have high digestibility (Becker, 2004). Some microalgae are rich source of ω3 and ω6 families of fatty acids. (Tonon et al, 2002). Composition of the microalgae used as food is shown in Table 1.1.

The blue-green microalga *Spirulina* has had a long history in human nutrition. *S. platensis* was consumed by the native population of the sub-saharan region of Kanem, northeast of Lake Chad. In 1964, health food was produced with microalgae cultivated in artificial media in Japan. In 1975, *Spirulina, Chlorella* tablets made from dry powder were sold in the markets, tablets were marketed (Liang et al, 2004). Spray-dried biomass is generally utilized for health foods, food additives and feed supplements. (Venkataraman et al, 1995; Yamaguchi, 1997).

Table 1.1. General composition of microalgae being used as food source (% dry weight)

Microalgae	**Protein**	**Carbohydrate**	**Lipid**
Anabaena cylindrica	43-56	25-30	4-7
Chlamydomonas reinhardtii	48	17	21
Chlorella vulgaris	51-58	12-17	14-22
Dunaliella salina	57	32	6
Porphyridium cruentum	28-39	40-57	9-14
Scenedesmus obliquus	50-56	10-17	12-14
Spirulina sp.	60-71	13-16	6-7
Synechococcus sp.	63	15	11

(Modified from Spolaore et al, 2006)

Spirulina is a rich natural source of protein, carotenoids, ω-3 and ω-6 polyunsaturated fatty acids, provitamins and other nutrients such as vitamin A, vitamin E, and selenium (Wu et al, 2005; Venkataraman et al, 1995). *Spirulina* has high protein efficiency ratio (PER) than those of cereals, vegetable and soya protein (Venkataraman, 1993). *Spirulina, Chlorella* are also utilized in the processing of common foods such as noodles, bread, green tea, health drink, candy (Liang et al, 2004).

Chlorella health foods in the form of tablets, granules and drinks entered the market in 1964 and met with increased sales during 1970. More than 70 companies have their *Chlorella* health foods registered at Japan Health Food Association and their annual sales are estimated to be above 40 billion yen (Yamaguchi, 1997). Beneficial health effects of *Chlorella*, preventive action against atherosclerosis, hypercholesterolemia, hypoglycemia in animal models has been reported (Jong-Yuh and Mei-Fen, 2005). β-carotene rich dried biomass of *Dunaliella* and its capsules and tablets are placed on the market as a health food (Metting, 1996). Microalgal oils have been commercially produced for incorporation into infant milk formulations, as dietary supplements and as food additives (Kyle and Gladue, 1996).

1.6. Bioactive compounds from microalgae

Microalgae have already been used as cheap and effective biocatalysts to obtain high added-value compounds including fine chemicals, vitamins, carotenoids, or polysaccharides (Holland, 1999; Harrigan and Goetz, 2002; Pulz and Gross, 2004). Microalgae such as *Phaeodactylum tricornutum, Isochrysis galbana, Crypthecodinium* sps., *Nannochloropsis* sps. are rich sources of polyunsaturated fatty acids (PUFA) - mainly Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA), (Apt and Behrens, 1999). DHA is important for proper brain and eye development in infants and has been shown to support cardiovascular health in adults (Kroes et al, 2003). The wide range of bioactive compounds produced by microalgae and their biological activity has been summarized in Table 1.2.

Table 1.2. Bioactive compounds from microalgae

Bioactive compound	**Organism**	**Activity**	**Reference**
Acetylated sulfoglycolipids	*Oscillatoria raoi*	antiviral	Reshef et al (1997)
Anabaenopeptin B	*Oscillatoria agardhii*	Protease inhibitor	Murakami et al (1997c)
Glycolipids	*Oscillatoria limnetica*	Antiviral (HIV-1)	Reshef et al (1997)
Glycolipids	*Oscillatoria trichoides*	Antiviral (HIV-1)	Loya et al (1998)
Oscillapeptin G	*Oscillatoria agardhii*	tryrosinase inhibitor	Sano and Kaya (1996)
Aeruginosin 102 A Aeruginosin 102 B	*Microcystis viridis*	thrombin inhibitor	Matsuda et al (1996)
Aqueous extract	*Microcystis aeruginosa*	Antiviral (influenza A)	Nowotny et al (1997)
Kawaguchipeptin B	*Microcystis aeruginosa*	bactericide	Ishida et al (1997c)
Lipid	*Microcystis aeruginosa*	Algicide	Ikawa et al (1996)
Microginin 299-A Microginin 299-B	*Microcystis aeruginosa*	leucin aminopeptidase inhibitor	Ishida et al (1997b)
Micropeptin 103	*Microcystis viridis*	chymotrypsin inhibitor	Murakami et al (1997a)
Micropeptin 478-A Micropeptin 478-B	*Microcystis aeruginosa*	plasmin inhibitor	Ishida et al (1997a)
Banyaside A and B	*Nostoc sps.*	trypsin and thrombin inhibitor.	Pluotno and Carmeli (2005)
Borophycin	*Nostoc linckia* *Nostoc spongiaeforme*	cytotoxic	Singh et al (2005)
Cyanovirin N	*Nostoc ellipsosporum*	Antiviral (HIV-1)	Boyd et al (1997)
Cryptophycin	*Nostoc sp.* ATCC 53789	Fungicide Cytotoxic	Singh et al (2005)

Continued..

Bioactive compound	Oraganism	Activity	Reference
Nostopeptin A Nostopeptin B	*Nostoc minutum*	elastase inhibitor	Okino et al (1997)
Microviridin	*Nostoc minutum*	elastase inhibitor	Murakami et al (1997b)
Tenuecyclamides A-D	*Nostoc spongiaeforme*	growth inhibitor	Banker and Carmeli, (1998)
Hydrophilic extract Lipophilic extract	*Nostoc*	Antibacterial cytotoxic	Piccardi (2000)
Nostocine A	*Nostoc spongiaeforme*	Cytotoxic	Hirata et al (2003)
Calcium spirulan	*Spirulina platensis*	antiviral	Hayashi et al (1996)
Phycocyanin	*Spirulina platensis*	Antiinflammatory	Romay (1999)
		Antioxidant	Bhat and Madyastha (2000)
		hepatoprotective	Vadiraja et al (1998)
Aqueous extract	*Spirulina*	Antioxidant antiproliferative	Wu et al (2005)
Circinamide	*Anabaena circinalis*	papain inhibitor	Shin et al (1997)
Dehydroradiosumin	*Anabaena cylindrica*	trypsin inhibitor	Kodani et al (1998)
Dendroamides	*Stigonema dendroideum*	reversing multidrug resistance	Ogino et al (1996)
Fischerellin A	*Fischerella muscicola*	fungicide	Hagmann and Jüttner, (1996)
Lyngbyastatin 1	*Lyngbya majuscula*	cytotoxic	Harrigan et al (1998b)
Nodulapeptin A Nodulapeptin B	*Nodularia spumigena*	protracted toxic	Fujii et al (1997)
Phytoalexin	*Scytonema ocellatum*	fungicide	Patterson and Bolis (1997)
Scyptolin	*Scytonema hofmanni*	Elastase inhibitor	Antonopoulou et al (2005)
Sulfolipids	*Phormidium tenue*	antiviral (HIV-1)	Falch et al (1995)
Symplostatin 1	*Symploca hydnoides*	cytostatic	Harrigan et al (1998a)
Polysaccharide	*Porphyridium*	antiviral	Huheihel et al (2002)
Polyunsaturated fatty acids (PUFA)	*Odontella aurita* *Isochrysis galbana*	DHA-Postnatal brain Development	Makrides et al (1995)
Polyunsaturated fatty acids (PUFA)	*Phaedactylum tricornutum* *Crypthecodinium*	*EPA*-Prevention heart disease, hypertriglyceridemia, blood platelet aggregation	Lebeau and Robert (2003) Singh et al (2005)
Curacin A	*Lyngbya majuscule*	Inhibitor of cell growth and mitosis	Burja et al (2002)
Polysaccharides	*Chlorella pyrenoidosa*	Immunomodulatory	Yang et al (2006)
Aqueous extract	*Chlorella vulgaris*	Antiviral, antibacterial	Hasegawa et al (1995)

(Modified from Skulberg 2000)

The US Company Mertek has 240 t annual production of DHA oil from microalga *Crypthecodinium cohnii.* Similarly OmegaTech, also owned by Martek produce DHA oil (Spolaore et al, 2006). The list of companies producing microalgae as a source of nutraceuticals is provided in Table 1.3.

Table 1.3. Companies producing microalgae as a source of nutraceuticals

Country	Company	Alga	Product	Activity
USA	Martek/Omegatec	*Crypthecodinium*	Docosahexaenoic Acid (DHA)	Brain development
Germany	Nutrinova/Celanese	*Ulkenia*	Docosahexaenoic Acid (DHA)	Treats brain, heart, mental disorder
USA	Cyanotec Corporation, USA	*Haematococcus*	Astaxanthin	Treating carpal tunnel syndrome
USA	Mera Pharmaceuticals Inc. USA	*Haematococcus*	Astaxanthin	Anti-inflammatory, treats muscle soreness
Canada	OceanNutrition	*Chlorella*	Carbohydrate extract	Immune system, anti-flu
France	InnovalG	*Odontella*	Eicosapentaenoic Acid (EPA)	Anti-inflammatory
Austria	Panmol/Madaus	*Spirulina*	Vitamin B_{12}	Helps immune system
UK	BSV	*Rhodophyta (mix)*	Biomass	Treats irritable bowel candidiasis

(Adapted from Pulz and Gross, 2004)

1.7. Carotenoids from microalgae

Microalgae produce wide spectrum of carotenoids. These carotenoids are associated with light incidence in addition to chlorophyll. Carotenoids protect the microalgae against solar radiation and related effects. The wide range of carotenoid present in prokaryotic as well as eukaryotic microalgae has been listed in Table 1.4.

β-carotene from the alga *Dunaliella salina* is the first high value algal product commercialized which is now being produced in Australia, USA and Israel (Spolaore et al, 2006). Most of the chlorophycean members contains multitude of

Table 1.4. Occurrence of carotenoids in microalgal sources

Algal class	Common name	Carotenoid pigment
Cyanophyceae	Blue green algae	1,3,12,13,16,19,22,55-62
Chlorophyceae	Green algae	1,7,10-14,16,17,18,19,20-22,24,28,29, 36,43,47,48,65-67
Charophyceae	Stoneworts	1,65
Euglenophyceae	Euglenoids	1,13,14,16,18,19,23,29,31,35,36,48,70,71
Phaeophyceae	Brown algae	1,13,14,16,18,19,23,29,31,35,36,48,70,71
Chrysophyceae	Golden and yellow green algae including diatoms	1,4,9,14,15,20,28,29,31,33,35,36
Pyrrophyceae	Dinoflagellates	1,2,18,26,32,35,37-39,44,45,51,53,54,64
Rhodophyceae	Red algae	1,10,12,21,26,28-30,40, 42,46,65,68,69
Cryptophyceae	Yellow algae	1,2,4,21,42,46,65
Raphidophyceae	Yellow algae	1,4,12-14,20,26,2829,36,51,63
Xanthophyceae		1,12,19,20,28,29,33
Eustigmatophyceae		1,4,12,20,26-29,35,36
Prasinophyceae and Loxophyceae	Green flagellates	1,7,10-14,16,18-20,22,24,28,29,43,47, 48, 65-67

(Adapted from Shahidi et al, 1998)

Note:1.β,β-carotene, 2. β,∈-carotene, 3. β,ψ-carotene, 4. ∈,∈- carotene, 5. β,β- carotene -2-ol, 6. β,β-carotene-2,2-diol, 7. β,∈-carotene -2-ol, 8. β- carotene epoxide, 9.β-carotene diapoxide, 10. Lutein, 11. Lutein epoxide, 12. Zeaxanthin, 13. Cryptoxanthin, 14. Cryptoxanthin epoxide, 15. Cryptoxanthin diapoxide, 16. Echinenone, 17. Lycopene, 18. Astaxanthin, 19. Canthaxanthin, 20. Antheraxanthin, 21. Alloxanthin, 22. 3-hydroxycanthaxanthin, 23. 3-hydroxy-echinenone, 24. 4-hydroxyechinenone, 25. 3'-hydroxyechinenone, 26. Fucoxanthin, 27. Fucoxanthinol, 28.Violaxanthin, 29. Neoxanthin, 30.Taraxanthin, 31. Heteroxanthin, 32. Dinoxanthin, 33. Vaucheriaxanthin, 34. Vaucheriaxanthin ester. 35. Diatoxanthin, 36. Diadinoxanthin, 37. Diadinoxanthin epoxide, 38. Peridinin, 39. Peridinol, 40. Auroxanthin, 41. Aurochrome, 42. Crocoxanthin, 43. Loroxanthin, 44. Phytoene, 45. Phytofluene, 46. Monadoxanthin, 47. Siphonaxanthin, 48. Siphonein, 49. Micronone, 50. Deepoxyneoxanthin, 51. 19'-hexanoyloxyfucoxanthin, 52.19-hexanoyloxyparacentrone-3-acetate, 53. Pyrrhoxanthin, 54. Pyrrhoxanthinol 55. Myxoxanthophyll, 56. Oscillaxanthin, 57. 4-ketomyxoxanthophyll, 58. Aphanizophyll, 59. O-methyl-methylpenthoside, 60.Mutachrome, 61.Caloxanthin, 62. Nostoxanthin, 63.19'-butanoyloxyfucocanthin, 64. Gyroxanthin, 65. ∝-carotene, 66. Prasinoxanthin, 67. Fritschiellaxanthin, 68.∝-cryptoxanthin, 69. β-cryptoxanthin, 70. Eutreptiellanone, 71.Anhydrodiatoxanthin.

carotenoids- neoxanthin, violaxanthin, lutein, zeaxanthin, and antheraxanthin (Jin et al 2003b). Due to its carotenoid rich nature, *Chlorella vulgaris* is being used as natural colour ingredient in animal feed (Gouveia et al, 1996a). Few green algae like *Haematococcus, Chlorococcum, Neochloris* produce carotenoid astaxanthin along with other carotenoids. Several carotenoids have been recognized as biotechnologically important (Table 1.5).

Table 1.5. Microalgal carotenoids of biotechnological importance

	Yield	**References**
Lutein		
Chlorella zofingiensis	21 µg/ml	Del Campo et al (2004)
Chlorella protothecoides	225 µg/ml	Shi et al (1999)
Muriellopsis sp.	35 µg/ml	Del Campo et al (2001)
Zeaxanthin		
Dunaliella salina	6 mg/g	Jin et al (2003a)
Microcystis aeruginosa	Not reported	Chen et al (2005)
Nannochloropsis	Not reported	Lee et al (2006)
Astaxanthin		
Haematococcus pluvialis	30 mg/g	Lorenz and Cysewski (2000)
Chlorella zofingiensis	<1 mg/g	Ip and Chen (2005)
β-Carotene		
Dunaliella salina	100mg/g	Garcia-Gonzalez et al (2005)
Dunaliella bardawil	>100mg/g	Lers et al (1990)
Canthaxanthin		
Chlorella emersonii	0.6 µg/ml	Arad et al (1993)

Modified from Bhosale and Bernstein (2005).

1.8. Astaxanthin

Astaxanthin is a ketocarotenoid or oxygenated derivative of carotenoid, widely used in salmonoid, lobsters and crustacean aquaculture as a pigmentation source. In the natural aquatic environment, astaxanthin is biosynthesized in the food chain within microalgae or phytoplankton at the primary production level. The microalgae are consumed by zooplankton, insects or crustaceans which accumulate astaxanthin and in turn, are ingested by salmonids. Astaxanthin cannot be synthesized by animals, hence must be

acquired through diet. Since these organisms do not have access to natural sources of astaxanthin, the desirable reddish orange colour is imparted by feeding the astaxanthin rich diet. Some of the natural sources of astaxanthin have been listed in table 1.6. Few attempts have been made to produce astaxanthin in higher plants. Ralley et al (2004) have illustrated the potential of two gene products [3,3′-β-hydroxylase (*crt*Z) and 4,4′-β-oxygenase (*crt*W)] from marine bacteria (*Paracoccus* species) to produce ketocarotenoids in plants. *Haematococcus* oxygenase (*crt*O) has been expressed in tobacco resulting in astaxanthin formation in the nectary tissue (Mann et al, 2000).

Adding to its pigmentation efficiency, astaxanthin has several essential biological functions including protection against UV light effects, immune response and reproductive behaviour in the aquatic animals in which it is found (Lorenz and Cysewski, 2000). This has led the researcher to explore the biological potentials of astaxanthin and prompted numerous research studies concerning its potential benefits to human and animals.

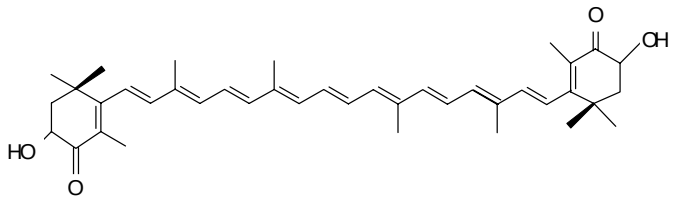
1.9. Chemistry of Astaxanthin

Astaxanthin was first chemically identified by Kuhn and Sorenson (1983). Astaxanthin is a complex molecule and the synthesis, being difficult, results in an expensive product costing approximately US $2000 (Olaizola, 2003). Astaxanthin is derived from lycopene and it contains two terminal ring systems joined by a chain of conjugated double bonds or polyene system. This molecule has two asymmetric carbons located at the 3, 3' positions of the benzenoid rings with hydroxyl group(-OH) and at 4,4′ positions with keto (=O) group on the either end of the molecule. Astaxanthin can exist in three configurational isomers: two enantiomers (3S, 3′S and 3R, 3′R) and a meso form (3R,3′S) (Figure 1.3),(Higuera-Ciapara et al, 2006). From all these isomers, the 3S,3′S is the most abundant in nature. Astaxanthin producing organisms, including *Haematococcus*, synthesize the (3S,3′S)-isomer, yeast *Xanthophyllomyces dendrorhous* produces the opposite isomer having the (3R,3′R)-configuration (Visser et al, 2003).

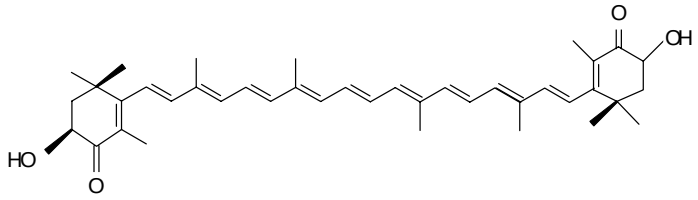
Table 1.6. Natural sources of astaxanthin

Organism	Content (% w/w dry wt)	Reference
Green algae		
Haematococcus pluvialis	2.0-3.0	Lorenz and Cysewski (2000)
Neochloris wimmeri	0.6	Orosa et al (2000)
Chlorococcum	< 0.2	Zhang et al (1997a)
Nannochloropsis gaditana	<0.3	Lubian et al (2000)
Scenedesmus vacuolatus	0.01	Orosa et al (2000)
Chlorella zofingiensis	<0.01	Ip and Chen (2005)
Chlamydomonas nivalis	0.04	Bidigare et al (1993)
Fungi		
Xanthophyllomyces dendrorhous (Phaffia rhodozyma)	0.4*	Jacobson et al (2000)
Yeast-*Candida utilis*	0.04	Miura et al (1998)
Bacteria		
Mycobacterium lacticola	0.003	Simpson et al (1981)
Agrobacterium aurantiacum	0.01	Yokoyama et al (1995)
Paracoccus carotinifaciens	Not reported	Tsubokura et al (1999)
Brevibacterium sp	0.003	Neils and Leenheer (1991)
Animals		
Shrimp-*Pandalus clarkii*	0.015	Meyers and Bligh (1981)
Shrimp-*Pandalus borealis*	0.014	Shahidi and Synowiecki (1991)
Backs snow crab *Chinoecetes opilio*	0.011	Shahidi and Synowiecki (1991)

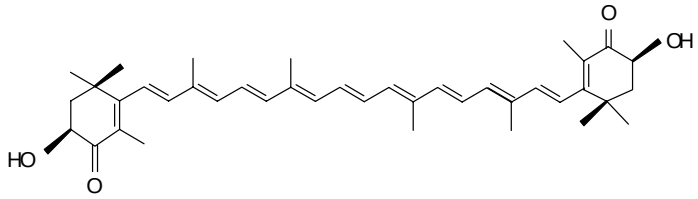
*Various researchers have reported different amount of astaxanthin



3S, 3´S Astaxanthin
(3,3´-dihydroxy β,β-carotene 4,4´-dione)



3R, 3´S Astaxanthin (*meso*)



3R, 3´R Astaxanthin

Figure 1.3. Configurational isomers of astaxanthin

Synthetic astaxanthin consists of a racemic mixture of the two enantiomers and the meso form - 1:2:1 of isomers of (3S, 3′S) (3R, 3′S) and (3R, 3′R) respectively. Depending on their origin, astaxanthin can be found in association with other compounds such as fatty acid. Thus the mono or diesters of astaxanthin with fatty acids such as palmitic, oleic, linoleic etc. in one or both hydroxyl groups may be found. It may also be found free with the hydroxyl groups without being esterified. Synthetic astaxanthin is in free form; algal is mixture of mono and diesters (Figure 1.4). On the other hand, crustacean is in free form and exists as the mixture of all the three isomers.

1.10. Health benefits of astaxanthin

The beneficial role of astaxanthin and its applications has been reviewed by Guerin et al (2003) and Higuera-Ciapara et al (2006). Research on the health benefits has mostly been performed *in vitro* or at the pre-clinical level with humans. One of the most important properties of astaxanthin is its antioxidant property which has been reported to surpass the known antioxidants. Using various direct and indirect methods of assaying antioxidant activity, several-fold stronger antioxidant activity of astaxanthin

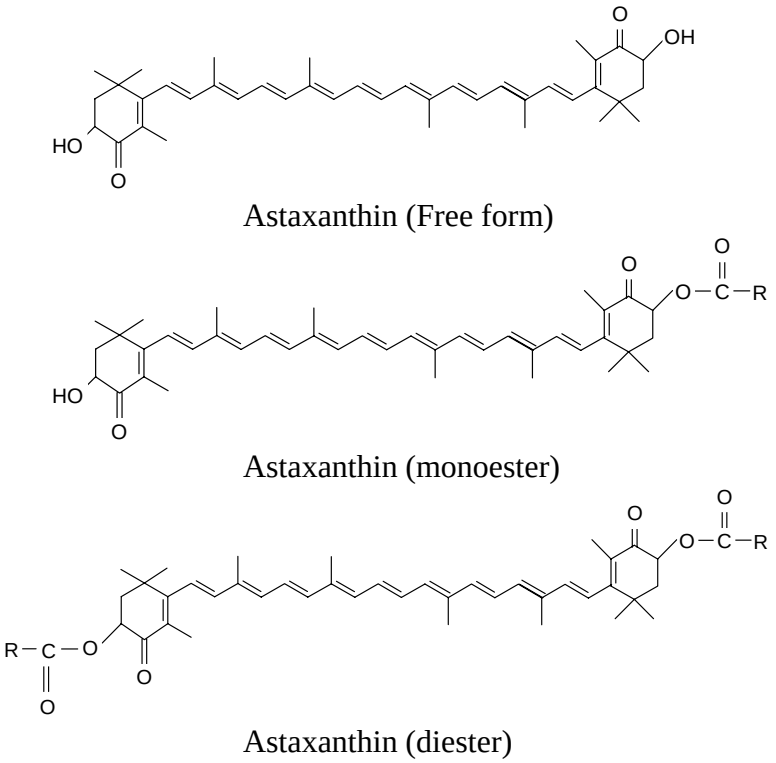


Figure 1.4. Free and esterified forms of astaxanthin
(R= saturated or unsaturated alkyl chains), Adapted from Miao et al, 2006

than vitamin E and β-carotene has been reported by various researchers (Miki, 1991; Lawlor and O'brien, 1995; Nakagawa et al, 1997; Naguib, 2000; Goto et al, 2001). Employing the fluorometric assay procedure, Naguib (2000) found that astaxanthin has a higher antioxidant activity than lutein, lycopene, ∝ and β-carotene, and ∝-tocopherol. Bell et al (2000) have reported that astaxanthin also functions as an antioxidant in membranes isolated from salmon, previously fed with astaxanthin. Astaxanthin has shown protection against the peroxidation in membranous phospholipids. (Palozza and Krinsky, 1992). The antioxidant properties of astaxanthin are believed to have a key role in the pharmaceutical, nutraceutical and food industry (Guerin et al, 2003).

The *in vitro* protective effect of astaxanthin against UV-induced photooxidation was stronger when compared with β-carotene and lutein (O'Connor and O'Brien, 1998). Astaxanthin supplementation helped in protecting the retinal photoreceptors in the eyes of rats exposed to acute UV-light injury (Tso and Lam, 1996). Astaxanthin-rich algal

meal showed an inhibitory effect on *Helicobacter pylori* infection in mice (Wang et al, 2000).

Astaxanthin increases the production of T-helper cells antibody and increases the number of antibody secretory cells from primed spleen cells (Jyonouchi et al, 1996). The effect of astaxanthin in the production of immunoglobulins *in vitro* by human blood cells was studied by these researchers (Jyonouchi et al, 1995) and found that it increases the production of IgA, IgG and IgM in response to T-dependent stimuli. They also proposed astaxanthin is devoid of pro-vitamin A activity.

Anti-cancer activity of astaxanthin has been demonstrated in several studies, Tanaka et al (1994) reported the inhibition of chemically induced carcinogenesis in mice urinary bladder by astaxanthin. Astaxanthin was also found effective in protecting rat against azomethane-induced colon cancer (Tanaka et al, 1995). Bertram and Vine (2005) have proposed the hypothesis that the retenoids/provitamin A carotenoids and the non-provitamin A carotenoids operate through separate mechanism. If these separate mechanisms can be activated, combined treatment with a retinoid and astaxanthin may result in effective chemoprevention against cancer without toxicity associated with retenoids.

Protection of rat liver damage induced by CCl_4 through the inhibition of lipid peroxidation and the stimulation of the cell antioxidant system was reported by Kang et al (2001). Tumor growth inhibition in mammary tissue of female mice by astaxanthin was demonstrated by Chew et al (1999). It has also been suggested that astaxanthin attenuates the liver metastasis induced by stress in mice thus promoting the immune response through the inhibition of lipid peroxidation (Kurihara et al, 2002). Jyonouchi et al (2000) suggested that the anticancer activity of astaxanthin might be due to the modulation of immune response against tumor cells.

A drink containing astaxanthin whose antioxidant action on low-density lipoprotein would be useful for the prevention of arteriosclerosis, ischemic heart disease or ischemic encephalopathy has been proposed by Miki et al (1998). Beneficial effect of astaxanthin to heart health by reducing the inflammation associated with the development of coronary heart disease has been reported by Tracy (1999).

The natural astaxanthin manufacturing companies have performed few clinical trials with voluntary patients (Mera Pharmaceuticals, 2003; Cyanotech, 2002). These safety studies of algae derived astaxanthin did not present any disease or intoxication to the patients.

1.11. *Haematococcus*

The green alga *Haematococcus pluvialis,* among the biological sources, has a high concentration of ketocarotenoid astaxanthin, up to 2.0-3.0% w/w on dry weight basis (Lorenz and Cysewski, 2000; Yuan and Chen, 2000).

Taxonomic classification:

Phylum	:	Chlorophyta
Class	:	Chlorophyceae
Order	:	Volvocales
Family	:	Haematococcaceae
Genus	:	*Haematococcus*
Species	:	*pluvialis*

Haematococcus pluvialis is a eukaryotic, unicellular, motile, biflagellate, green fresh water alga capable of both photoautotrophic and heterotrophic growth (Sarada et al, 2002b, Kang et al, 2005). Under favourable growth conditions, it exists as a single biflagellate swimmer capable of photosynthetic autotrophic growth. During unfavourable growth conditions, *H. pluvialis* initiates carotenogenesis and undergoes morphological transformation from green vegetative cells to deep-red, astaxanthin-rich, immotile aplanospores (Harker et al, 1996a). Thus, a distinct two morphological phases viz 'green motile vegetative phase' and 'red nonmotile carotenoid accumulated encysted (aplanospore) phase' exists in the life cycle of *H. pluvialis*. The conditions such as nutrient limiting condition, oxidative stress, elevated temperature, intense light and salinity represent the unfavourable growth conditions, also referred as stress factors or inductive conditions (Kobayashi et al, 1993; Tjahjono et al, 1994a; Sarada et al, 2002a; Jin et al, 2006). The life cycle of *Haematococcus pluvialis* is shown in Figure 1.5.

During the morphological transformation, a trilaminar sheath and acetolysis-resistant material is formed and thickened, coinciding with massive accumulation of

astaxanthin in extra-plastidic lipid vesicles and expansion of cell volume (Johnson and Shcroeder, 1995; Montsant et al, 2001). Astaxanthin enables *Haematococcus* to acclimate to high light by dissipating the excessive light energy, shielding the photosynthetic apparatus (Wang et al, 2003). Subsequently, after being exposed to a favourable environment, cysts revert to the motile phase. Astaxanthin exists mainly as free astaxanthin in the red yeast *Phaffia rhodozyma* (Parajo et al, 1998) and as astaxanthin esters in the green algae *H. pluvialis* (Johnson and An, 1991). Astaxanthin biosynthetic pathway in *Haematococcus* is shown in Figure 1.6.

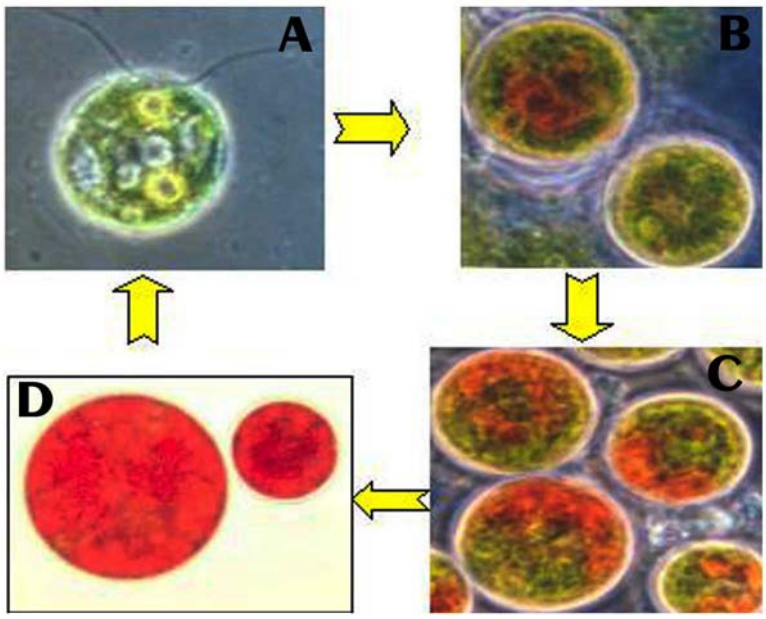


Figure 1.5. Life cycle of *Haematococcus pluvialis*

A. Green motile vegetative phase B. Intermediate non-motile phase
C. Intermediate carotenoid accumulating phase D. Carotenoid rich encysted phase.

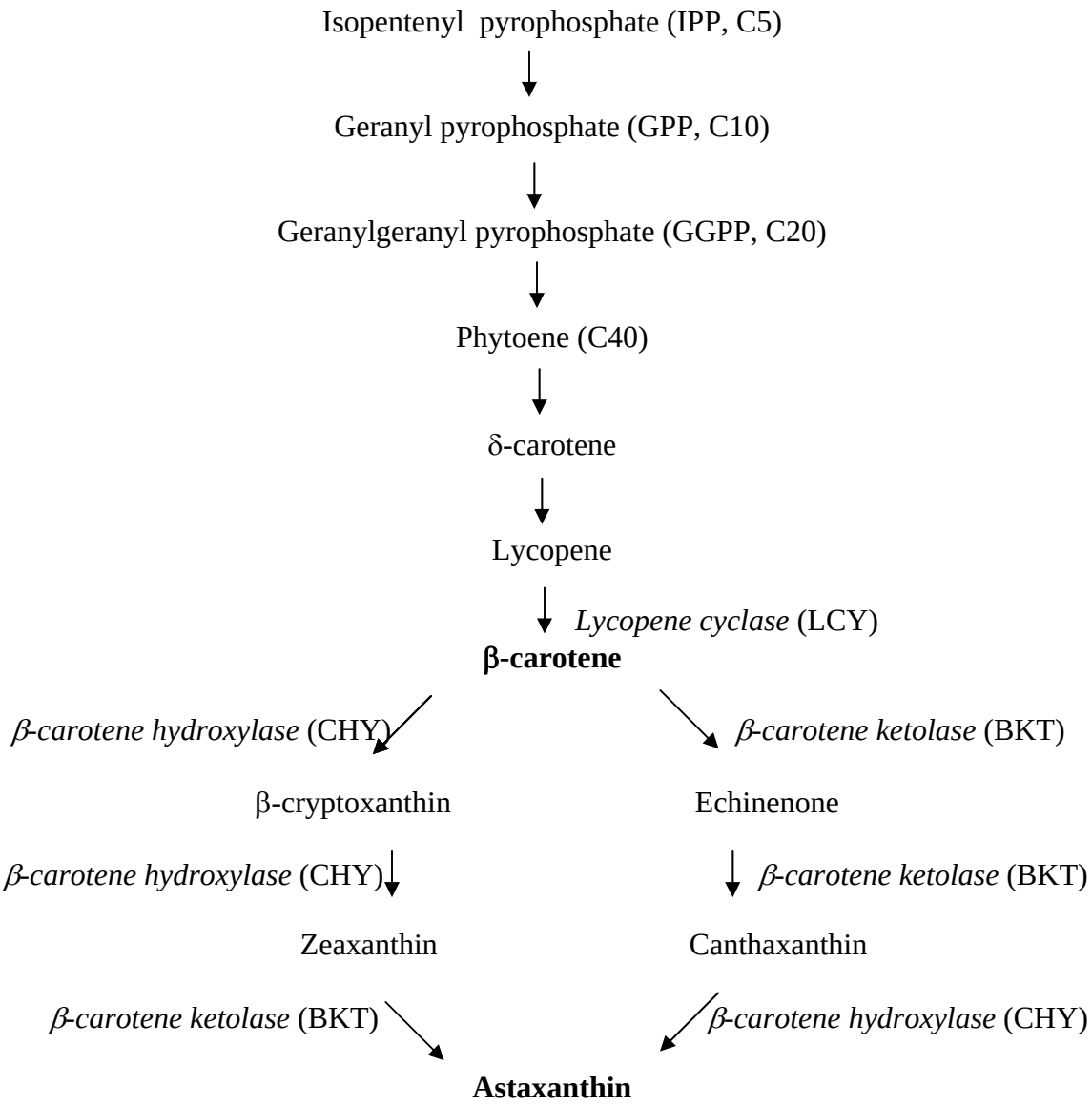


Figure 1.6. Possible biosynthetic pathway for astaxanthin formation in *Haematococcus* (Adapted from Bhosale and Bernstein, 2005)

1.12. Microalgal culture condition for growth and carotenogenesis

Considerable amount of research has been done on the physiology and growth conditions for producing the compounds of interest from microalgal form. Determining the exact and narrow range for each parameter without restriction on growth is difficult since the optimum conditions for a given algal strain varies considerably. Droop (1954) defined the culture condition for formation of astaxanthin in *Haematococcus* for the first time.

Culture condition for indoor cultivation of *Haematococcus* and astaxanthin production has been reported by many authors (Sarada et al, 2002b; Orosa et al, 2001; Kobayashi et al, 2001; Fabregas et al, 2003; Boussiba, 2000)

1.12.1. Autotrophic and heterotrophic system for growth

Many microalgae, including *Haematococcus*, are capable of autotrophic as well as heterotrophic growth (Sarada et al, 2002b; Kang et al, 2005). Heterotrophic cultivation has a potential for achieving high cell concentration and it has been demonstrated for production of *Chlamydomonas* biomass (Chen and Johns, 1996). Due to the problems of maintaining sterile conditions, heterotrophic system is not suitable for growth of most other microalgae. Few researchers have reported cultivation of microalgae in mixotrophic system where acetate is used as carbon source (Orosa et al, 2001; Martinez, 1997; Gong and Chen, 1997). Sequential heterotrophic-photoautotrophic cultivation of a green alga, *Haematococcus* was reported by Hata et al (2001) where the algae was grown heterotrophically to high cell concentration, followed by illumination of the culture for astaxanthin accumulation. Astaxanthin production by *Haematococcus* in autotrophic, mixotrophic and heterotrophic medium was reported by Tripathi et al (1999). Martinez and Orus (1991) have reported the glucose uptake and photosynthetic and respiratory performance in light by *Chlorella*. They have concluded CO_2 as the major carbon species taken from the medium by *Chlorella vulgaris* UAM 101.

1.12.2. Gas –liquid mass transfer

In terms of amounts, carbon is the dominant nutrient which constitutes 45-50% of the dry organic weight. Carbon is stored in liquids as dissolved CO_2, bicarbonate and carbonate. Mass transfer is important to maximizing dissolution and minimizing outgassing of CO_2 in addition to its role in stripping of photosynthetically- derived oxygen. Primarily, pH determines the relative amount carbonate, bicarbonate and dissolved CO_2 (Weissman et al, 1988). CO_2 concentration is reflected in the culture pH changes (Livansky and Bartos, 1986)

1.12.3. Nutrients

The major inorganic elements which must be supplied for algal growth, other than C, are N, K, P, Mg, Ca, S and Fe (Oh-Hama Miyachi, 1987). Some elements are required in minute quantity, which are also referred as micronutients or trace elements, are Zn, Mn,

Br, B, Mo, Cd, Ni, V, W, Al, Cu, Co, and I. Deficiency of certain inorganic elements facilitates induction of carotenoid accumulation. Fabregas et al (1998) have reported the stimulation of astaxanthin biosynthesis in *Haematococcus pluvialis* under nitrogen and magnesium deficient conditions. Deficiency of phosphate is also known to induce astaxanthin accumulation (Harker et al, 1996a).

1.12.4. Light

The amount of light energy received by a photosynthetic culture over a finite interval is a function of the photon flux density measured at the surface of the culture and the illuminated surface area. Productivity of photosynthetic culture is governed by the availability and intensity of light. In the light limited linear growth phase of an algal culture where all photosynthetically available photons are absorbed, the biomass output rate is determined by the area to volume ratio (Pirt et al, 1980; Lee et al, 1995). Light intensity act to accelerate nitrogen consumption and astaxanthin synthesis (Fabregas, et al 2003). In *Haematococcus* culture, Park and Lee (2001) obtained a high cell density of 2.7g/L at 75μE m^{-2} s^{-1}. Cultivation of *Haematococcus pluvialis* under illumination with red light emitting diodes (LEDs) without induction of astaxanthin, and then switching to illumination with blue LEDs at a high light intensity to induce a high level of astaxanthin has been reported (Katsuda et al, 2004; Lababpour et al, 2004). Tripathi et al (2002) have observed a 4 fold enhancement in production of astaxanthin with multidirectional illumination compared to unidirectional illumination. Choumont and Thepenier (1995) have demonstrated stimulation of carotenoid accumulation in *Haematococcus* from the first hours of sunlight illumination. Steinbrenner and Linden (2003) have examined the light regulation of carotenoid biosynthesis in *Haematococcus* and detected increased transcript levels for carotenoid biosynthetic genes under blue and red light conditions.

1.12.5. Temperature

The temperature requirement of *Haematococcus* for growth phase and carotenoid phase is different. *Haematococcus* cells in vegetative phase are susceptible for large temperature fluctuations; hence most of the literature on cultivation aspects is based on indoor experiments. Based on increase in cell number and increase in chlorophyll concentration, Fan et al (1994) have found that the temperature of 25°-28°C to be optimal for growth. High temperature has been demonstrated to induce astaxanthin biosynthesis

(Tjahjono et al, 1994a). Tripathi et al (2002) have reported optimum temperature of 25°C for growth and 35°C for astaxanthin accumulation under heterotrophic condition.

1.13. Photobioreactors

To increase the biomass productivity is the main challenge for the cultivation of microalgae. Though microalgae can grow naturally in biotopes, the axenic culture demands the dedicated cultivation systems. Multiple technical approaches and systems of cultivation of microalgae have been developed based on targeted end product. The basic types of open systems are widely accepted for production of microalgal biomass. The configurations of open pond system currently used for large-scale outdoor cultivation are aimed at low-cost biomass production. With regard to the production of high value compounds, closed and semiclosed system has its own advantages over open system. A comparative description of open and closed cultivation system is provided in Table 1.7. Various photobioreactors for cultivation of microalgae have been investigated by researchers worldwide. Different configuration of photobioreactor reported in the literature has been listed in Table 1.8. The aim of photobioreactors include effective and efficient provision of light, supply of CO_2 with minimal losses, removal of photosynthetically produced O_2 and effective control of temperature (Weissman et al, 1988).

1.14. Strain improvement by mutation

Algal strain with improved growth rate and enhanced carotenoid accumulation makes the commercial process of astaxanthin production more feasible. Induction and selection of mutants has been widely employed technique for strain improvement as well as for studying mechanisms of metabolic processes (Fischer, 1998). Different methods have been used to introduce random mutations, e.g. chemical mutagenesis using nitrous acid, hydroxylamine, N-methyl-N′nitro-N-nitrosoguanidine (NTG), bisulphate, ethyl methane sulphonate (EMS) and physical methods using X-ray irradiation, UV irradiation etc.(Walton et al, 1991; Miura et al, 2004; Lai et al, 2004; Wang et al, 2005).

Mutants of *Phaffia rhodozyma* have been obtained by UV exposure and by EMS or NTG treatment for hyperproduction of astaxanthin (Chumpolkuwong et al, 1997). Using EMS induced mutagenesis, a mutant of *Dunaliella salina,* lacking neoxanthin,

Table 1.7. Advantages and disadvantages of open and closed algal cultivation plants

Parameter	**Open pond (raceway ponds)**	**Closed system (Photobioreactor system)**
Contamination risk	Extremely High	Low
Space required	High	Low
Water losses	Extremely High	Almost none
CO_2–losses	High	Almost none
Biomass quality	Satisfactory	high
Variability as to cultivatable species	Cultivation possibilities are restricted to a few algal varieties	High, nearly all microalgal varieties may be cultivated
Flexibility of production	Change of production between the possible varieties nearly impossible	Change of production without any problem
Reproducibility of production parameters	Dependant on exterior conditions	Possible within certain tolerances
Standardization	Limited scope	Possible
Weather dependence	Absolute, production impossible during rain	Insignificant, because closed configurations allow production also during bad weather
Period until net production is reached after start or interruptions	Long, approximately 6 -8 weeks	Relatively short, approximately 2–4 weeks
Biomass concentration during production	Low, approximately 0.1–0.2 g/L	High, approximately 2–8 g/L
Efficiency of treatment processes	Low, time consuming, large volume flows due to low concentrations	High, short time, relatively small volume flows

(Adopted from Pulz, 2001).

Table 1.8. Various configurations of photobioreactor reported for cultivation of microalgae

Photobioreactor	**Microalgae**	**Parameter**	**Growth rate/ biomass yield**	**Reference**
Bubble column	*Haematococcus*	Light	5.2×10^5 cells/ml	Choi et al (2003)
Air-lift	*Haematococcus*	Autotrophic medium	1.6g/ L [a]	Harker et al (1996b)
Modular flat-panel	*Nannochloropsis*	Light	1.45 g/ L/d	Zittelli et al (2000)
Stirred tank	*Dunaliella*	Dissolved oxygen	0.119 h^{-1}	Li et al (2003)
Closed tubular	*Dunaliella*	Outdoor culture	2 $g/m^2/d$	Garcia-Gonzalez (2005)
Closed	*Chlorococum*	Outdoor -Fluid dynamics and flashing light	20.5 $g/m^2/day$	Sato et al (2006)
Stirred tank	Microalgae	Light	7g/L	Ogbonna and Tanaka (2000)
Tubular airlift	Microalgae	Hydrodynamics and mass transfer	1.2g/L/d [b]	Babcock et al (2002)
Flat panel airlift	*Chlorella*	Flashing light	0.11 g/L/ h	Degen et al (2001)
three-stage serial tubular	*Spirulina*	Biofixation of CO_2	0.22 g/ L/d,	de Morais and Costa (2007)
Closed solar	*Spirulina*	High irradiance	0.5 g/L/d	Masojidek, et al (2003)
Vertical flat-plate	*Synechocystis*	CO_2 fixation	31 $g/m^2/d$,	Zhang et al (2001a)
Closed tubular	*Synechocystis*	Autotrophic medium	0.11 g/L/d	Hai et al (2000)
Tubular airlift	*Phaeodactylum*	Solar irradiance and CO_2	2.47 g/L/d	Sobczuk et al (2000)
Helical tubular	*Phaeodactylum*	Outdoor-Fluid dynamics and mass transfer	1.4 g/L/d,	Hall et al (2003)

[a] after 90 days of growth

[b] Theoretical yield

violaxanthin and antheraxanthin but accumulating zeaxanthin was isolated by Jin et al (2003a). Wang et al (2005) have reported isolation of a nitrate reductase -deficient mutant

of *Chlorella ellipsoidea* nrm-4 using X-ray mutagenesis. Meireles et al (2003) have isolated mutant of microalga *Pavlova lutheri*, using UV-light as mutagen, with the higher yields of eicosapentaenoic and docosahexaenoic acids than parent strain. Using UV irradiation, isolation of a starchless mutant of *Chlorella pyrenoidosa* STL-PI with a high growth rate, high protein and polyunsaturated fatty acid content has been reported by Ramazanov and Ramazanov (2006). Tripathi et al (2001a) reported the enhancement of astaxanthin production in *Haematococcus* mutants using the mutagen UV and EMS, screened using inhibitors of the carotenoid biosynthetic pathway. A *Chlorococcum* mutant with enhanced accumulation of secondary carotenoid has been isolated by Zhang and Lee (1997b) using azide, an inhibitor of electron transport in photosynthetic oxygen generation reaction.

1.15. Current status and astaxanthin market

Annual growth of the global market for astaxanthin for human use is thought to be at least 15 per cent, with current estimates valuing the market at $15-20m (€12.4-16.6m) per year (www.nutraingredients-usa.com). Astaxanthin is referenced in the US Code of Federal Regulations Title 21 part 73—listing of color additives exempt from certification—Subpart A-Foods (Sec. 73.35 Astaxanthin). Upon approval of *Haematococcus* alga by the US Food and Drug Administration (21 CFR 190.6) and clearance for marketing as a new dietary ingredient, various products of *Haematococcus* have entered the world market. US based Cyanotech Corporation, Mera Pharmaceuticals Inc., Israel based Algatechnologies Ltd, Sweden based BioReal AB are the major producers of *Haematococcus* algae meal and astaxanthin products. Mera Pharmaceuticls Inc. employs a fully enclosed 25,000L computer-controlled outdoor photobioreactor for large-scale biomass production of *Haematococcus*. (Olaizola, 2000). BioReal (Sweden) AB has the BioDome technology for cultivation of *Haematococcus* (www. fujihealthscience.com). In India, Chennai based Parry Nutraceuticals is engaged in development of process for production of astaxanthin from *Haematococcus* (www.parrynutraceuticals.com).

The range of astaxanthin products produced and marketed by various companies has been listed in Table 1.9. Significant price difference exists between the synthetic and

natural astaxanthin, synthetic variety costs $2000 per kg where as the natural astaxanthin is still expensive at between $10,000 - 15,000 per kg (www.nutraingredients-usa.com).

Table 1.9. *Haematococcus* algae meal and astaxanthin products in world market

Product	**Company**	**Particulars**	**Website**
AstaFactor®	Mera Pharmaceuticals Inc. USA.	Astaxanthin packaged as soft gel; dietary supplement derived from *Haematococcus*	www.astafactor.com
AstaPure™	Algatechnologies Ltd, Israel.	Dry algal biomass, astaxanthin beadlets and oleoresin	www.algatech.com
AstaXin® AstaCarox®	BioReal (Sweden)AB	Dietary supplement containing *Haematococcus* - crushed and dried algae meal	www.bioreal.se
AstaEquus®	BioReal (Sweden)AB	Feed supplement for horses	www.bioreal.se
AstaREAL®	BioReal (Sweden)AB	Super critical fluid-oil extract derived from crushed algae	www.bioreal.se
BioAstin®	Cyanotech Corporation, USA	*Haematococcus* extract – packaged in soft gel, beadlets; dietary supplement	www.cyanotech.com
Britaxan®	Britannia Health Products Ltd. UK	Astaxanthin complex with other carotenoids packaged as capsule- dietary supplement	www.britannia-health.co.uk
NaturAsta™	Jingzhou Natural Astaxanthin Inc,China	Dry algal biomass and astaxanthin soft gel	www.asta.cn
Naturose™	Cyanotech Corporation, USA	*Haematococcus* algae meal; pigmentation source for ornamental fish and animals	www.cyanotech.com
Novaasta®	BioReal (Sweden)AB	A feed supplement or animals	www.bioreal.se
Stazen	Stazen Inc., USA	Dietary supplement containing *Haematococcus* - crushed and dried algae meal	www.stazen.com
Zanthin®	Valensa International, USA	*Haematococcus* extract, soft gel, beadlets	www.usnutra.com

The health and nutraceutical applications of astaxanthin is the chief market driving force, hence most of the companies hold the intellectual property rights or patents on the production as well as benefits of astaxanthin. Selected patents on astaxanthin and its production are listed in Table 1.10.

Table 1.10.Selected patents on *Haematococcus* astaxanthin.

Title	Assignee	Patent No.
Procedures for large scale production of astaxanthin from *Haematococcus*	Ben Gurion University of Negev Research and Development authority	US6022701
An improved process for the preparation of carotenoids from encysted *Haematococcus* cells.	Council of Scientific and Industrial Research/Central Food Technological Research Institute	IN 2006 191585
Process to produce astaxanthin from *Haematococcus* biomass	Parry Nutraceuticals Ltd	WO03027267
An improved culture medium useful for carotenoid production	Council of Scientific and Industrial Research/Central Food Technological Research Institute	IN 2006 192829
Use of astaxanthin for retarding and ameliorating central nervous system and eye damage	US Nutra	EP0786990
Use of astaxanthin for treatment of autoimmune diseases, chronic viral and intracellular bacterial infections	Astacarotene	EP1217996
Astaxanthin-containing food or drink	Suntory and Itano	JP10276721
Method of retarding and ameliorating carpal tunnel syndrome	Cyanotech	US6258855
Stable astaxanthin-containing powdery compositions and process for producing the same	Fuji Chemical Co Ltd	WO02/077105
A process for production of carotenoid rich microalgae	Council of Scientific and Industrial Research/Central Food Technological Research Institute	490/DEL/2004

1.16. Objectives and scope of the present investigation

The health benefits derived from the ketocarotenoid astaxanthin has warranted its production through natural sources. *Haematococcus* being the rich source of astaxanthin has been prospected for mass production. Heterotrophic cultivation of *Haematococcus* has its limitations for economical commercial production of astaxanthin. Hence autotrophic system is advantageous and necessitated detailed study. From industrial point of view, high yielding and strains adoptable to wide environmental conditions are desirous.

In view of the growing demand for natural astaxanthin, detailed studies on growth and astaxanthin production by *Haematococcus* were taken up with the objectives given below.

- Optimization of autotrophic culture conditions for growth and carotenogenesis by *Haematococcus* in open and closed prototype bioreactor.
- Isolation of *Haematococcus* mutant for high biomass and astaxanthin production.
- Characterization of mutants by biochemical methods.
- To study bioactivity of astaxanthin in *in vitro* and *in vivo* models.

Chapter 2
Materials and Methods

2.1. Materials

2.1.1. Green alga Haematococcus

The *Haematococcus pluvialis* (SAG-19a) culture was obtained from Sammlung von Kulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen, Germany.

2.1.2. Glassware

All the glassware, such as conical flasks, test tubes, culture tubes, measuring cylinders, pipettes etc., used for the experiments, were from Vensil Ltd., Mumbai or Borosil Glass works Limited, Mumbai, India. The rectangular glass tanks were fabricated locally and the tubular glass photobioreactor was fabricated at Super Scientific Co. Bangalore.

2.1.3. Plasticware

The microcentrifuge tubes, microtips and screwcap centrifuge tubes were from Tarsons Products Pvt. Ltd. Kolkata. The polyethylene (LDPE) bags and tubes were procured from local market. Carboys were purchased from Genetix Biotech Asia Pvt. Ltd., Bangalore.

2.1.4. Chemicals

All the media chemicals used for the experiments were analytical grade, obtained from companies - Sisco Research Laboratories Pvt. Ltd., Mumbai; HiMedia Laboratories Pvt. Ltd., Mumbai; Ranbaxy Fine Chemicals Ltd., New Delhi; Thomas Baker (Chemicals) Pvt. Ltd., Mumbai; Loba Chemie Pvt. Ltd., Mumbai. Qualigens Fine Chemicals, Mumbai. Commercial carbon dioxide and nitrogen gas cylinders were procured from Kiran Corporation, Mysore.

Authentic standards and fine chemicals such as astaxanthin, β-carotene, MSX, azaserine, BAP, GA, methyl viologen, NTG, EMS, DPPH, DCPIP, lipoxygenase, linoleic acid, Glutathione, ATP, NADPH, omeprazole were obtained from Sigma-Aldrich Chemicals, USA.

Solvents used for the experiments were analytical grade or HPLC grade obtained from companies - Qualigens Fine Chemicals, Mumbai., Merck Ltd. Navi Mumbai, Ranbaxy Fine Chemicals Ltd., New Delhi; Sisco Research Laboratories Pvt. Ltd., Mumbai.

The fertilizers - Suphala was obtained from Rashtriya Chemicals and Fertilizers Ltd. Mumbai and Diammonium phosphate from Gujarat State Fertilizers Ltd. Gujarat.

The rat feed was obtained from Sai Durga Feeds, Bangalore. The ingredients for the layer diet and fish diet were procured from local market and mixed accordingly.

2.2. Maintenance of stock culture

Stock cultures of *H. pluvialis* were maintained on both semisolid and liquid autotrophic Bold's basal medium (BBM), (Kanz and Bold, 1969). The composition of the BBM is provided in Table 2.1. The ingredients were dissolved in distilled water.

Table 2.1. Composition of Bold's basal medium

Components	**g/L**
$NaNO_3$	0.25
K_2HPO_4	0.075
KH_2PO_4	0.175
$MgSO_4.7H_2O$	0.073
$CaCl_2.2H_2O$	0.024
NaCl	0.025
$FeSO_4.7H_2O$	0.005
EDTA	0.05
KOH	0.031
Trace elements*	1 ml/L
pH	7.0
***Trace elements** (for preparation of 1L stock solution)	
	g/L stock
H_3BO_3	11.42
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2$	0.49

The above medium was solidified with agar (15g/L) to prepare slants.

The media was distributed into 150ml conical flasks, closed with cotton plugs and sterilized by autoclaving at 121°C for 20 min and allowed to cool at room temperature before inoculation. Inoculation was carried out under aseptic conditions in laminar air flow hood. The *H. pluvialis* slants and the liquid cultures were subcultured at every 4 week and 2 week intervals respectively.

2.2.1. Normal growth condition

The inoculated slant and liquid cultures were incubated in culture room under controlled temperature at 25±1°C and light intensity of 1.5 klux. Light was provided by cool white fluorescent set of lamps (40W; Phillips India Ltd, Kolkata, India) and the light intensity was measured using lux meter (TES 1332, Taiwan).

2.2.2. Growth in CO_2 enriched condition

The two-tier vessel consisting of two 250ml narrow neck Erlenmeyer flasks (Husemann and Barz, 1977) was used for enriching CO_2 in the culture environment. Upper compartment of the flask contained 50ml culture and the lower compartment of the flask contained 100ml of 3M buffer mixture ($KHCO_3/K_2CO_3$) at specific ratio, which generated a partial pressure of CO_2 at 2% in the two-tier flask (Tripathi et al, 2001b). The mouths of the upper and lower compartments were sealed tightly using cotton plug and parafilm.

Figure 2.1. Two-tier flask used for CO_2 enrichment

2.3. Growth measurement

2.3.1. Cell number

Algal cell number was determined by counting algal cells using Neubauer haemaocytometer (Thoma, Germany) and expressed as cells/ml.

2.3.2. Dry weight

Known volume of culture was centrifuged; the algal biomass was washed with distilled water and dried in a hot air oven (Sanyo, Electrical Biomedical Co. Ltd., Japan) at 60°C till constant weight was attained. Biomass weight was expressed as g/L.

2.3.3. Growth rate

H. pluvialis growth rate (μ) was determined according to Zlotnik et al (1993) using the equation, $\mu = [\ln (N_2/N_1)]/t_2\text{-}t_1$, where$N_2$ and N_1 are mean cell numbers at days t_2 and t_1 respectively.

2.4. Chlorophyll estimation

Chlorophyll content in the *H. pluvialis* culture and dry biomass was determined by the method of Lichtenthaler (1987).

Known quantity of *H. pluvialis* culture was centrifuged at 5000 rpm for 15 min and the pellet was used for extraction of chlorophyll. This pellet or dry biomass was extracted in acetone by homogenizing in mortar and pestle. The extract was centrifuged and absorbance of the supernatant was recorded at 645nm and 661.5nm using spectrophotometer (Shimadzu 160A). Chlorophyll content (% w/w) was calculated using the following equations,

Chlorophyll a = 11.24 × Abs 661.5 – 2.04 × Abs 645

Chlorophyll b = 20.13 × Abs 645 – 4.19 × Abs 661.5

Chlorophyll a +b = 7.05 × Abs 661.5 +18.09 × Abs 645

2.5. Carotenoid and astaxanthin estimation

Total carotenoid content was estimated by the method of Lichtenthaler (1987). The cells were homogenized using acetone. The extract was centrifuged and the absorbance at 470 nm was recorded spectrophotometrically for carotenoid estimation. Astaxanthin content was determined at 480 nm using an absorption coefficient, A1% of 2500 by the method of Davies (1976).

2.6. Separation of carotenoids by thin-layer chromatography

The *H. pluvialis* extract were analysed using TLC aluminium sheets (20×20cm) precoated with silica gel 60 (Merck Ltd, New Delhi). *H. pluvialis* extract was spotted on TLC sheet and developed using solvent system acetone:hexane (3:7; Fiksdahl et al, 1978). The developed plates were allowed to dry at room temperature and carotenoids were identified by comparing with authentic β-carotene and astaxanthin standards.

2.7. Separation of carotenoids by HPLC

The total extract as well as the fractions separated from TLC plates were analyzed by HPLC (Shimadzu LC-10AT) using reversed phase C18 (Supelco) 25 cm × 4.6 mm column. Gradient solvent system consisting of acetone and 90% (v/v) methanol at a flow rate of 1.25 ml/minute was used. The separated carotenoids and astaxanthin peaks were identified using photodiode array detector (Shimadzu, SPD-M10AVP) by comparing with authentic standards of β-carotene, astaxanthin and lutein and canthaxanthin.

2.8. Optimization of culture conditions

2.8.1. Influence of ammonium salts on growth of H. pluvialis and astaxanthin production

Bold's basal medium containing ammonium chloride (0.25-1.0g/L, equivalent to 4.67-18.68mM in terms of nitrogen), urea (0.15- 1.0 g/L or 4.99-33.26mM) ammonium carbonate (0.15-1.0 g/L or 2.86-19.06mM), ammonium sulphate (0.15-1.0 g/L or 2.27-15.33mM) and ammonium acetate (0.25-1.0 g/L or 3.24-12.96) as nitrogen sources were independently prepared. The experiments were carried out in 150 ml conical flasks containing 50 ml medium. Effect of ammonium salts, urea and commercial salts such as suphala (N:P:K 15:15:15), diammonium phosphate (DAP) was studied on *H. pluvialis* growth and astaxanthin production in presence of 2% carbon dioxide using two tier flasks (Section 2.2.2). While adding commercial salts, the concentration of nitrogen and phosphate were estimated using APHA (1998) and AOAC procedures (1999) accordingly the amounts were adjusted. A 10-day old *H. pluvialis* autotrophic culture was used as inoculum for all the experiments so as to have an initial cell count of 12×10^4 cells/ml. BBM with 0.249g/L of sodium nitrate (equivalent to 2.93mM of nitrogen) was considered as control.

All the flasks were incubated at 25 ± 1°C in a culture room under light intensity of 1.5klux. After two weeks of growth, one set of flasks were harvested for cell count and biomass estimation while second set of flasks were incubated further for two weeks under high light (3.5 klux) for carotenoid accumulation. Data was represented as an average of three replicates in two repetitive experiments.

2.8.2. Supplementation of plant growth regulators

Plant growth regulators- 6-benzyl aminopurine (BAP) and Gibberellic acid (GA_3) were added to the culture medium both at 1.25mg/L concentration independently and in combination. At regular intervals, cell count was recorded. At the end of the experimental period the biomass and carotenoid contents were analyzed and compared to control.

2.9. Effect of stress conditions

2.9.1. Salinity stress

Two weeks old culture of *H. pluvialis* (100ml) was supplemented with 42mM sodium chloride and 10mM sodium acetate, individually and in combination. Culture without sodium chloride and sodium acetate was used as control. The cultures were exposed to light intensity of 3.5klux for 15 days duration and the temperature was maintained at 25± 1°C. Total carotenoid and astaxanthin contents were analyzed as given in section 2.5.

2.9.2. Light stress

Two weeks old culture of *H. pluvialis* (100ml) in LDPE transparent pouches were used for the experiment. Sodium acetate and CO_2 (2% v/v mixture with air) were provided individually as carbon source. Separate sets of cultures were exposed to sunlight (maximum of 32-36 klux as measured by lux meter) and the culture pouches were kept immersed in water jacket to prevent rise in temperature. One set of cultures was harvested after 7 days and another after 15 days of exposure to sunlight. The total carotenoid and astaxanthin contents were estimated as given in section 2.5.

2.9.3. Oxidative stress

Two weeks old culture of *H. pluvialis* (100ml) in LDPE transparent pouches were used for the experiment. CO_2 (2% v/v mixture with air) was provided as carbon source. Oxidative stress was induced by addition of methyl viologen (0.01nM). Cultures were exposed to light intensity of 3.5klux and harvested after 7 days. The total carotenoid and astaxanthin contents were estimated as given in section 2.5.

2.10. Cultivation of *H. pluvialis* in open prototype bioreactor

2.10.1. Medium: For both open and closed type bioreactor, modified Bold's basal medium (MBBM) was used in which sodium nitrate of BBM (Table 2.1) was replaced with ammonium carbonate (0.15g/L).

2.10.2. Inoculum development

The culture maintained in the slants was inoculated into liquid MBBM (100ml) and allowed to grow for 2 weeks in indoor condition as explained in the section 2.2.1. Then subcultured in 400ml, and then to 1 liter flasks and allowed to grow for 2 weeks.

2.10.3. Bioreactor: Glass tanks of the dimension 45×22×22cm (Length×Width×Height) and 75×30×30cm were used for cultivation of *H. pluvialis*.

The rectangular glass open bioreactors with different designs for cultivation of *H. pluvialis* were,

Design 1. With float.

Design 2. With CO_2 bubbling

Design 3. With stirrer

Design 4. With float and stirrer

The schematic diagrams of the above designs with provisions for CO_2 supply has been shown in Figure 2.2.

2.10.4. CO_2 supply: Air supplied through air compressor (Metro Machinery Manufacturers, Coimbatore, India) was mixed in a connecting jar with 2% (v/v) carbon dioxide from cylinder. The amount of CO_2 inflow was regulated and monitored through flow meter (Fischer & Porter GMBH, Germany). This air plus CO_2 mixture was supplied to culture in two ways.

a) *Float*: A cubical transparent plastic device, to provide CO_2 enriched mass transfer area of ~0.02m^2, open on one side and a gas inlet on the opposite side. It was filled with 2% (v/v mixed with air) carbon dioxide and allowed floating on the culture surface. The gas phase inside the float was replaced with fresh CO_2 mixed with air, twice a day.

b) *Bubbling*: Air+CO_2 2-4% (v/v) mixture was directly bubbled into the culture at a rate of 1L/min through a glass tube. In this way, CO_2 was supplied for 10 min, twice a day.

2.10.5. Stirrer: Provision for agitation of culture was made using motor (U.P.National Manufacturers, Varanasi, India) with shaft and agitating blade. Culture was agitated at speed of 30 rpm for 10 min, twice a day.

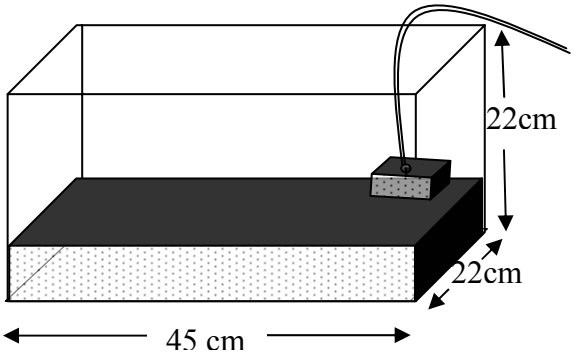
2.10.6. Raceway Tank

Raceway tank constructed of cement with dimension 1.2×0.6×0.3m (Length×Width×Height) with 150 L capacity was used for cultivation of *H. pluvialis*. Paddle wheel (locally designed with stainless steel, driven by induction motor) was provided for agitation of the culture and CO_2 (2% v/v mixed with air) was supplied through float.

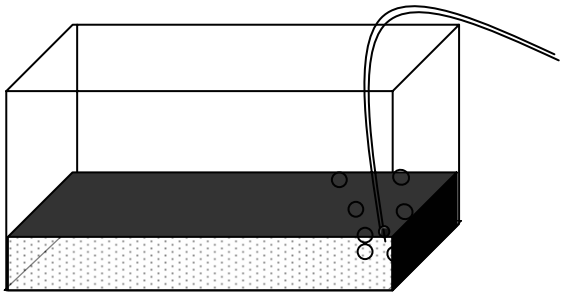
2.11. Cultivation of *H. pluvialis* in closed system

H.pluvialis was cultivated in carboys, polyethylene (LDPE; guage ~ 340) pouches of 4 L capacity, bags (0.68m×0.48m; Length×Width) and tubular sleeves (1.5m × 0.065m; Length × diameter). Photobioreactor consisting of glass tubular modules (0.5m length × 0.14m diameter×10 loops) combined with glass rectangular bioreactor was evaluated for cultivation of *H.pluvialis* (Figure 2.3). CO_2 (2% v/v mixture with air) was supplied through float. Culture was circulated in photobioreactor using peristaltic pump (Murhopye Scientific Company, Mysore) with a flow rate of 40-50ml/min. The reactor volume, culture volume, CO_2 supply has been detailed in table 2.2.

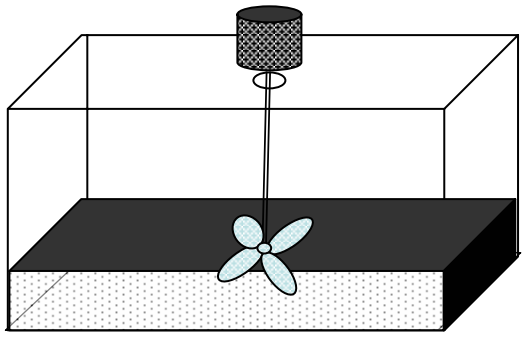
In the closed systems, 2% (v/v) CO_2 mixed with air was passed through a 0.2μm size filter (Midisart 2000, Sartorius, Germany) and also through 10% (v/v) formaldehyde solution , 5% (w/v) copper sulphate solution and sterile water to prevent possible air borne contamination.



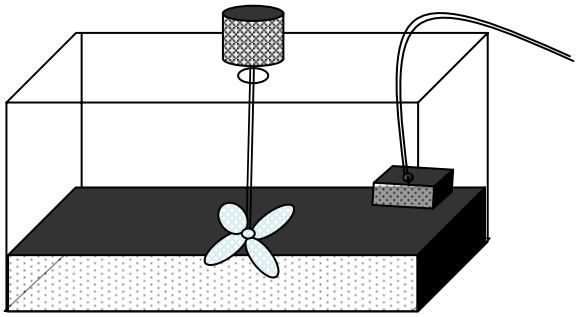
Design 1. Reactor with CO_2 float



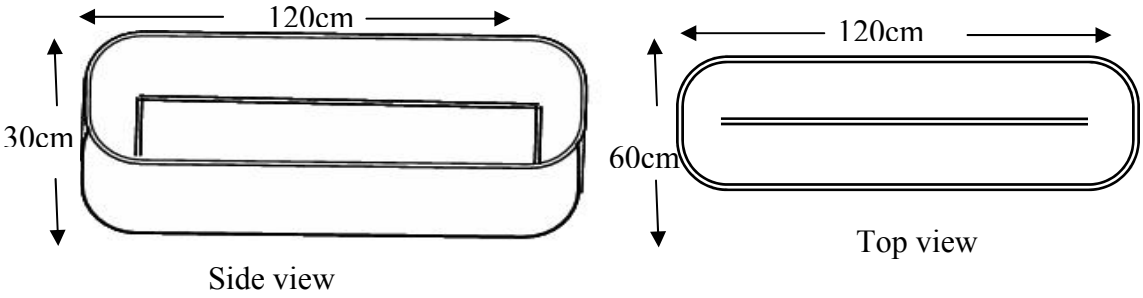
Design 2. Reactor with CO_2 bubbling



Design 3. Reactor with stirrer



Design 4. Reactor with CO_2 float and stirrer



Open raceway prototype

Figure 2.2. Schematic representation of different designs of open prototype bioreactors used for growing *H. pluvialis.*.

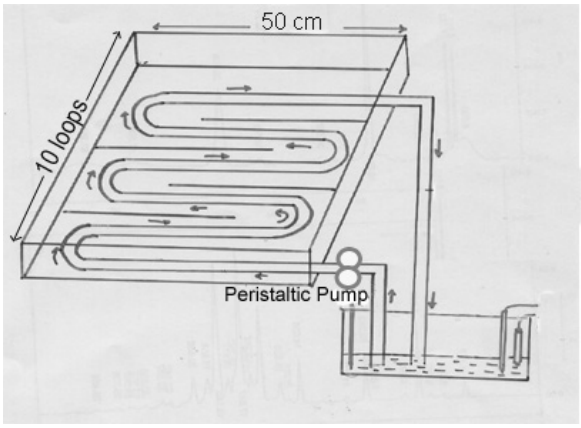


Figure 2.3. Schematic diagram of closed photobioreactor used for growing *H. pluvialis*.

Table 2.2 Various reactors employed for closed cultivation of *H. pluvialis*

Bioreactor	Reactor design	CO_2 supply	Bioreactor Volume	Culture volume	CO_2 enriched gas - liquid mass transfer area
Bioreactor B	Tubular-Polyethylene	Airspace	5L	3L	$0.09m^2$
Bioreactor C	Rectangular-Polyethylene	Airspace	10L	5L	$0.3m^2$
Bioreactor D	Photobioreactor	Ambient +CO_2 float	20	5L	$0.02m^2$
Bioreactor E	Raceway-tank	Ambient+CO_2 float	150L	40L	$0.02m^2$

2.12. Harvesting of *H. pluvialis* biomass

2.12.1. Gravity settling

Since the encysted *H. pluvialis* cells get enlarged, the cells were harvested by gravity settling. The rate of cell settling was recorded by withdrawing the culture sample and estimating cell number for every 10 minutes.

2.12.2. Centrifugation

The culture was harvested by centrifugation (C 24; Remi Instruments Ltd, Mumbai) at 5000 rpm for 10 min.

2.13. Drying of biomass

In order to determine the relative effectiveness of various drying processes, different techniques were studied, so that the process provides most stable product suitable for its further use.

Oven drying

The harvested *H. pluvialis* cells were dried at 50°C for nearly 7 hours in hot air oven (Sanyo Electrical Biomedical Co.Ltd., Japan), to get constant weight.

Spray drying

The harvested cells were dried by mini spray drier (JISL, LSD-48, Mumbai, India) inlet temperature 140°C with the feed rate of 0.5L/hour.

Freeze drying

Harvested cells were dried in a freeze dryer (Heto, FD3, Denmark) at -20°C for 8 hours.

2.14. Storage stability studies of *H. pluvialis* cells

Known quantity of *H. pluvialis* biomass was packed in transparent polyethylene and metallized polyester polypouches. These pouches were filled with nitrogen gas to create inert atmosphere. These pouches were stored at different temperatures viz. 7°C, –20°C, ambient temperature in light and dark conditions. Colour of the cells was monitored at 15 days interval, the carotenoid content was analyzed at initial period and at the end of 60 days. Stability of cells was also evaluated by mixing butylated hydroxy anisole (BHA) with *H. pluvialis* biomass at 100ppm level and kept at room temperature in dark condition.

2.15. Digital Image processing for estimating the carotenoid content

2.15.1. Extraction and analysis of pigments

H. pluvialis culture (50ml) was centrifuged and known quantity of freeze dried biomass was taken for extraction. The cells were homogenized and carotenoids were extracted with acetone. Total carotenoid, astaxanthin and chlorophyll contents were analysed as detailed in the section 2.4 and 2.5. *H. pluvialis* cells at various stages of carotenoid formation ranging from green vegetative phase to red encysted phase (10 different stages) were analyzed for carotenoid content and expressed in terms of %(w/w) dry weight basis.**.**

Digital image processing adopted encompassed a broad range of hardware, software, and theoretical underpinnings. This involves image acquisition and a series of image processing steps as shown in Figure 2.4. (Gonzalez and Woods, 1992). The problem domain referred is the images of *H. pluvialis* containing different amount of carotenoids.

2.15.2. Image acquisition

Image acquisition involves capturing the image by means of a Camera-monochrome or colour. Charge Couple Device (CCD) cameras are usually employed. These cameras have discrete imaging elements called 'photosites', which give out a voltage proportional to the light intensity. A frame grabber card (FlashBus FBG 4.2, 1996, Integral Tech, Inc.) was used to convert the analog image signal into the digital form.

The analysis of carotenoid content was achieved by exploiting the colour based method. In this method the sample images were captured using CCD camera (Watec, WAT202D version) and the captured images were processed and analyzed by making use of DIP tools.

Fundamental algorithms for colour to gray conversion, threshold, filtering, segmentation, were implemented using the C programming language (Lindley, 1990). These steps were aimed at extracting the colour and intensity information from the images. The schematic representation of the steps involved in image processing is shown in Figure 2.4.

The image of algal cells was grabbed by the CCD camera and the same was first converted to the gray scale. Threshold was carried out for convenient processing and to get a uniform background and shape information of the image. The boundary of the object was detected and the region within the boundary was filled to achieve clear distinction between the object and the boundary. Hue being a colour attribute, describes the pureness of the colour and is expressed as an angle with reference to the colour triangle. Based on the detected boundary information, the Hue values for each of the original colour image were computed by converting them from Red Green Blue (RGB) model to Hue Saturation Intensity (HSI) model.

Hue (H) is calculated using the equation :

$$H = \cos^{-1}\left[\frac{\tfrac{1}{2}[(R-G)+(R-B)]}{[(R-G)^2+(R-B)(G-B)]^{1/2}}\right]$$

where R, G, B are red, green and blue values of the pixels in the image (Gonzalez and Woods, 1992).

The concept of Artificial Neural Networks (ANN) was used (Schalkoff, 1997) to relate hue values to carotenoid/chlorophyll content. An Artificial Neural Network is an information-processing paradigm that is inspired by the way biological nervous systems, such as the brain, process information. The key element of this paradigm is the novel structure of the information processing system. It is composed of a large number of highly interconnected processing elements (neurons) working in unison to solve specific problems.

The Hue value so obtained was categorized to 28 classes depending on its distribution in the various stages and fed as input values to the neural network. The topology of the back propagation neural network model used was:

- 28 input Hue units (0-360°)

A1 to A6	0-30° in intervals of 5°
A7	30°-105°
A8	105°-150°
A9 to A17	150°-195° in intervals of 5°
A18	195°-240°
A19 to A21	240°-255°in intervals of 5°
A22	255°-330°
A23 to A28	330°-360° in intervals of 5°

- 1 hidden layer with 12 units

- 2 output units representing % carotenoid and % chlorophyll (target)

The network devised to achieve the desired output had an output threshold of 0.5, learning rate of 0.6, momentum of 0.9 and an error margin of 0.0001.

The neural network was accomplished on a computer with Pentium 2 processor, 550 MHz. The network was trained to obtain the target values utilizing 27 learning sets. Neural network software, Neuroshell Utility™ (Rel 4.01, Ward System Group Inc. USA) was used for the purpose. Figure 2.5 depicts the neural network model devised for the purpose. The network devised to achieve the desired output had an output threshold of 0.45, learning rate of 0.6, momentum of 0.9 and an error margin of 0.0001.

The weight matrix W_{IJ} between the 28 units of input layer (I) and 12 units of hidden layer (J) was:

$$W_{ij} = \begin{pmatrix}
-0.39 & -0.32 & -2.97 & -0.35 & -0.7 & -0.21 & -0.48 & -0.42 & 0.39 & -0.47 & -20.1 & -1.06 \\
-0.21 & 0.2 & -0.7 & 0.25 & 0.61 & -0.61 & 0.81 & -0.39 & -0.27 & 0.15 & -13.4 & 1.37 \\
-0.18 & 0.18 & 0.1 & -0.14 & 0.27 & -0.01 & 0.02 & 0.17 & 0.12 & 0.12 & -0.19 & 0.07 \\
-0.03 & -0.32 & -0.67 & 0.12 & -0.17 & -0.08 & -0.36 & 0.01 & 0.3 & -0.14 & 1.01 & 0.1 \\
-0.23 & -0.03 & 0.06 & -0.16 & -0.48 & -0.01 & -1.12 & 0.03 & 0.3 & -0.35 & 4.27 & -0.62 \\
-0.18 & -0.13 & -0.87 & -0.2 & -0.52 & -0.71 & -0.9 & -0.58 & -0.47 & -0.23 & -8.14 & -0.27 \\
-0.1 & -0.15 & -2.95 & -0.19 & -0.08 & -1.04 & 0.39 & -0.71 & -0.19 & 0.27 & 11 & -0.36 \\
-0.59 & -0.07 & 0.29 & -0.09 & -0.63 & -0.08 & 0.12 & -0.32 & -0.11 & -0.49 & -1.28 & -0.28 \\
0.26 & 0.28 & 0.25 & 0.25 & -0.06 & 0.11 & -0.12 & 0.02 & 0.12 & 0.14 & 0.25 & 0.19 \\
-0.2 & -0.05 & -0.75 & -0.5 & -0.73 & -0.24 & -0.7 & 0.05 & 0.12 & -0.6 & -0.2 & -0.27 \\
0.57 & 1.19 & -3 & 0.5 & -1.34 & -0.89 & -0.66 & 0.44 & -0.96 & -0.31 & -1.03 & -0.49 \\
-0.39 & 0 & -2.16 & -0.49 & -0.26 & -0.56 & -0.21 & -0.13 & -0.62 & -0.32 & 17.5 & 0.7 \\
-0.48 & -0.76 & 0.04 & -1.06 & 1.43 & -0.06 & -0.58 & -0.92 & 0.58 & -0.33 & 43.3 & -0.03 \\
0.51 & -0.22 & 1.29 & 0.83 & -0.03 & -0.45 & 2.44 & -0.23 & -0.02 & 0.02 & -22.4 & 0.35 \\
-0.57 & -0.37 & 1.04 & -0.34 & 0.48 & -0.07 & 0.88 & -0.42 & -0.1 & 1.23 & 0.75 & 1.14 \\
-0.19 & -0.02 & -0.11 & 0.34 & 0.48 & -0.34 & 0.68 & 0.04 & -0.55 & 0.27 & 1.03 & 0.45 \\
0.57 & -0.01 & -2.5 & 0.33 & -0.29 & 0.37 & 0.57 & 0 & -1.17 & -0.13 & 0.74 & -0.99 \\
1.58 & -0.88 & 3.93 & -1.28 & -2.7 & -0.6 & -1.8 & -0.59 & 1.14 & -1.37 & 5.15 & -4.56 \\
0.55 & 0.11 & 0.17 & -0.47 & -1.83 & -0.38 & -1.53 & 0.28 & 0.96 & -1.17 & -4.49 & -3.2 \\
-0.95 & -1.22 & 6.97 & -1.08 & -1.19 & -0.42 & -1.03 & -0.59 & -1.04 & -1.52 & -34.7 & 1.95 \\
-1.5 & -0.58 & 2.9 & -0.22 & 1.64 & -0.35 & 1.33 & -1.06 & -1.78 & -0.35 & -16.1 & 4.03 \\
-0.55 & -0.17 & -0.21 & -0.06 & 0.55 & 0.72 & -0.01 & 0.25 & -0.23 & -0.31 & 31.7 & 0.88 \\
-0.01 & 0.02 & -0.05 & 0.11 & -0.09 & -0.04 & -0.33 & 0.14 & 0.22 & -0.12 & 0.21 & -0.14 \\
-0.07 & -0.07 & -0.24 & 0.09 & -0.06 & -0.06 & 0.18 & 0.27 & -0.1 & 0.32 & -0.05 & 0.06 \\
0.31 & 0.1 & -0.82 & 0.4 & 0.82 & -0.4 & 0.9 & 0.12 & -0.18 & 0.94 & -0.32 & 1.74 \\
0 & 0.02 & 0.03 & 0.25 & -0.09 & -0.08 & 0.11 & -0.26 & 0.55 & 0.32 & -2 & 0.86 \\
-0.02 & -0.11 & -0.1 & -0.03 & 0.81 & -0.08 & 1.26 & 0.22 & -0.14 & 0.57 & -1.49 & -0.72 \\
-0.56 & 0 & -2.65 & -0.34 & -0.72 & -0.06 & -0.02 & 0.12 & -0.23 & -0.59 & -2.21 & 1.11
\end{pmatrix}$$

The weight matrix W_{JK} between the 12 units of hidden layer (J) and 2 units of output layer (K) was :

$$W_{jk} = \begin{pmatrix} 2.03 & 0.19 \\ 0.85 & 0.31 \\ -3.33 & -0.3 \\ 0.76 & -0.38 \\ 0.81 & -1.46 \\ -0.79 & -0.61 \\ 0.99 & -1.68 \\ 0.55 & -0.34 \\ 1.2 & 1.99 \\ 0.9 & -0.57 \\ -7.96 & -0.01 \\ 2.41 & 0.51 \end{pmatrix}$$

The threshold values for the three layers of the neural network model were:

Input Layer:

{27.8,19,1.6,3.3,7.7,13.2,17.4,4.4,2.1,4.4,11.4,23.4,49.6,28.8,7.4,4.5,7.7,25.6,15.1,52.7, 31.9,35.6,1.5,1.6,7.1,4.6,5.5,8.6}

Hidden Layer: {-2.3,-3.6,-3.48,-3.57,-3.3,-2.83,-3.42,-3.18,-2.62,-3.63, 10.1,-2.99}

Output Layer: {5.42,-2.08}

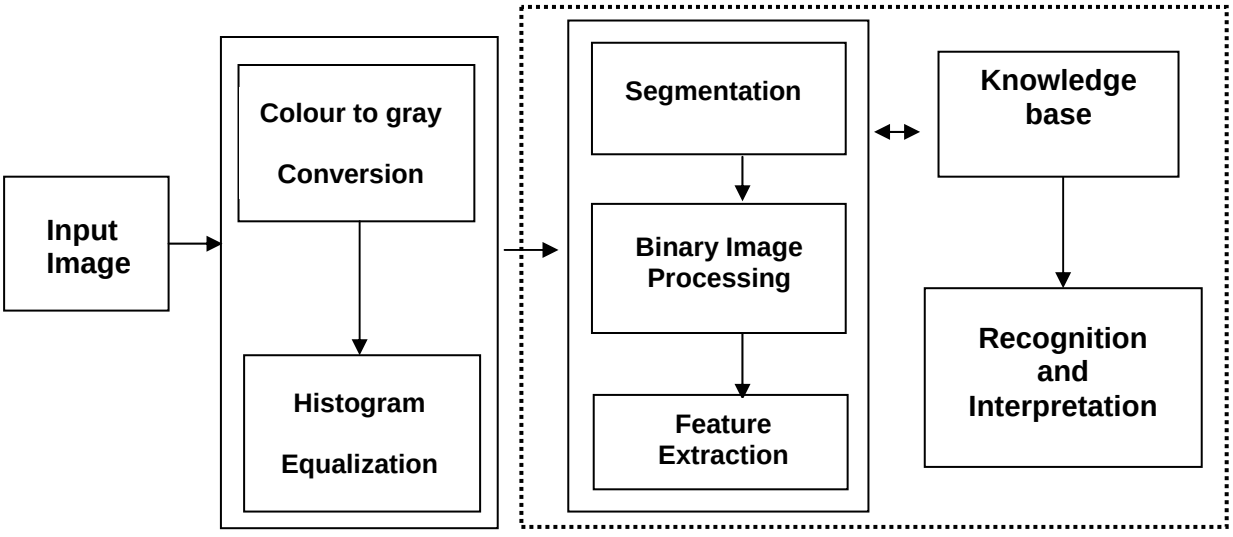


Figure 2.4. Schematic representation of the steps involved in image processing

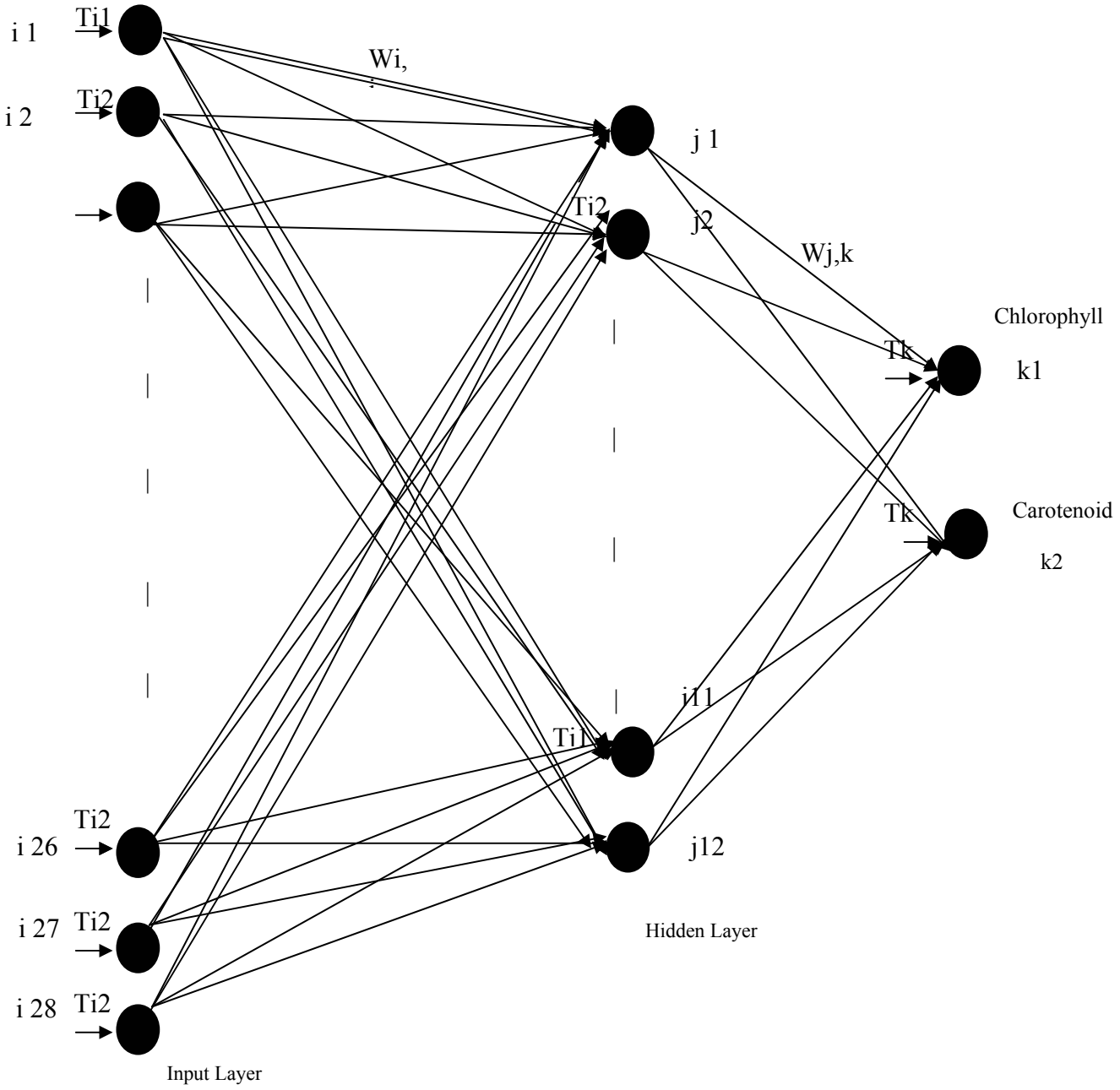


Figure 2.5. Back-propagation Neural Network Model

2.16. Strain improvement by mutagenesis

2.16.1. Isolation of H. pluvialis mutants

Mutation with UV: Five ml culture of the *H. pluvialis* with cell count of 1×10^6 cells/ml in logarithmic growth phase was exposed to UV irradiation (254nm) in an open petri dish using UV lamp (CAMAG). Two set of cells, one for 15 and another for 30-minute duration, were irradiated from a distance of 10 cm.

Mutation with ethyl methane sulphonate (*EMS):* One ml culture of *H. pluvialis* (cell number of 1×10^6 cells/ml) was washed with 0.2 M phosphate buffer and treated with EMS in the concentration range of 0.1M to 0.2M level for 30 minutes.

Mutation with 1-methyl 3-nitro 1-nitrosoguanidine (NTG): One ml culture of *H. pluvialis* (cell number of 1×10^6 cells/ml) was washed with 0.2 M phosphate buffer and treated with NTG at 0.1mM to 0.6M concentration for 60 minutes. After the treatment with chemical mutagen, the cells were washed thrice with 0.2M phosphate buffer. Both the irradiated and mutagen treated cells were stored overnight at 7°C. Later the cultures were grown in BBM for 3 days under 1.5 klux light intensity. The cells grown were plated on BBM incorporated with 28μM herbicide glufosinate [2-Amino 4(hydroxy methyl phosphinyl) butanoic acid]. The cultures were incubated at 25 ±1°C under 1.5 klux light intensity.

The colonies grown in petri dishes were transferred onto the BBM slants based on colony characteristics and colour. After growth for 15 days in slants the cultures were transferred to liquid BBM. Their growth was monitored in CO_2 enriched medium as explained in the section 2.2.2.

After two weeks of growth, one set of flasks were harvested for cell count and biomass estimation while second set of flasks were incubated further two weeks under high light (3.5 klux) for carotenoid accumulation.

2.16.2. Analysis of carotenoid profile

The carotenoid from mutants was extracted, estimated and analysed by TLC and HPLC as explained in the section 2.5, 2.6 and 2.7.

2.16.3. Analysis of carotenoid profile under normal and stress conditions

The mutants were grown in Bold's basal medium in 150ml conical flask (culture volume -50ml) under normal growth conditions as explained in the section 2.2.1.

After growth for 15days, one set of cultures was supplemented with 42mM NaCl and subjected to stress condition as detailed in section 2.9.1. At the end of 15 days, carotenoid content was analyzed and compared with that of wild strain.

2.16.4. Measurement of photosynthetic activity

Photosynthetic activity was determined according to the method modified from Schwelitz et al (1972). To 5 ml of the algal suspension (equivalent to 25µg chlorophyll), 2 ml of dye solution [0.1mM of the dye 2, 6-dichlorophenol-indophenol and 0.01M KCl in 0.4 M potassium phosphate buffer (pH 6.5)] was added. This suspension was exposed to cool white fluorescent light (1.5 klux) and absorbance was recorded at 620nm every minute upto 12 minutes.

2.16.5. Fluorescence profile in presence of herbicide

The effect of herbicide glufosinate on the chlorophyll fluorescence of the mutants was tested. The chlorophyll content in the growing culture of mutants was estimated, centrifuged and normalized to get 25µg of chlorophyll per ml of culture. To this culture (3ml) herbicide glufosinate was added as to get final concentration of 200µM. Excitation wavelength was set at 400nm and emission was recorded in the range of 400-800nm up to 40min using spectrofluorophotometer (Shimadzu RF-5301PC). The variable fluorescence (Fv) was calculated according to Constant et al (1997), using the equation Fv= (Fmax-Fo)/Fo where Fmax is fluorescence intensity at particular time interval and Fo is initial fluorescence intensity. Fv of 1.6 was considered as 100% for calculations.

2.16.6. Determination of lycopene cyclase activity

Lycopene cyclase activity in the mutant cells was determined by the method of Schnurr et al (1996). *H. pluvialis* cells grown for 8 days (in the vegetative phase) and 15 days old stress induced [Stress induction by NaCl (42mM) and high light (3 klux) for 60 hours] cells were harvested and cell extract was used in the reaction mixture for enzyme assay. The culture was centrifuged and the wet biomass was extracted with 0.2M Tris-Maleate buffer (pH 6.8). The extract was centrifuged and supernatant was used for enzyme assay. The reaction mixture (final volume 0.5ml) contained 50µl of 5mM NADH and 150µl of lycopene in soybean oil (as substrate) and 300µl of enzyme. The incubation was done in dark at 30°C for 4 hours. Reaction was terminated by adding methanolic KOH. The mixture was extracted with diethyl ether and petroleum ether (1:1) mixture. Reaction

products were analyzed by HPLC as explained in the section 2.7. Lycopene cyclase activity was calculated in terms of β-carotene formed and was expressed as nmole of β-carotene formed/mg protein/hour. Protein content in the cell free extract was estimated by the method of Lowry et al (1951).

2.17. Expression analysis of carotenoid biosynthetic genes

2.17.1. RNA isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

To one week grown culture of wild and mutant strains, sodium chloride (34 mM) and Sodium acetate (4.4 mM) were added to induce carotenoid formation. The cultures were incubated under continuous light intensity of 3.5 klux for 48 hours. Thereafter, 1×10^8 cells from each culture were harvested, frozen under liquid nitrogen and subsequently powdered. Then total RNA was extracted using RNAqueous® kit according to instruction manual (Ambion, Austin, TX). Possible contaminant genomic DNA in RNA extract was removed using TURBO DNA-free™ kit (Ambion, Austin, TX). The concentration of total RNA was determined spectrophotometrically at 260 nm. Integrity of RNA was checked by electrophoresis in formaldehyde denaturing gels stained with ethidium bromide. The gene specific primers for phytoene synthase (PSY), phytoene desaturase (PDS), lycopene cyclase (LCY), β-carotene ketolase (BKT) and β-carotene hydroxylase (CHY) were designed using Primer3 software (Rozen and Skaletsky, 2000) (Table 2.3) and synthesized (Sigma - Genosys, Bangalore, India). First-strand cDNAs were synthesized from 0.2 µg of total RNA in 20 µl final volume, using M-MuLV reverse transcriptase and oligo-dT (18-mer) primer (Fermentas GmbH, Germany).

PCR amplifications were performed using PCR mixture (15 µl) which contained 1 µl of RT reaction product as template, 1× PCR buffer (Bangalore Genei, Bangalore, India), 200 µM dNTPs (Fermentas GmbH, Germany), 1 unit (U) of Taq DNA polymerase (Bangalore Genei, Bangalore, India), 0.1 µM of each primer depending on the gene. PCR was performed at initial denaturation at 94°C for 4 min, 30 or 22 cycles (1 min at 94°C; 1 min at 55 or 60°C or 20 s at 61°C; 1 min at 72°C) and final elongation (10 min at 72°C) using a thermal cycler (Eppendorf Thermal cycler, Germany). The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%) and documented in a gel documentation system (Herolab GmbH Laborgerate, Germany). The size of the amplification products was estimated from 100 bp DNA ladder

Table 2.3. Specific primers, annealing temperatures and total numbers of amplification cycles used for RT-PCR

Primer	Primer sequence (5'–3')	Annealing temperature (°C)	Total number of amplification cycles	GenBank ID / reference	Amplicon size (bp)
PSY-forward	ATGTACCATCCCAAGGCAAG	60	30	AY835634	402
PSY-reverse	CTGGACCAGGCCTACGAC				
PDS-forward	TCCATGATCTTTGCCATGC	60	30	AY768691	462
PDS-reverse	CGGGAGTTGAACATGAGGTC				
LCY-forward	CTTCTTCTCCGCCTTCTTCA	60	30	AY182008	565
LCY-reverse	GCATCCTACCGCTCAAAGAA				
BKT-forward	CATCTCCTTGTACGCCTGGT	55	30	X86782	423
BKT-reverse	CAGTGCAGGTCGAAGTGGTA				
CHY-forward	CTACACCACAGCGGCAAGTA	55	30	AF162276	521
CHY-reverse	GCCTCACCTGATCCTACCAA				
ACT-forward	AGCGGGAGATAGTGCGGGACA	61	22	Huang et al (2006)	200
ACT-reverse	ATGCCCACCGCCTCCATGC				

(Fermentas GmbH, Germany). The band intensity of each gel was checked using the Herolab E.A.S.Y Win32 software (Herolab GmbH Laborgerate, Germany). Transcript levels of each gene in control cells were taken for comparison in calculating the transcript abundance of respective genes of mutants under stress conditions. To normalize the RT-PCR data, each gene was compared with actin transcript, whose expression was constant under all culture conditions (Eom et al, 2005; Huang et al, 2006).

2.18. *In vitro* and *in vivo* biological activity of astaxanthin

2.18.1. Preparation of astaxanthin samples

Total Carotenoid from *H. pluvialis* biomass was extracted by homogenizing with acetone as described in the section 2.5. This cell free extract was designated as total carotenoid. TC was subjected to preparative thin layer chromatography using the solvent system Acetone: Hexane (3:7, v/v), (Fiksdahl et al, 1978) and separated ester bands were scraped from TLC plates and resuspended in acetone. This fraction was designated as astaxanthin esters. Astaxanthin ester in acetone was saponified according to the method modified from Yuan and Chen (1999), to obtain free astaxanthin with equal volume of diethyl ether and 2% methanolic KOH for 4 hours at 0°C. To this suspension, 10% NaCl was added and repeatedly extracted with diethyl ether. Astaxanthin containing ether layer was collected and concentrated by vacuum pressure to remove residual ether. This fraction was redissolved in acetone and designated as saponified astaxanthin. Astaxanthin content in the above fractions was quantified as explained in section 2.5. All the above extractions were carried out in dark condition. The fraction containing vials were flushed with nitrogen and stored at 0°C till further use. Separation of astaxanthin esters from total carotenoid and complete saponification was confirmed by analyzing the samples by HPLC (section 2.7).

2.19. Animals and experimental groups

Healthy Albino Wistar rats of both sexes (175±25 g) used for the experiments were maintained under standard conditions of temperature, humidity, light and were provided with standard rodent pellet diet (M/s Sai Durga Feeds, Bangalore, India) and tap water *ad libitum.* The study was approved by the institutional ethical committee, which follows the

guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. 49, 1999), Government of India, New Delhi, India. All animals were grouped into 14 groups of 6 animals each and their body weight was checked regularly. Dosage of astaxanthin or omeprazole (a known antiulcer drug; protects ulcer by inhibiting H^+,K^+-ATPase enzyme) was calculated based on the body weight of animals and administered orally at specified concentrations. Astaxanthin was dissolved in groundnut oil (the vehicle) immediately before use at a volume of 1ml/kg b.w. The astaxanthin sample or normal saline were fed for a duration of 21 days. Group 1 – untreated healthy control groups were administered with normal saline throughout the experiment; Group 2 received ethanol without any treatment, hence considered as ulcerous group; Group 3, 4 and 5 were fed with total carotenoid - 100, 250 and 500µg/kg b.w. respectively and received ethanol at the end of 21 days. Group 6 and 7 were fed with total carotenoid- 250 and 500µg/kg b.w. respectively as total carotenoid controls. Group 8, 9 and 10 were fed with astaxanthin esters - 100, 250 and 500µg/kg body weight respectively and received ethanol at the end of 21 days. Group 11 and 12 were fed with astaxanthin esters - 250 and 500µg/kg b.w. respectively as astaxanthin ester controls. Group 13 and 14 were fed with omeprazole (20mg/kg body weight and groundnut oil (1ml/kg b.w.) respectively and received ethanol.

2.19.1. Gastric ulcer induction by ethanol and assessment of gastric mucosal injury

At the end of 21 days, all the rats were fasted for 18 h with free access to drinking water. Gastric ulcers were induced except healthy control and sample control groups by orally administering 100% ethanol (5 mL/kg b.w.) for 1 hour (Lee et al, 2006). All the animals were sacrificed by cervical dislocation. Sacrificing was done under ether anesthesia to ensure negligible stress to the animals. The stomach was removed, opened along the greater curvature, washed with normal saline stretched and flattened on a piece of cardboard. The inner surface was examined for mucosal integrity and occurrence of ulcers. The total number of mucosal lesions per stomach was counted, mean ulcer scores of each experimental group was calculated and expressed as ulcer index (UI), (Srikanta et al, 2007). Briefly lower to higher grading was assigned to milder to severe symptoms

respectively. Ulcer Score: 0.5 - red coloration, 1.0 - spot ulcers, 1.5 - hemorrhagic streaks, 2.0 - ulcers more than 3 mm and less than 5 mm, 3.0 – ulcers more than 5 mm.

2.19.2. Determination of gastric mucin content

Gastric wall content of mucin was determined in control rats as well as treated, ulcer induced groups according to the method described by Corne et al (1974). The glandular part of each stomach (0.5g) was placed 10ml of 1% alcian blue solution in 0.16 M sodium acetate (pH 5.8) for 2 h. The dye complex was extracted with 0.5M magnesium chloride solution, centrifuged and measured spectrophotometrically at 580nm.

2.19.3. Histopathological studies

Gastric tissue samples were fixed in 10% buffered formalin for 24 hours. The processed tissues were embedded in paraffin blocks and sections were made. These sections were stained with hematoxylin and eosin dye (Sibilia et al., 2003). The histochemical sections were evaluated by light microscopy (Leitz, Germany) at 10x magnification.

2.20. Determination of *in vivo* antioxidant enzyme activity

2.20.1. Determination of Superoxide dismutase (SOD) activity

The activity of SOD was assayed using Nitroblue tetrazolium (NBT) as the substrate. (Flohe and Otting, 1984). 100µl of 5% homogenate in phosphate buffer (pH 7.4) or serum was taken in Beckman quartz cuvette of 1cm path length. To this, 1ml of 50mM sodium carbonate, 0.4ml of 24µM NBT, and 0.2ml 0.1mM EDTA were added. The reaction was initiated by adding 0.4ml of 1 mM hydroxylamine hydrochloride. Zero time absorbance was taken at 560nm followed by recording the absorbance after 5 min at 25°C. The control was simultaneously run without homogenate or serum. One unit of the SOD enzyme activity is equivalent to 50% inhibition in the formazan formation in 1 min at room temperature and the results are expressed as units per mg of protein.

2.20.2. Determination of catalase activity

Catalase activity was determined according to the method of Aebi (1984). Decomposition of H_2O_2 on addition of 5% homogenate or serum was followed at 240 nm. One unit of catalase was defined as the amount of enzyme required to decompose 1µmole of H_2O_2

per minute at 25°C at pH 7.0. Results are expressed as units of catalase activity per milligram of protein.

2.20.3. Determination of glutathione peroxidase (GPx) activity

Glutathione peroxidase activity was estimated according to the method of Flohe and Gunzler (1984). The mixture containing 0.1ml homogenate or serum, 0.1ml of 10mM glutathione reductase (0.24U) and 0.1ml of 10mM glutathione was preincubated for 10min at 37°C and thereafter 0.1ml of NADPH solution was added. The hydroperoxide independent consumption of NADPH was monitored for 3 min. Overall reaction was started by adding 0.1ml of prewarmed hydroperoxide solution and the decrease in absorption at 340nm was monitored for 3min and the activity was expressed as nM of NADPH oxidized/min/mg protein.

2.20.4. Measurement of lipid peroxidation

Lipid peroxidation products (Thiobarbituric Acid Reactive Species; TBARS) in liver, stomach homogenates and serum were determined according to the method of Buege and Aust (1978). Thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a diadduct which can be detected spectrophotometrically at 532nm. To 1 ml of the above homogenates, 1 ml of 0.15M KCl and 0.1ml of 0.2mM ferric chloride was added to initiate peroxidation and incubated for 37°C for 30min. The reaction was terminated by adding 2 ml of an ice-cold mixture of TCA-TBA-BHT (15% Trichloroacetic acid, 0.3% Thiobarbituric acid, 0.05% Butylated hydroxy toluene in 0.25N HCl) and was heated at 80°C for 60 min. The reaction mixture was cooled, centrifuged and the absorbance of the supernatant was measured at 532nm. The results were expressed as MDA equivalents per mg of protein, which was calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

2.21. Determination of *in vitro* H^+, K^+- ATPase activity

Gastric membrane containing H^+, K^+- ATPase was prepared (Cheon et al, 2001) from mucosal scrapings of stomach of sheep obtained from local slaughter house, was homogenized in 20mM Tris-HCl buffer (pH 7.4). Homogenate was centrifuged for 10min at 5000×g and the resulting supernatant was subsequently centrifuged at 5000×g for 20

min. and the parietal cell extract thus prepared was used to determine the H^+, K^+- ATPase inhibition.

H^+, K^+- ATPase activity was assayed in the presence and absence of different doses of astaxanthin. The reaction mixture (1ml) contained enzyme in 20mM Tris-HCl pH7.4, 2mM $MgCl_2$, 2mM KCl. After preincubation for 2 minutes at 37°C, the reaction was initiated with the addition of 2mM ATP and the incubation was continued for 30min at 37°C. The reaction was terminated by the addition of ammonium molybdate and trichloroacetic acid mixture followed by centrifugation at 2000×g. The amount of inorganic phosphate released from ATP was determined spectrophotometrically (Yoda and Hokin, 1970) percent inhibition was calculated against maximal stimulation, and IC_{50} values were obtained from a typical dose response curve.

2.22. Determination of antioxidant activity *in vitro*

2.22.1. Determination of reducing power

The reducing power of astaxanthin fractions was determined according to the method of Yen and Chen (1995). Aliquots of astaxanthin were added to equal volume of 0.2M phosphate buffer (pH 6.6) and 1% potassium ferricyanide. The mixture was incubated at 50°C for 20min. Equal volume of 10% TCA was added to the mixture, which was then centrifuged at 3000×g for 10min. The supernatant was mixed with distilled water and 0.1% ferric chloride at a ratio of 1:1:2 (v/v/v). The absorbance was recorded at 700nm.

2.22.2. Determination of free radical scavenging ability

The free radical scavenging ability of astaxanthin fractions was determined by method of Lai et al (2001). Aliquots of astaxanthin samples were mixed with 0.8ml of 100mM Tris-HCl (pH 7.4) and then with 1 ml of 500μM 1,1-Diphenyl 2-picrylhydrazyl in methanol. The mixture was shaken vigorously and allowed to stand at room temperature for 20 min. Changes in absorbance were measured at 517nm.

2.22.3. Lipoxygenase inhibitory assay

Lipoxygenase activity was measured according to the method of Shobana and Naidu (2000). The reaction mixture (final volume -1 ml) in the sample cuvette contained 134μM linoleic acid, 150U soybean lipoxygenase enzyme in 50mM borate buffer (pH 7.4). Increase in absorbance was measured spectrophotometrically at 234nm. Astaxanthin

fractions- total carotenoid, astaxanthin esters, saponified astaxanthin and synthetic astaxanthin were added as DMSO solutions (final DMSO concentration of 1.6%); DMSO alone was added in control experiments. The enzyme solution was kept on ice, and controls were measured at intervals throughout the experimental periods to ensure that the enzyme activity was constant. Soybean lipoxygenase enzyme was pre-incubated for 2 minutes with different concentrations of astaxanthin samples prior to initiation of the reaction with substrate, linoleic acid. Appropriate corrections were made for dilutions and for absorbance of the reaction product. The IC_{50} value was determined by plotting a graph with astaxanthin concentration versus percent inhibition of lipid peroxidation.

2.23. Pigmentation efficiency of astaxanthin in egg yolk

2.23.1. Preparation of poultry feed

Isonitrogenous and isocaloric basal diet was prepared as detailed in Table 2.4 and the process of feed preparation is as follows. The ingredients were ground and weighed individually and mixed in feed mixer for 15 minutes. A portion of the feed mix was supplemented with dry *H. pluvialis* cells and pulverized in dark conditions. A known quantity of the powdered feed supplemented with *H. pluvialis* cells containing astaxanthin equivalent to 2 and 4 mg/kg diet was packed into white coloured capsules and fed orally to layers. This step was taken to ensure the feeding of total quantity of algal cells with required carotenoid content. Astaxanthin extract, instead of *H. pluvialis* cells, was mixed in the diet and fed at 0.5 mg/kg level as mentioned above. The composition of each diet was: Diet 1- Basal diet (control); Diet 2- Basal diet + *H. pluvialis* biomass (carotenoid equivalent) 2 mg/kg ; Diet 3- Basal diet + *H. pluvialis* biomass (carotenoid equivalent) 4mgkKg ; Diet 4- Basal diet + *H. pluvialis* extract (carotenoid equivalent) 0.5mg/kg.

2.23.2. Pigmentation test on laying hens

Twenty four laying hens (Single comb white leghorn layers) of 20 weeks old were randomly divided into four groups and housed in wire cages with three birds in each cage. The cages were situated in a shed in which 16h light per day was provided including day light. Feed and water were provided at *ad libitum*. The feeding and watering of hens were done during the morning hours and eggs were collected thrice a

day. All the groups were fed with respective experimental diets for a period of 4 weeks. Eggs were collected after 2 weeks of commencement of feeding for internal egg quality evaluation.

Table 2.4. Composition of basal layer diet

Ingredient	**g/100g**
Yellow maize	58
Deoiled groundnut cake	7
Deoiled soya meal	2
Deoiled sunflower meal	12
Fish meal	12
Shell grit	4.925
Mineral mix	3.0
Salt	0.25
Dicalcium phosphate	0.8
Synthetic L- lysine	0.01
Synthetic DL-methionine	0.015

2.23.3. Internal egg quality evaluation

Eggs from each treatment were evaluated for their physical characteristics and internal qualities like egg weight, shell thickness, albumen index, Haugh Unit score, USDA grade percent thin and thick albumen, yolk color score and yolk index. The eggs were broken, using an egg breaking table with a glass pan, on which albumen height, yolk height, widths and air cell height were measured with the help of AMES micrometer (B.C. AMES, Co, Waltham, Mass, USA), and the width with vernier calipers (Mitu toys, Japan). Haugh units score and USDA grade were calculated using interior egg quality calculator for eggs (Catalog 4-4200, American Instruments, Co, Inc, Silver spring, MD, USA). Similarly, yolk color was measured by a Roche yolk color fan (Roche, Australia; Vuillenmier, 1969), shell thickness by AMES shell thickness measuring gauge (B.C. AMES, Co, Waltham, Mass, USA)

2.23.4. Colouration of egg yolk

Colour of the egg yolk is an important parameter in determining the quality of the egg. Hence the egg yolk colour was measured by subjective colour evaluation and reflectance colorimetry as described below.

By subjective colour evaluation

Fresh yolks were placed in a glass petri dish over a white background and colour was determined visually by comparison with the Roche yolk colour fan (colour range 0-14).

By reflectance colorimetry

Glass petri dish having the fresh egg yolk was placed on the port of colorimeter (Lab Scan XE) with port size 1.2 inch, C illuminant and 2° view angle. Readings were taken at three positions by rotating the petri dish 90° each time.

2.23.5. Carotenoid analysis

Carotenoid content in the *H. pluvialis* biomass, used in feed preparation, was estimated as explained in the section 2.5.

Egg yolk was carefully separated from the albumen, weighed and one gram of yolk was taken for carotenoid extraction. Yolk was repeatedly extracted with acetone, pooled and the carotenoid content was estimated by AOAC method (1999).

2.24. Pigmentation in ornamental fish

Skin pigmentation is important factor in ornamental high value species such as Koi carp and gold fish together with body shape, size and fin shape. Hence, the efficacy of the astaxanthin rich *H. pluvialis* biomass in producing skin colouration was tested.

2.24.1. Preparation of experimental diet

The experimental fish feed was prepared by homogenizing encysted dry *H. pluvialis* cells equivalent to 5ppm and 25ppm carotenoid level with mixture of rice bran and groundnut cake (1:1).

2.24.2. Experimental design and fish rearing system

The koi carp fishes -*Cyprinus carpio* (approximately 6 weeks old) were collected from Alanahalli, a local fish farm, Mysore, Karnataka. Each group consisted of 12 fishes. These fishes were allowed for acclimatization in 70L capacity glass tank for 2 weeks before commencement of experiment. Water temperature was maintained at 25° ± 2 and

natural photoperiod was used during the trial. The experimental feed was fed *ad libitum* to fishes for a period of 3 months. Weight of the fishes was recorded every 15 days.

2.24.3. Colour measurement

At the end of 12 weeks of feeding the experimental diet, fishes were taken for carotenoid estimation. Skin colouration was measured using colour measurement system (Lab Scan XE) with port size 1.2 inch, C illuminant and 2° view angle.

2.25. Statistical analysis

All data values were expressed as means (± SD). Statistical evaluation of *in vivo* and pigmentation experiments was carried out with two-way analysis of variance (ANOVA). The significance ($p < 0.05$) of the variables studied was assessed by simple student 't' test using Microsoft® Excel. The mean separations were performed by Duncan's multiple range test for segregating means where the level of significance was set at $p \leq 0.05$ (Harter, 1960).

Chapter 3
Results and Discussion

Growth and carotenoid production under autotrophic condition.

Background

Microalgae of commercial importance are generally cultivated in autotrophic conditions to exploit their photosynthetic potential for production of value added products in an economical way. Although some species of microalgae are able to grow in autotrophic, mixotrophic and heterotrophic conditions, their growth in autotrophic conditions is more advantageous because the organism can be grown with minimal contamination, growth can be achieved in outdoor conditions and it is more economical than heterotrophic conditions. The adaptability of the organism to different environmental conditions varies among different microalgae. Therefore every organism of commercial importance has to be studied in detail to understand the influence of both cultural and environmental factors for achieving maximum growth and metabolite production. *Haematococcus pluvialis*, a green microalga is able to grow in both heterotrophic and autotrophic conditions. It has two distinct phases in its life cycle (a) motile vegetative growth phase and (b) immotile encysted carotenoid accumulation phase. Both the phases differ in their optimum culture conditions. Therefore it is relevant to study the influence of different culture conditions for growth and carotenoid accumulation in *H. pluvialis* under autotrophic conditions. The present study focused on various aspects of growth, influence of different ammonia salts on growth and carotenoid formation, effect of plant growth factors, influence of stress factors on carotenoid formation, cultivation of *H. pluvialis* in open and closed prototype bioreactors, drying of harvested biomass, storage stability of algal biomass and image processing based method for estimating the carotenoid content in *H. pluvialis* cells.

3.1. Maintenance of *Haematococcus pluvialis* stock culture

Haematococcus pluvialis stock cultures were maintained on agar slants of Bold's basal medium (BBM) as explained in section 2.2. (Fig 3.1A). Liquid cultures were maintained under normal culture condition (section 2.2.1; Figure 3.1B) and after 15 days of growth period, exposed to 3.5 Klux to induce carotenoid accumulation (Figure 3.1C).

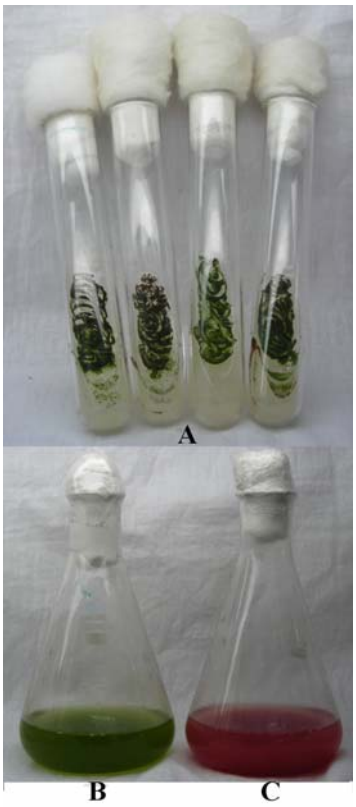


Figure 3.1. Maintenance of *Haematococcus pluvialis* stock culture

A. Slant culture
B. Vegetative growth phase
C. Carotenoid accumulated encysted phase

3.2. Effect of ammonium salts on *H.pluvialis* growth

Utilization of ammonium salts as a nitrogen source by *H. pluvialis* was studied in autotrophic Bold's basal medium (BBM) and BBM with $NaNO_3$ served as control. Growth profile of *H. pluvialis* under the influence of ammonium chloride, ammonium carbonate, ammonium sulphate and urea at ambient CO_2 are shown in Figure 3.2.

The cells were found to be motile upto 48 hours at all concentrations of ammonium chloride tested, and later at higher concentrations, 50% of the cells lost motility. Only at 0.25 g/L concentration, the growth in terms of cell number (21×10^4 cells/ml) was comparable to control and at higher concentrations the cell number per ml decreased (Figure 3.2). Ammonium carbonate supported growth comparable to control at 0.5g/L concentration, while decrease in the cell number was observed at higher concentration.

Supplementation of culture with ammonium acetate rendered algal cells immotile and cell lysis was observed within 48 hours. Thus ammonium acetate was not found suitable for growth at ambient CO_2 level, whereas in ammonium sulphate fed cultures the cell number was found to be 2 to 3 fold high compared to control at 0.15g/L concentration. However at higher concentrations, growth was inhibited. At 0.15g/L level of urea, the growth was comparable to control and a two fold increase was observed at the end of experimental period. At higher concentrations of urea (0.5 –1.0g/L), the cells lost motility, entered the encystment phase and growth was significantly affected (Figure 3.2).

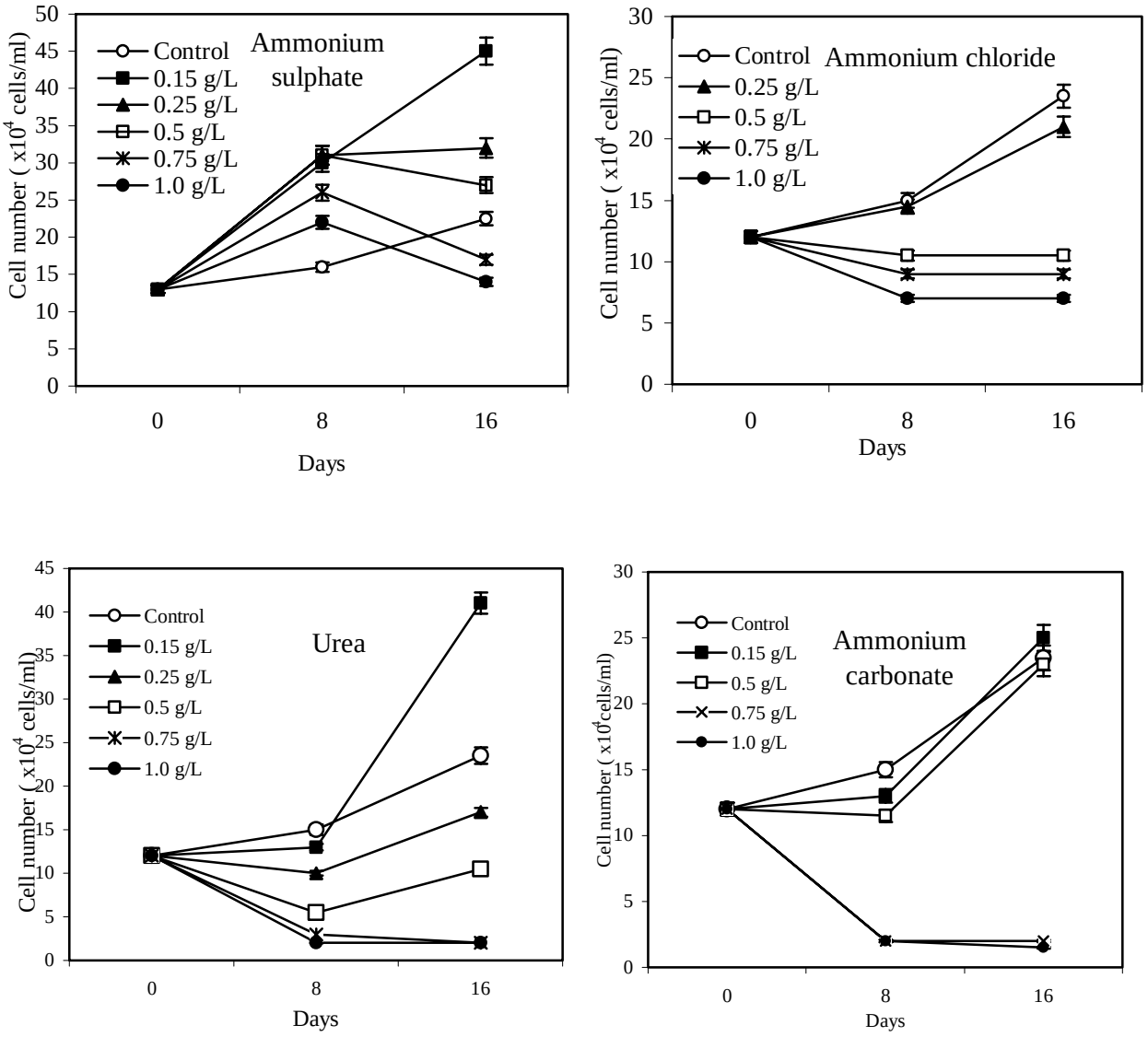


Figure 3.2. *H. pluvialis* growth profile in different concentrations of ammonium salts at ambient CO_2.

The results obtained for *H. pluvialis* growth with different ammonium salts in CO_2 enriched environment are shown in Table 3.1 and Figure 3.3A. Cell number of 80-100× 10^4 cells/ml was obtained with different ammonia salts (ammonium carbonate, ammonium acetate) which is 8-10 fold increase over initial cell count (Table 3.1) when compared to only 2-4 fold increase in cell number observed at ambient CO_2 level (Figure 3.2). The ammonia concentration in the *H. pluvialis* culture was found to be decreased to 9% of the initial level, which shows almost 90% of NH_3 utilization. The cells grown in urea turned brownish yellow colour after 6 days indicating early induction of carotenoid formation. In the second set of flasks, which were exposed to high light, the cells were encysted and accumulated carotenoids.

Table 3.1. Total carotenoids and astaxanthin content in *H. pluvialis* cells grown in media containing different ammonium salts in the presence of 2% CO_2.

	Cell number (×10^4cells/ml)	**Total carotenoids (% w/w)**	**Astaxanthin (% w/w)**
BBM	80 ± 2.29	2.27 ± 0.05	1.97 ± 0.05
Ammonium carbonate (0.15g/L)	94 ± 2.25	2.12 ± 0.04	1.85 ± 0.03
Ammonium acetate (0.25g/L)	100 ± 2.71	1.57 ± 0.04	1.36 ± 0.03
Ammonium sulphate (0.15g/L)	55 ± 2.25	0.43 ± 0.04	0.36 ± 0.02
Urea (0.15g/L)	77 ± 1.26	1.02 ± 0.03	0.82 ± 0.03
Suphala (1.0g/L)	64 ± 1.80	2.36 ± 0.04	2.1 ± 0.06
Diammonium phosphate (1.0g/L)	82 ± 1.5	2.12 ± 0.05	1.87 ± 0.04

Initial cell number- 12 ×10^4 cells ml^{-1}.

Data recorded after 4 weeks

The biomass obtained in different ammonium salts was in the range of 2.1 - 3.1 g/L which was comparable to control (Figure 3.3A) except in urea (0.15 g/L level) and ammonium sulphate (0.15 g/L level) where the biomass yield was significantly less. In the case of DAP there was 10% increase in biomass production compared to control. Although, the cell number was high (100×10^4 cells/ml) on ammonium acetate supplementation (Table 3.1), there was no corresponding increase in the biomass.

Total carotenoid content in ammonium carbonate, suphala, DAP supplemented cultures was found to be in the range of 2.1 –2.3 % w/w (Table 3.1) which was comparable to control. It was significantly less in urea (1.02%) and ammonium sulphate (0.43%) supplemented cultures. However, the astaxanthin content was in the range of 85 - 88% of the total carotenoid in all the cultures.

Total carotenoid and astaxanthin productivity was high (63-66mg/L) in BBM and commercial salts followed by ammonium carbonate (46mg/L) and ammonium acetate (37mg/L)and least in urea (3.6mg/L; Figure. 3.3B).

3.3. Utilization of ammonia and influence of L- methionine DL- sulfoximine (MSX) and azaserine on *H.pluvialis* growth and astaxanthin production

This study was carried out to know the possible pathway of ammonia assimilation by *H. pluvialis*. MSX and azaserine are inhibitors of glutamine synthetase (GS) and 2-oxoglutarate amido transferase (GOGAT) respectively, the enzymes involved in ammonia assimilation. MSX and azaserine were added individually(at 1mM level) to the 3 day old cultures of *H. pluvialis* in ammonium carbonate (0.15g/L) supplemented culture and allowed to grow in CO_2 rich atmosphere as described in section 2.2.2.

Both MSX and azaserine inhibited the growth of *H. pluvialis* at 1mM level. However, at 0.1mM concentration biomass yield obtained was that of 32-40% of control (Table 3.2). As in control, the cells accumulated carotenoid with 85% astaxanthin. But when compared to control, carotenoid content was found to be 25-40% less in MSX and azaserine respectively (Table 3.2).

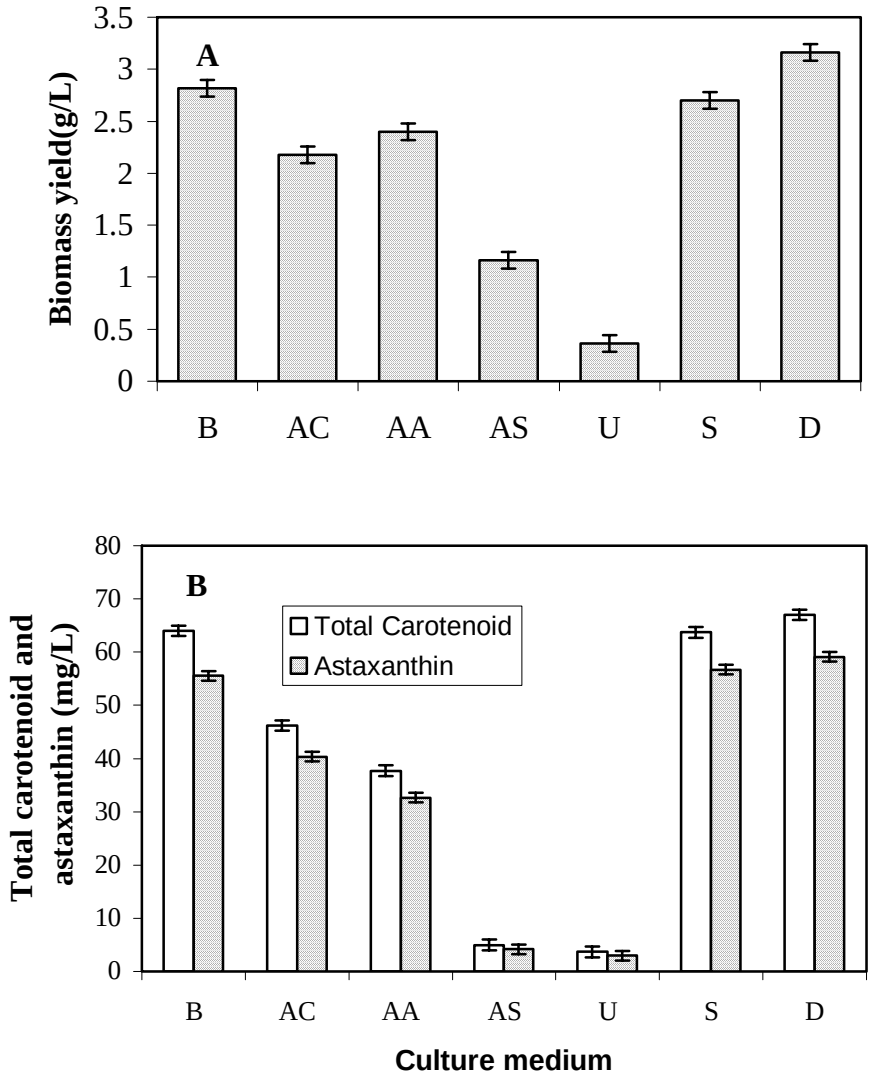


Figure 3.3. Growth and carotenoid production by *H. pluvialis* on CO_2 supplementation

A. Biomass yield

B. Total carotenoids and astaxanthin production of *H. pluvialis*

B-BBM, AC- Ammonium carbonate (0.15g/L), AA-Ammonium acetate (0.25g/L), AS- Ammonium sulphate (0.15g/L), U-Urea(0.15g/L), S- Suphala (1.0g/L), D - Diammonium phosphate (1.0g/L)

Table 3.2. Effect of L-methionine DL-sulfoximine(MSX) and azaserine on *H. pluvialis* growth and carotenoid production.

	Control	Azaserine	MSX
Biomass (g/L)	2.7 ± 0.08	0.88 ± 0.02	1.1 ± 0.02
Total carotenoid content (% w/w)	2.27 ±.0.05	1.43 ± 0.04	1.66 ± 0.05
Astaxanthin (% w/w)	1.97 ± 0.06	1.2 ± 0.03	1.5 ± 0.03
Total carotenoid production (mg/L)	52 ± 1.82	12.58 ± 0.51	18.26 ± 0.64
Astaxanthin production (mg/L)	47 ± 1.18	10.56 ± 0.35	16.5 ± 0.42

Data recorded after 4 weeks.

3.4. Supplementation of plant growth regulator

The effect of supplementing plant growth promoters such as 6-benzyl amino purine, gibberellic acid on *H. pluvialis* cells growth and carotenoid formation was evaluated. The biomass yield obtained by the use of plant growth promoters is shown in Figure 3.4. There was 23% increase in the biomass with BAP while 18% increase was observed with BAP and GA (each at 1.25mg/L) in combination as compared to control. Total carotenoid and astaxanthin content was found to be 35% higher with combination of BAP and GA compared to control without growth regulators

3.5. Effect of stress factors on carotenoid production

This study was taken up to understand the influence of stress factors on carotenogenesis. After the growth phase of *H. pluvialis*, different levels of the salinity stress and light requirements were studied in presence of carbon source.

The two week old *H. pluvialis* cultures exposed to 3.5 Klux light intensity and treated with both sodium acetate (10mM) and NaCl (42mM) accumulated 42% higher astaxanthin as compared to control (Figure 3.5). However, only 17-20% increase was observed when cultures were treated with sodium acetate and NaCl independently. When trials were conducted using different concentrations of NaCl, cell rupturing and damage was observed at higher concentrations (>85mM). Growing cells were found to be more sensitive to higher concentrations of NaCl compared to stationary phase cells.

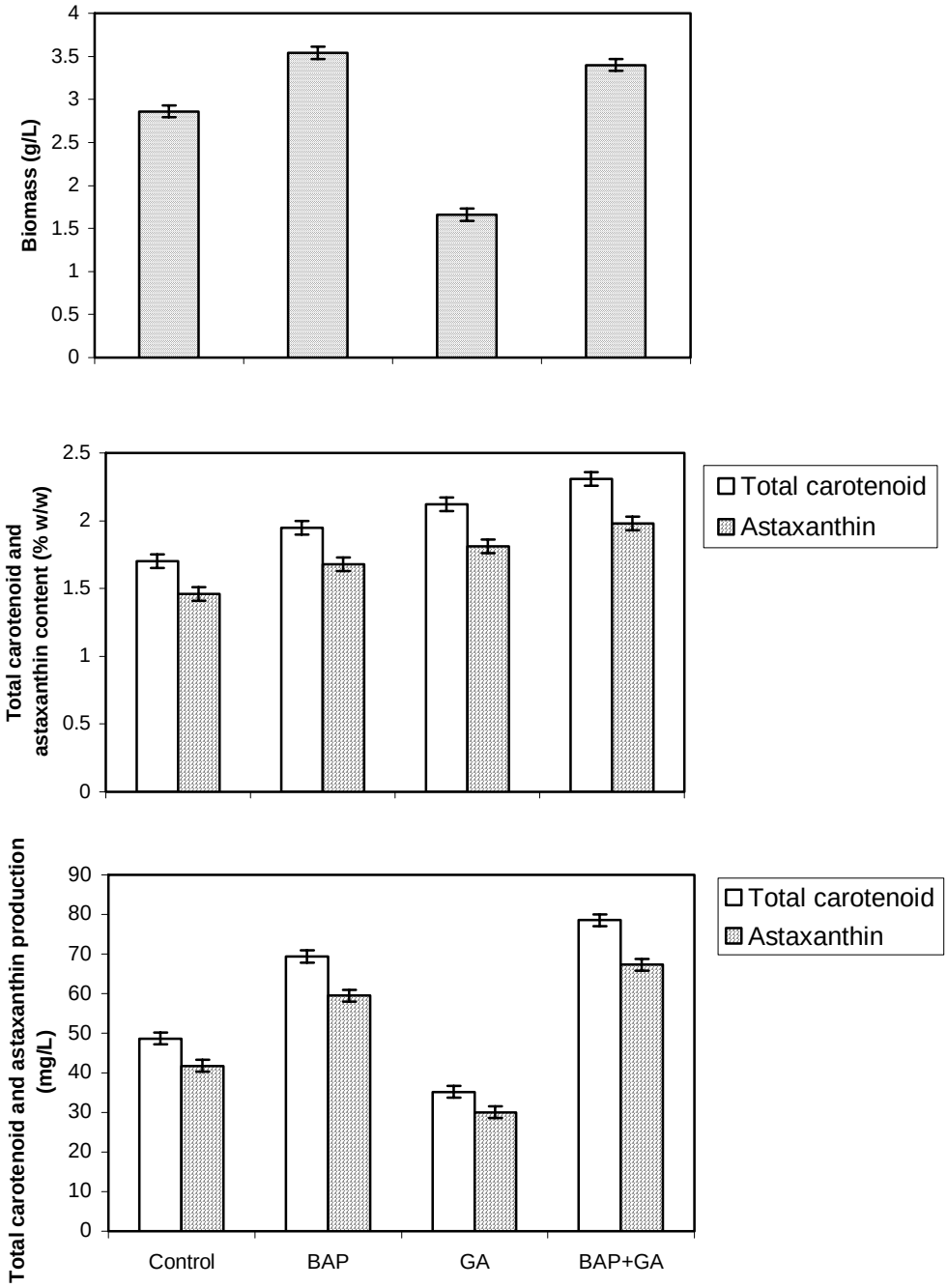


Figure 3.4. Growth and carotenoid production in *H.pluvialis* in presence of plant growth promoters- benzyl amino purine (BAP; 1.25mg/L) and gibberellic acid (GA_3;1.25mg/L).

Stress was also induced by addition of reactive-oxygen-generating reagent methyl viologen (MV) which was used at a final concentration of 0.01nM. The effect was evaluated in presence and absence of NaCl (42mM). The oxidative stress generated by MV supplementation was comparable with NaCl stress (Figure 3.7). MV along with NaCl produced 1.39%(w/w) total carotenoid which did not differ much in comparison to NaCl supplemented culture(1.45 %w/w).

The two week old *H. pluvialis* cultures were exposed to sunlight and supplemented with sodium acetate (10mM) or CO_2 (2% v/v mixed with air). As shown in Figure 3.6, higher total carotenoid (1.49%w/w) and astaxanthin (1.27%w/w) content was obtained in cultures, which utilized CO_2 as carbon source. CO_2 absorption appeared to be time dependent as reflected by 37% increase in astaxanthin content in 15 days period than that in 7 days period (Figure 3.6).

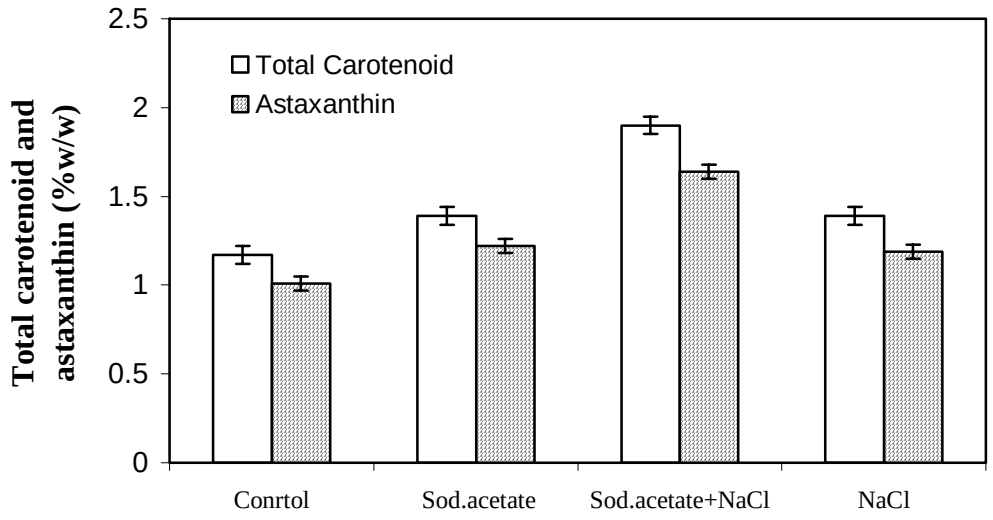


Figure 3.5. Total carotenoid and astaxanthin content in *H.pluvialis* under salinity stress.

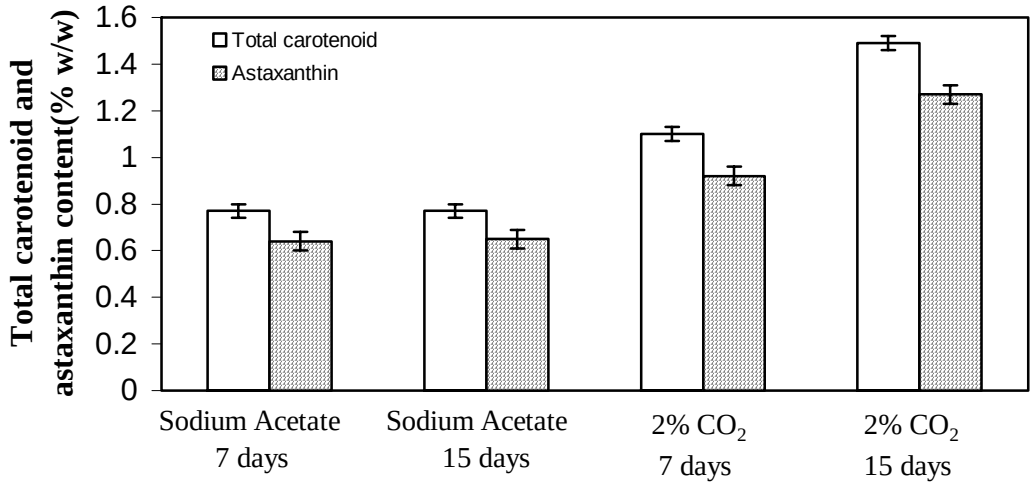


Figure 3.6. Total carotenoid and astaxanthin content in *H.pluvialis* exposed to sunlight.

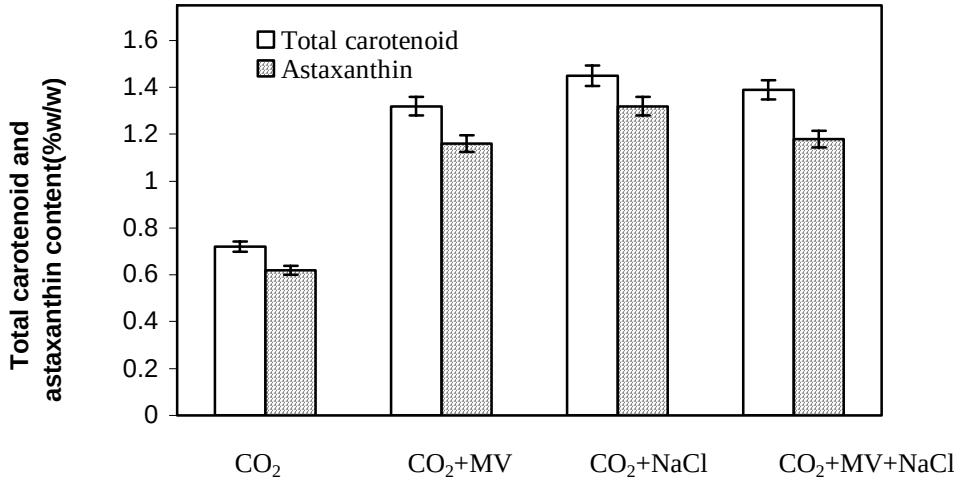


Figure 3.7. Carotenoid content under stress conditions
NaCl-(42mM), MV= Methyl viologen (0.01nM)

3.6. Cultivation of *H. pluvialis* in open and closed prototype bioreactor

The feasibility of open and closed prototype bioreactors for cultivating *H. pluvialis* was evaluated in the present study. The inoculum was developed in 500ml conical flasks and then in 10 L capacity carboys (Figure 3.8). Modified BBM was used for cultivation. CO_2 supply system was studied as a parameter since inorganic carbon intake is major

limitation in algal cultures. The prototype with different designs has been shown in Figure 3.9.

The bioreactor provided with CO_2 float (Design 1) was found useful for efficient gas-liquid mass transfer as indicated by maximum cell number (49×10^4cells/ml). The design 1 and 4 showed almost 3-4 fold growth in terms of cell number during a span of 12 days (Figure 3.10a and d). The growth rate of *H. pluvialis*, biomass and carotenoid content obtained in different designs is shown in Table 3.3. CO_2 bubbling (~2%v/v mixed with air) caused significant variation in the pH of the culture. This pH variation has affected the cell growth as indicated by the low cell number and less biomass yield (Figure 3.10b and Table 3.3). Settling of the cells was also observed at the bottom of the bioreactor due to pH variation.

Figure 3.8. Inoculum development in 10L capacity carboy

Stirrer was provided in the design 3 and 4 (Figure 3.9C) with the purpose to facilitate gas-liquid mass transfer. It was intended that it would help in removal of dissolved O_2 produced by photosynthetic activity and ambient CO_2 could be absorbed into the culture. The design 4 with float along with stirrer was useful for growth as indicated by maximum growth rate (μ) of 0.09 d^{-1} (Table 3.3). Though occasional stirring

of the culture prevented settling and adhesion of the cell to the reactor walls (Design 3), the growth rate (μ) and biomass productivity was less in comparison to other designs.

H. pluvialis was cultivated in closed prototype bioreactors- polyethylene bags, polyethylene tubular sleeves and tubular glass photobioreactor (Figure 3.11). The air space was filled with 2% (v/v) CO_2 mixed with air. The growth profile of *H. pluvialis* in these bioreactors is shown in Figure 3.12A-C.

H. pluvialis was cultivated in prototype raceway tank of 150 L capacity tank with culture volume of 40 L. Occasional stirring and carbon dioxide (~2%v/v mixed with air) was provided through a float. This prototype produced maximum cell number of 46×10^4cells/ml (Figure 3.12D)

3.7. Astaxanthin formation under outdoor conditions

After growth phase for 12-15 days, the cells were exposed to sunlight for carotenoid formation for a period of 5 – 8 days. The cells were subjected to salinity stress (42mM) along with sodium acetate (10mM). The cells were exposed to sunlight. Water circulation was provided around the bioreactors to prevent increase in culture temperature due to sunlight and to maintain ambient temperature. The biomass, growth rate of *H. pluvialis* and carotenoid content obtained in prototype reactors is shown in Table 3.4.

The prototype provided with CO_2 float along with stirrer (Design 4), produced maximum biomass of 0.72g/L which is almost 2 fold higher in comparison to design 3. Since the algal cells were found encysted and settling at the bottom of the in design 1, stress conditions were induced on 12^{th} day. Maximum total carotenoid (1.75 %w/w) and astaxanthin content (1.51 %w/w) was obtained in this design.

Figure 3.9. Cultivation of *H. pluvialis* in open prototype bioreactors
A. Design 1-CO_2 float B.Design 2- CO_2 bubbling
C. Design 4-Stirring +CO_2 float D. carotenoid accumulation in outdoor condition
E. Raceway tank (growth phase) F.Raceway tank (carotenoid accumulation phase)

Table 3.3. Carotenoid production by *H. pluvialis* grown in CO_2 supplemented prototype bioreactors

Design	**Growth rate (μ; day^{-1})**	**Biomass (g/L)**	**Biomass productivity ($g/m^2/day$)**	**Total Carotenoid content (% w/w)**	**Astaxanthin content (% w/w)**	**Astaxanthin productivity (mg/L)**
Design 1- CO_2 float	0.08	0.64 ± 0.03*****	2.66	1.75 ± 0.09*****	1.51 ± 0.08*****	9.7
Design 2- CO_2 bubbling	0.06	0.47 ± 0.02	1.68	1.68 ± 0.08	1.44 ± 0.07	6.8
Design 3- Stirring	0.05	0.38 ± 0.02	1.35	1.22 ± 0.06	1.05 ± 0.05	4.0
Design 4- Stirring +CO_2 float	0.09	0.72 ± 0.04	2.4	1.61 ± 0.08	1.41 ± 0.06	10.2

* Data recorded after 12 days of growth phase and 6 days of encystment phase design 1 Data recorded after 14days of growth phase and 6 days of encystment phase for rest of the designs

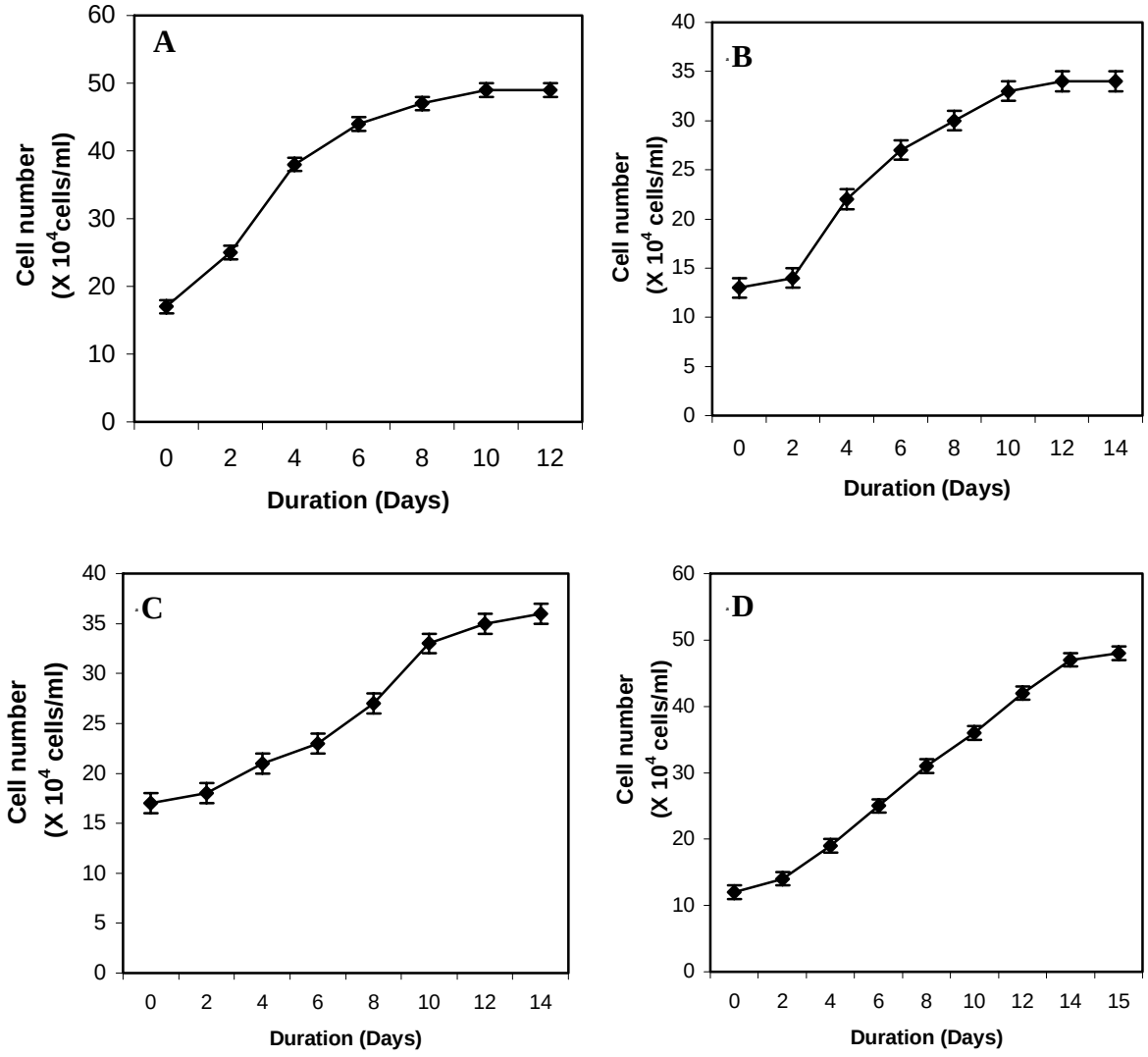


Figure 3.10. Growth profile of *H. pluvialis* grown in open bioreactor.

A. Design 1-CO_2 float B. Design 2- CO_2 bubbling

C. Design 3- Stirring D. Design 4- Stirring +CO_2 float

Figure 3.11. Cultivation of *H. pluvialis* in closed prototype bioreactors

A. Tubular polyethylene prototype B. Photobioreactor–Growth phase
C. Photobioreactor- carotenoid accumulation phase

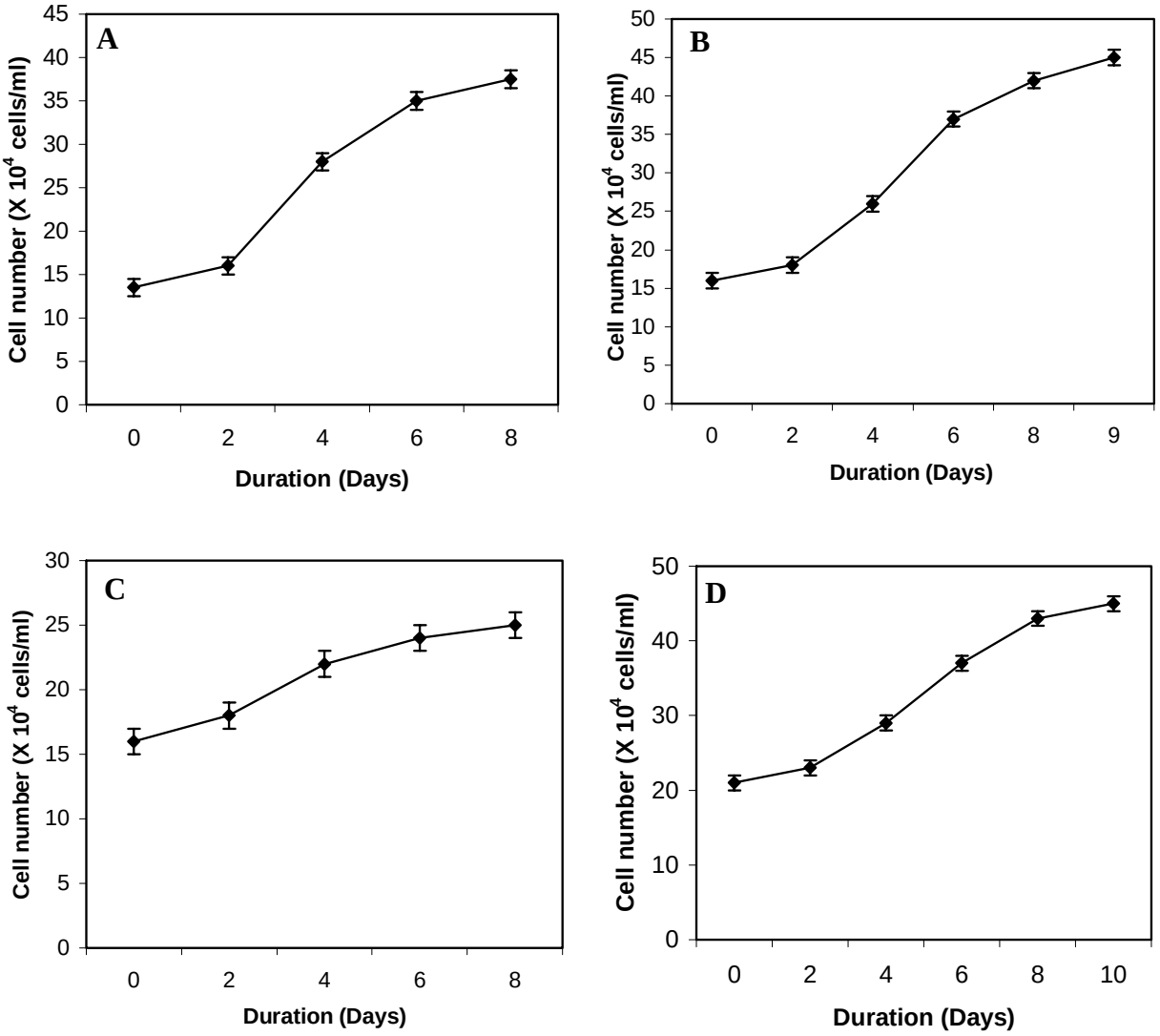


Figure 3.12. Growth profile of *H. pluvialis* grown in prototype bioreactors.

A. Tubular Polyethylene (Closed) B. Rectangular Polyethylene (Closed)

C. Photobioreactor (Closed) D. Raceway (Open)

Table 3.4. Growth and carotenoid production in open and closed prototype bioreactors

Reactor Type	Growth rate (μ; day^{-1})	Biomass (g/L)	Biomass productivity ($g/m^2/day$)	Total Carotenoid content (% w/w)	Astaxanthin content (% w/w)	Astaxanthin productivity (mg/L)
Bioreactor B-Tubular polyethylene	0.13	0.89 ± 0.04	1.48	2.11 ± 0.09	1.8 ± 0.09	16.0
Bioreactor C-Rectangular polyethylene	0.12	0.81 ± 0.04	1.36	1.91 ± 0.09	1.62 ± 0.08	13.1
Bioreactor D-Photobioreactor	0.05	0.24 ± 0.03	1.35	1.65 ± 0.06	1.43 ± 0.06	3.4
Raceway E-raceway tank	0.08	0.51 ± 0.04	2.22	1.63 ± 0.08	1.40 ± 0.07	7.2

Changes in the chlorophyll and carotenoid profile during the encystment process under sunlight is shown in Figure 3.13. The chlorophyll content, before exposure of culture to sunlight, was 1.61% (w/w) and it decreased to 0.45%(w/w) during a span of 5 days whereas almost 2.5 fold increase in total carotenoid content was observed with concomitant decrease in chlorophyll content.

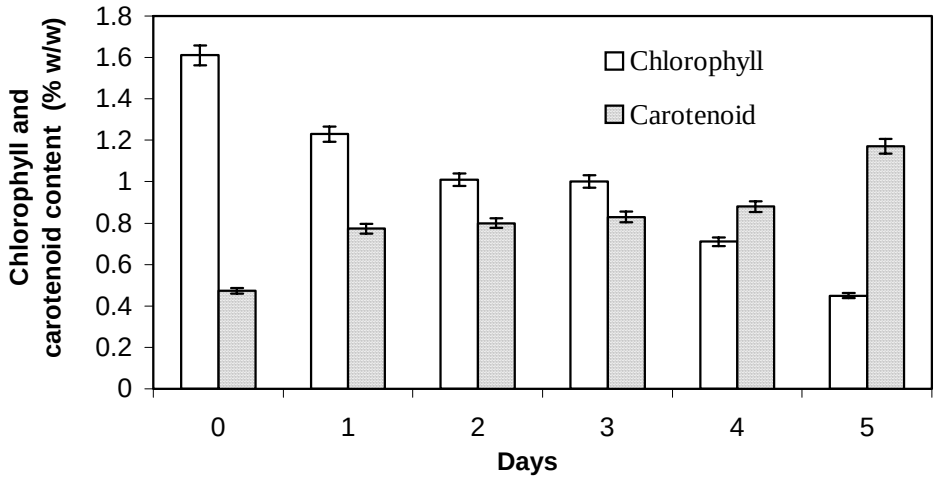


Figure 3.13. Changes in the profile of chlorophyll and carotenoid in *H. pluvialis* during second phase in outdoor conditons. (Stress induction by Sodium Chloride – 42mM and 2% CO_2)

3.8. Harvesting by gravity sedimentation and centrifugation

The *H. pluvialis* cells, during transition to encystment phase, get enlarged from 5μm to > 30μm and settled at the bottom of the reactor. This attribute has been well utilized for harvesting of *H. pluvialis* cells. The time taken by *H. pluvialis* cells to sediment at the bottom container is shown in Figure 3.14 and 3.15. Once the major portion (~90-95%) of cell free medium was removed, the rest of the culture was centrifuged at 5000rpm to completely harvest the biomass.

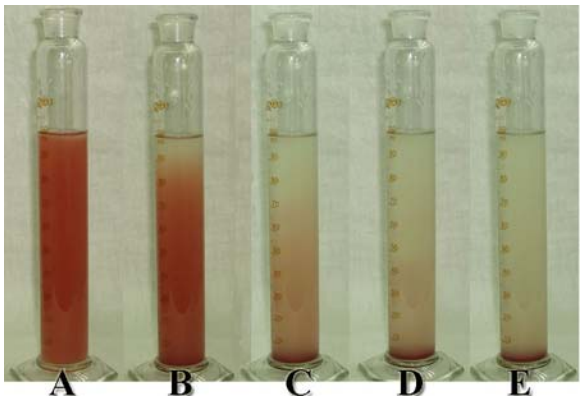


Figure 3.14. Gravity sedimentation of *H. pluvialis* biomass

A. 0 minutes B.10 minutes C.20 minutes D.30 minutes E. 40 minutes

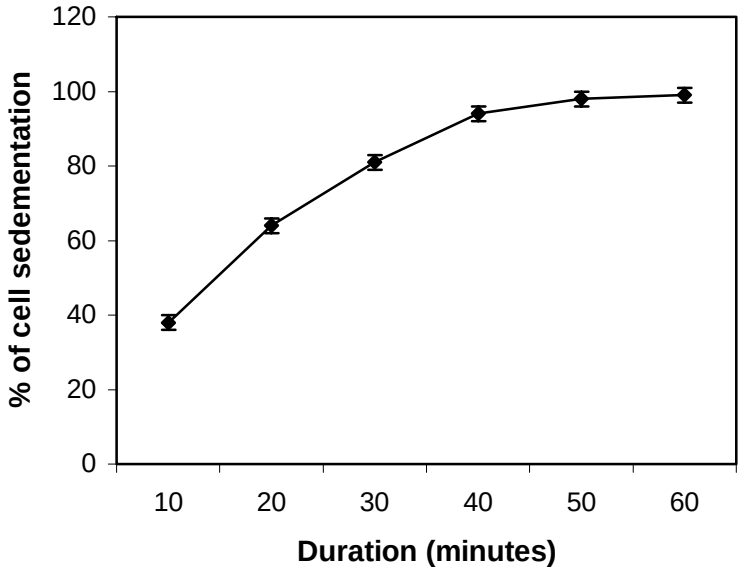


Figure 3.15. Relative sedimentation rate of encysted *H. pluvialis* biomass

3.9. Drying of *H. pluvialis* biomass

Drying the harvested biomass is crucial step since it is required to store the biomass for longer period and can be used for various purposes. It is desired that the drying method should not alter the carotenoid and colour qualities. In the present study, effect of drying methods such as spray drying and oven drying were evaluated as explained in section 2.13 and carotenoid content and colour values were compared with that of freeze dried sample.

The colour of the dried biomass was measured in terms of 4 parameters namely Hunter 'L', 'a', 'b' values and total colour difference –'DE'. The L value of the sample representing the lightness of the samples did not change significantly (Table 3.5). Positive values for 'a' indicates the redness of the sample and the negative value indicates greenness of the sample. The redness of the oven dried biomass was drastically reduced (almost 3 fold; Table 3.5) and it appeared brownish red in comparison with spray dried and freeze dried biomass. Positive values for 'b' indicate yellowness of the sample while negative value indicates blueness of the sample. The 'b' value also showed considerable difference in yellowness; however, the total colour difference 'DE' remained same among these biomass.

The results reveal that oven drying method is effective to obtain dry *H. pluvialis* biomass without loss of carotenoid content (Figure 3.16). Oven drying at 50°C for 6-7 hours has resulted in ~4% loss in the carotenoid content while it was 17% in spray dried biomass.

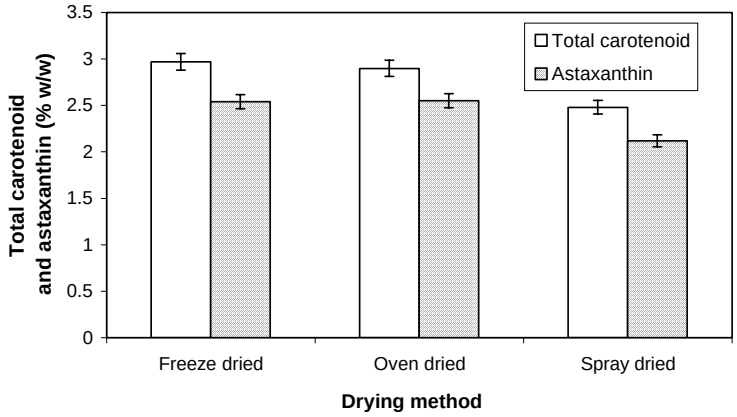


Figure 3.16. Effect of different drying methods on carotenoid content in

H. pluvialis biomass

Table 3.5. Hunter colour values of dried *H. pluvialis* biomass

	L	a	b	DE
Freeze dried	30.26[a]	9.08[a]	3.11[a]	68.61[b]
Oven dried	28.35[b]	3.06[b]	0.73[b]	70.27[a]
Spray dried	30.31[a]	9.02[a]	3.19[a]	68.72[b]

*Values following the same alphabets within a column are not significantly different.

3.10. Storage stability studies of *H. pluvialis* cells

The stability of the harvested *H. pluvialis* cells was analysed in terms of carotenoid contents and colour values. The samples were packed in polyethylene and metalized polyester poly pouches and stored as explained in the section 2.14. The colour values measured in terms of Hunter L a b values are shown in Table 3.6. It is evident from the Table 3.6 that cells stored at room temperature (in transparent polyethylene pouch) bleached within 15 days while those stored at -20°C retained colour values especially 'a' value which indicates redness of the cells. As shown in Figure 3.17, the red cell turned greenish yellow at room temperature. At the end of 60 days of storage, 19% reduction in 'a' value and ~40 % loss in carotenoid content were observed at 7°C while both 'a' value and carotenoid content remained same at -20°C. Samples stored at room temperature indicated drastic decrease in red colour and by 45 days they turned green (as reflected in 'a' negative value). In samples stored at room temperature there was increase in yellowness of the sample while the samples stored at low temperature did not show any significant change. It may be concluded that storing cells at room temperature resulted in loss of colour as well as carotenoid content thereby the cells appeared yellowish green in colour. The change in the colour appearance of cells also reflected in colour values and carotenoid content (Figure 3.18). The carotenoid analysis by HPLC clearly indicated the changes in its profile (Figure 3.19) in samples stored at room temperature.

The stability of *H. pluvialis* cells at room temperature and in dark condition was

increased by incorporating BHA at 100 ppm level (Table 3.7).

Stored at -20°C Stored at room temperature

Figure 3.17. Stability of *H. pluvialis* biomass stored at different temperatures. (Storage duration- 60 days)

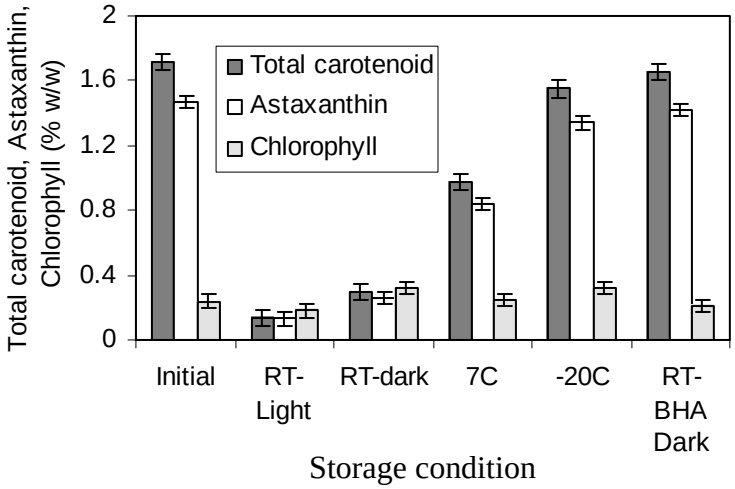


Figure 3.18. Pigment profile in the *H. pluvialis* biomass stored at different temperatures (Storage duration - 60 days).

Table 3.6. Colour values of *H. pluvialis* cells stored at different temperatures

		L	a	b	DE
RT-Light	Initial	24.11^{g}	$\mathbf{11.83}^{a}$	9.04^{b}	68.36^{a}
	15 days	35.56^{d}	$\mathbf{0.82}^{e}$	13.46^{ab}	56.64^{g}
	45 days	41.91^{b}	$\mathbf{-1.78}^{d}$	15.42^{ab}	50.96^{j}
	60 days	42.64^{a}	$\mathbf{-1.64}^{d}$	16.00^{ab}	50.45^{j}
RT-Dark	Initial	24.11^{g}	$\mathbf{11.83}^{a}$	9.04^{b}	68.36^{abc}
	15 days	34.21^{d}	$\mathbf{1.51}^{de}$	13.16^{ab}	57.92^{f}
	45 days	39.90^{c}	$\mathbf{-1.66}^{d}$	15.6^{ab}	52.93^{h}
	60 days	41.29^{b}	$\mathbf{-1.5}^{d}$	16.39^{a}	51.86^{i}
7°C	Initial	24.11^{fg}	$\mathbf{11.83}^{a}$	9.04^{ab}	68.36^{ab}
	15 days	24.80^{fg}	$\mathbf{10.64}^{b}$	9.09^{ab}	67.46^{cd}
	45 days	25.46^{ef}	$\mathbf{9.98}^{b}$	9.23^{ab}	66.72^{d}
	60 days	26.34^{e}	$\mathbf{9.61}^{c}$	9.00^{b}	65.77^{e}
- 20°C	Initial	24.11^{g}	$\mathbf{11.83}^{a}$	9.04^{b}	68.36^{ab}
	15 days	24.82^{fg}	$\mathbf{11.63}^{a}$	9.09^{b}	67.43^{bc}
	45 days	23.94^{g}	$\mathbf{11.60}^{a}$	9.35^{ab}	68.46^{a}
	60 days	24.24^{fg}	$\mathbf{11.38}^{a}$	9.02^{b}	68.12^{abc}

*Values following the same alphabets within a column are not significantly different.

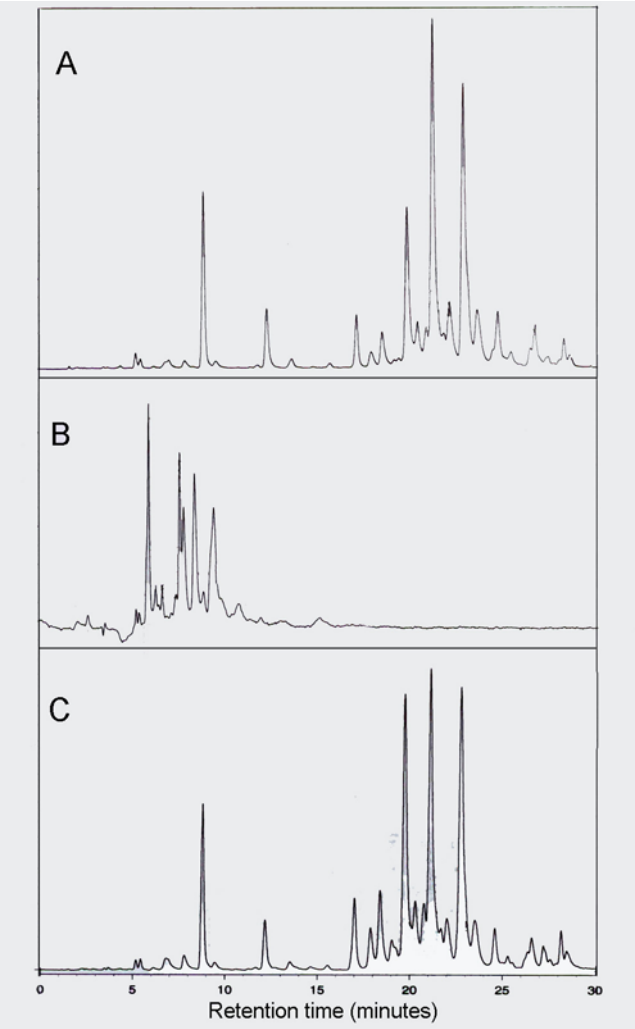


Figure 3.19. HPLC profile of carotenoids from *H. pluvialis* cells stored at different temperatures (Storage duration -60days)
A. Initial B. at room temperature C. at -20°C

Table 3.7. Stability of *H. pluvialis* biomass after treatment with butylated hydroxyl anisole (BHA).

		L	a	b	DE
Initial		23.41^{c}	7.45^{a}	9.59^{a}	65.46^{b}
After 1 week	Control	22.21^{c}	7.13^{ab}	7.29^{b}	69.3^{a}
	A	26.22^{a}	7.39^{a}	9.66^{a}	65.65^{b}
	B	26.89^{bc}	7.32^{ab}	9.95^{a}	65.03^{b}
After 3 weeks	Control	21.51^{c}	6.92^{b}	7.01^{b}	69.93^{a}
	A	22.33^{c}	7.16^{ab}	7.32^{b}	69.19^{a}
	B	21.79^{c}	7.02^{ab}	7.14^{b}	69.69^{a}

A- Treated with BHA(100 mg/kg) and kept in dark
B- Treated with BHA(100 mg/kg) and exposed to light.
*Values following the same alphabets within a column are not significantly different.

3.11. Digital Image processing based method for carotenoid estimation

DIP, which involved image acquisition, preprocessing, segmentation, feature extraction and the final recognition and interpretation was done using a knowledge base specifically created for the analysis of the problem domain. Also, a supervised Artificial Neural Network (ANN) was used to correlate colour information to carotenoid and chlorophyll content in the alga.

H. pluvialis cells in different growth phases were selected for carotenoid and chlorophyll estimation and the cells were photographed, processed by digital image processing. The images were captured by a CCD camera and processed using image processing techniques. As the culture grows, there will be limitation for nutrients which induces cyst formation and the stress condition enhances the accumulation of carotenoids. The Hue values for the green motile phase 53.24° and for the carotenoid accumulated phase were in the range 293.4°. The neural network model developed (Figure 2.5) was applied to compute the carotenoid and chlorophyll content in the algal cells.

The analytically estimated values were correlated with predicted value for carotenoid and chlorophyll contents in *H. pluvialis* cells. A good correlation of R^2 =0.967 was observed in case of carotenoid (Figure 3.20A). A similar correlation of R^2= 0.997 was observed for chlorophyll (Figure 3.20B). These results clearly showed

that digital image processing method could be applied to estimate carotenoid pigment content in *H. pluvialis* cells.

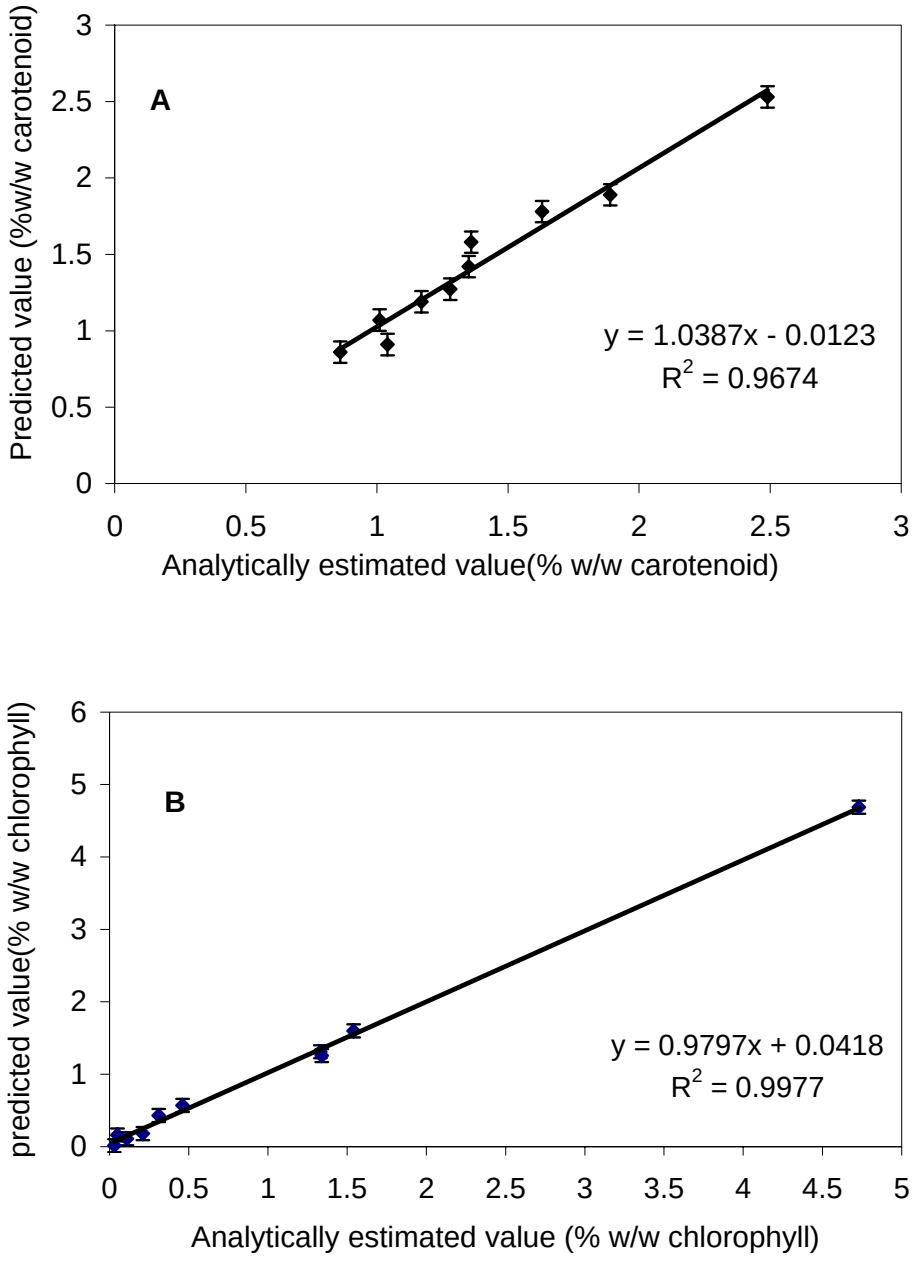


Figure 3.20. Correlation of analytically estimated carotenoid (A), chlorophyll (B) and predicted content.

3.12. Discussion

Microalgae, in general, have the ability to use different organic and inorganic nitrogen compounds as nitrogen source. Fabregas et al (2000), Hata et al (2001) and Grunewald et al (1997) have used potassium nitrate as nitrogen source. Dominguez-Bocanegra et al (2004), Choi et al (2003) and Torzillo et al (2003) have reported the use of sodium nitrate and Rio et al (2005) have used calcium nitrate as a source of nitrogen under autotrophic conditions. The contribution of other sources, e.g. urea, commercial fertilizer has been reported only in a limited number of cases. In the present study, different ammonia salts were studied for their influence on *Haematococcus pluvialis* growth, since little information is available on ammonia assimilation.

Some benthic microalgae are highly versatile in assimilating various sources of nitrogen. Flynn and Wright (1986) found that high concentrations of L-arginine and ammonia are utilized simultaneously by microalgae *Phaeodactylum tricornutum.* Abeliovich and Azove (1976) reported unionized ammonia is more toxic to various photosynthetic organisms and ammonia among other weak electrolytes, causes swelling and osmotic lysis of the cells in the case of *Prymnesium parvum*. Swelling and cell lysis was also observed in the present study when ammonium acetate and urea were used in the medium at higher concentrations.

Sarada et al (2002b) reported slow growth of *H. pluvialis* when ammonium nitrate was used as nitrogen source under heterotrophic conditions. There was a significant increase in cell number (8-10 fold) in cultures grown in presence of CO_2 compared to those in the absence (2-4 fold) with different ammonium salts (Figure 3.2 and Table 3.1). Ammonium acetate assimilation seems to be CO_2 dependent, which has influenced the growth phase (Table 3.1) but not the encysted phase in presence of CO_2. This explanation is supported by the findings of Lara and Romero (1986) which has established the clear distinction between ammonium utilization by microalgae *Anacystis* and nature of their dependence upon CO_2 fixation. The results indicated that *H. pluvialis* can utilize ammonia salts as nitrogen source in the presence of CO_2 for photoautotrophic growth and it could not utilize ammonium acetate without CO_2. Urea supplemented cultures showed early induction of carotenoid but both the content and production of carotenoid were less.

It was of interest to know that the transfer of *H. pluvialis* culture to medium containing DAP significantly increased the biomass yield (3.1 g/L) and carotenoid production (66 mg/L) with 2.1% astaxanthin content (Table 3.1 and Figure 3.3). It has also been observed that, upon subculturing, the growth in DAP and Suphala supplemented cultures, the growth was not satisfactory. Thus, for the batch cultivation, these commercial salts are reliable nitrogen sources. The continuous cultures were maintained in ammonium carbonate (0.15g/L) supplemented medium.

Occurrence of various ammonium assimilating enzymes such as glutamine dehydrogenase, glutamine synthetase, glutamate synthase, alanine dehydrogenase and carbamoyl phosphate synthetase in algae were reported by Kaplan et al (1986) and Stewart (1980). Flynn (1990) has suggested that the incorporation of ammonium occurs mainly through the action of glutamic dehydrogenase or GS/GOGAT. The latter is considered more important when the N-source is at low concentration because the substrate affinity is higher. L- methionine DL-sulphoximine was reported to inhibit GS activity while azaserine was reported to inhibit GOGAT activity (McAuley, 1995). Inhibition of *H. pluvialis* growth in the presence of L- methionine DL- sulfoximine and azaserine (Table 3.2) suggests that *H. pluvialis* might assimilate ammonia through GS/ GOGAT pathway.

The effect of phytohormones 6-benzyl amino purine (cytokinin) and Gibberellic acid (Gibberellins) was studied on growth and carotenoid production by *H. pluvialis*. Little information is available on use of these growth promoters on microalgae. Bajguz and Czerpak (1998) studied the influence of growth regulator- brassinosteroids and reported the stimulation of the growth and metabolites production by these brassinosteroids. Since cytokinins are involved in promoting cell division and differentiation, and Gibberellins are involved in stem elongation and other physiological processes, it was expected that its supplementation in microalgal culture would enhance growth and/or carotenoid production. When used in combination, BAP and GA produced 35% higher total carotenoid and astaxanthin content in comparison with unsupplemented culture (Figure 3.4).

In response to the stress factors, morphological and physiological changes occur

coinciding with carotenoid accumulation. Hence developing the optimum conditions for carotenogenesis is necessary for maximum yield. Torzillo et al (2003) have reported sunlight as the important factor for carotenoid production. Elevated temperature has also been reported as stress factor by Tjahjono et al (1994a). But the rise in temperature due to sunlight has resulted in cell lysis or bleaching in the trails conducted during the present experiments. Sarada et al (2002a) have found >1.0% of NaCl was lethal for heterotrophically grown *H. pluvialis* and age of the culture was crucial for stress induced astaxanthin production.

Effect of sunlight on carotenoid accumulation and changes in chlorophyll profile is shown in Figure 3.13. It is evident from the figure that there is a considerable increase in carotenoid content with concomitant decrease in chlorophyll content. The most significant physiological change in *Haematococcus* during the accumulation of astaxanthin is the decrease in photosynthesis as demonstrated by Hagen et al (1992) and Zlotnik et al (1993). The amount of cellular astaxanthin is inversely related to the cellular photosynthetic activities, although the amount of chlorophyll and PSII reaction centre remain stable during astaxanthin accumulation (Boussiba et al, 1999; Tan et al, 1995)

Light, temperature and inorganic carbon availability are the most important factors controlling the photosynthetic productivity of algal system. Carbon is the major element (40-50%) in algal biomass; gas-liquid mass transfer is crucial which in turn meets the requirement of inorganic carbon. The complex interactions occurring in the pH-CO_2-HCO_3 system (Yue and Chen, 2005; Rubio et al, 1999; Rodriguez-Maroto et al, 2004; Talbot et al, 1991) and photosynthetic utilization of inorganic carbon (Qiu and Gao, 2002) has been studied by many researchers. Carbon is stored in liquids as dissolved CO_2, bicarbonate or carbonate. The relative amount of each species is determined primarily by pH (Weissman, 1988). In the present study, due to bubbling, CO_2 concentration is reflected in the culture pH changes, and this phenomenon has also been observed by Livansky and Bartos (1986). This variation in pH has resulted in low cell number and less biomass yield (Fig 3.10b and Table 3.3). Excessive shearing action might also have played a role in obtaining less biomass yield. In sparged photobioreactors, such as bubble column and flat panels, though shearing action is required for mixing, excessive shear can lead to impaired cell growth, cell damage and

eventually cell death. As reported by Barbosa (2003), this is a key problem in culture of microalgae in sparged photobioreactor.

Hydrodynamics or mixing characteristics are function of reactor geometry and operating conditions, and are principal determinants of the light regime experienced by culture (Babcock et al, 2002). In the open bioreactors, stirrer was provided to create turbulence which facilitates mass transfer phenomenon between the culture medium and the atmospheric CO_2. Though the absorption of atmospheric CO_2 is not selective, stirrer was found useful for removal of photosynthetically produced O_2 which is also detrimental for growth of algae. Air bubbling also provides turbulence in the culture medium but in the present experiment, air bubbling did not support efficient mass transfer as indicated by low biomass yield (Table 3.3). Loss of major portion of CO_2 during bubbling appears to be the chief reason for this low biomass yield. This view is also supported by the Rodriguez-Maroto et al (2004) who have reported the experimental data and process modeling on loss of carbon during air bubbling.

The CO_2-float design was employed to reduce the CO_2 loss and to provide efficient gas-liquid mass transfer. This design also served as pH-control system, without producing huge variations in the pH which was observed in case of CO_2 bubbling. Maximum growth rate of 0.08-0.09 day^{-1} and biomass productivity of 2.4- 2.6 $g/m^2/day$ was obtained (Table 3.3) in the designs provided with CO_2 float. These results are in agreement with the report of Sobczuk et al (2000) who have observed pH gradient in bubbled culture and enhanced the biomass productivity by reducing CO_2 loss. Astaxanthin productivity was also increased in this design (9.7-10.2mg/L) in proportion with biomass.

In the attempts to cultivate *H. pluvialis* in open tanks under outdoor conditions, complete bleaching and cell damage was observed in 1-2 days. This can be attributed to the phenomenon of photoinhibition at early stages of growth wherein the key components of photosystem II (PSII) are destructed, thus capacity of microalga to harvest light is lost. Photolimitation usually occurs in dense cultures, whereas photoinhibition can occur at considerable irradiance. Grima et al (1996) have reported the photolimitation and photoinhibition of growth in dense cultures of *Isochrysis* under indoor conditions. Some microalgae can overcome the effect of photoinhibition to certain extent. Fernandez et al

(1998) have explained the co-existence of photoinhibition and photolimitation in *Phaeodactylum* culture. Due to the light gradients inside the culture, both light-limited growth and photoinhibiting conditions could occur simultaneously, each one to a different extent depending on the external irradiance. For *H. pluvialis*, the light intensity below 3 klux was found insufficient for pigmentation phase and above is photoinhibitory for growth.

In addition to photoinhibition, inability of the organism to acclimatize to outdoor condition and contamination with other microorganisms, were the other obstacles faced during open cultivation of the *H. pluvialis*. The vegetative cells were sensitive to temperature fluctuations and growth was significantly affected below 20°C and above 26°C. Algal cell lysis or encystment was observed when the temperature was above 28°C. Though the cultivation system was autotrophic, bacterial, fungal and protozoan contamination was frequently encountered.

The shallow algal culture system using polythene bags for cultivation of freshwater microalgae, *Ankistrodesmus* and *Scenedesmus* has been reported by Martinez-Jeronimo and Espinosa-Chavez (1994). The dense culture which are employed in shallow systems require less concentration at harvesting, reducing the associated cost. The tubular polyethylene bags used in the present study were found useful in producing maximum biomass of 0.89g/L and astaxanthin productivity of 16.0mg/L (Table 3.4).

Multistage systems that not only utilize autotrophic growth and stress components, but also combined autotrophic/heterotrophic systems are thought to provide solution to specific production requirements (Grobbelaar, 2000). Since *H. pluvialis* growth phase requires stringent culture conditions like controlled temperature and light, two-stage system involving growth in closed indoor conditions and carotenoid accumulation in open outdoor condition was found suitable to attain maximum astaxanthin productivity. Based on the results obtained, the culture conditions optimized for *H. pluvialis* cultivation system has been summarized in Table 3.8.

Recovery of biomass from liquid cultures can be difficult and pose problem when cells are in small size (<20μm). Large volumes can be processed through centrifuge in relatively shorter span of time, but in dilute culture, energy requirement would be higher. Grima et al (2003) have suggested centrifugation as a preferred method for recovering

Table 3.8. Optimization of culture conditions for *H. pluvialis* growth and astaxanthin production

	For algal growth	**For carotenoid formation**
Cultivation system	Closed	Open
Medium	Modified Bold's Basal medium	-
Sodium chloride	-	42mM
Light	0.8 - 1.5klux	25-30 klux
Temperature	26 ± 1°C	20 - 30°C
Carbon source	2% CO_2 supplied with air	2% CO_2 supplied with air
pH	7.2 ± 0.2	7.0-8.0
Duration	13-15 days	6 days

microalgal biomass. Since encysted cell of *H. pluvialis* sediment easily, centrifugation of entire culture is not required. About 95% of the encysted biomass is settled in 40-50 minutes (Figure 3.15), the sedimented culture can be centrifuged to obtain wet biomass. This approach has the advantage over other harvesting techniques such as flocculation, wherein the residual chemical flocculant may not be desirable in food source.

The employment of specific drying method depends on the intended final product and expected shelf life. Though freeze drying is method of choice to produce premium quality biomass without loss of algal components, it is too expensive for use in large scale. Spray drying is common practice for microalgal processing, but in some cases, it is reported to deteriorate the algal components (Grima et al, 2003). Ben-Amotz and Avron (1987) have obtained satisfactory results in terms of uniformity of the biomass powder and stability of β-carotene in microalga *Dunaliella* biomass after spray drying, freeze drying and drum drying. As shown in Figure 3.16, spray drying resulted in 17% loss in carotenoid content in comparison with freeze dried biomass. Though the external colour and redness did not match with the spray dried powder (Table 3.5), the present result reveal that oven or thermal drying is the suitable and relatively low cost method to obtain

dry *H. pluvialis* biomass without loss of carotenoid content.

On industrial scale, shelf-life of microalgae is extended by combination of techniques like addition of non-toxic preservatives, low temperature storage and low density storage (Heasman et al, 2000). Low temperature storage (-20°C) of *H. pluvialis* for 60 days was found satisfactory without loss of external red colour and carotenoid content (Figure 3.18 and Table 3.6). Since this is energy requiring technique, alternate method using the preservative butylated hydroxy anisole (BHA; 100 mg/kg) was attempted. The result suggests that inclusion of BHA in *H. pluvialis* biomass has extended its stability at room temperature; hence it can be a suitable method for storage without loss of desired characteristics. The biochemical differences among the biomass, dried and stored by the above methods, and their utility in food and/or nutraceutical formulation is subject matter of further study.

The present work on digital image processing aims at demonstrating the applicability of technique as a tool for quality control of biotechnological processes. It was established that digital image processing method helped in analyzing the carotenoid content from microalgal cells such as *H. pluvialis* eliminating the conventional homogenization of cells and extraction with solvents. It also helped in manipulating the culture conditions to enhance carotenoid content and thereby facilitating easy and immediate analysis of carotenoid and chlorophyll contents in the cells. The technique could be used for online monitoring of pigment contents in a variety of cultured cells.

The carotenoid accumulated cyst cell consists of thick hard cell wall made of sporopollenin like material with (Hagen et al, 2002), which hinders solvent extraction and cracking of the cell requires high pressure homogenization at low temperature. A conventional method like homogenization results in the loss of pigment. All the reported methods suggest cell disruption (Zlotnik et al, 1993) or extraction with dimethyl sulfoxide (Boussiba and Vonshak, 1991) at high temperature which involve loss of carotenoid. Therefore the technique such as digital image processing (DIP) system would be useful to quantify the redness of the encysted cell and to estimate the carotenoid content without disrupting the cell wall.

During carotenogenesis, the chlorophyll content significantly decreases (Figure 3.13) and the decrease in green colour relating to chlorophyll is seen clearly in the DIP

also. Image processing technique has been applied for quantifying adulteration in roast coffee powder by Sano et al (2002). Coupled with Neural Network model this technique could be used for online monitoring of the carotenoid content just by observing the cells under microscope, capturing the image by CCD camera, for further processing by DIP.

Estimation of pigment content in microalgal cells is an integral part of algal cultivation process. The method explained is useful in analyzing the carotenoid content of more number of algal samples in short span of time. Requirement of very small quantity of sample for analysis is the advantage of this method.

Isolation and characterization of *H. pluvialis* mutants

Background

Isolation and selection of high yielding mutants has been widely used technique for strain improvement. *Haematococcus* is slow growing microalgae with stringent growth conditions. There are no reports on availability of strain of Indian origin. Hence it was intended to obtain *H. pluvialis* mutant through mutagenesis. In the present study, UV, EMS and NTG were used to induce mutation in order to obtain high yielding strains of *H. pluvialis*. The herbicide employed is a broad spectrum herbicide –glufosinate, which is a substrate analogue of glutamate and strong inhibitor of glutamine synthetase causing toxic levels of ammonia to build up in treated cell. Thus it was expected that herbicide resistance would be a selection marker for isolation of mutants.

The isolated mutants were evaluated for growth and carotenoid production, carotenoid profile, response to stress condition, effect of herbicide on photosynthetic activity and fluorescence profile, lycopene cyclase activity and expression of carotenoid biosynthetic genes.

3.13. Mutagenesis and screening

H. pluvialis culture in the logarithmic phase of growth was selected for mutagenesis. The appropriate level of each mutagen was determined by treating the algal cells in a wide concentration range. The treated cells were grown in the medium containing herbicide-glufosinate. Green colonies of *H. pluvialis* were observed after 9 days of incubation. The survival rate of algae after treatment with UV, EMS and NTG is shown in Table 3.9.

Table 3.9. Survival rate of *H. pluvialis* cells obtained after treatment with mutagen.

Treatment	**Survival rate (%)**
UV (15 min)	11.8
UV (30 min)	5.9
EMS (0.1M)	17.3
EMS (0.2M)	15.4
NTG (0.1mM)	33.6
NTG (0.2mM)	18.7

The survival rate was found to be concentration dependent. A wide range of concentration of herbicide was first tested to examine its inhibitory levels. Lower concentration of mutagen (<0.1M EMS and <0.1mM NTG) and herbicide often produced large number of colonies (>10,000) with the low possibility of producing mutant. Hence suitable concentration of mutagen as well as herbicide was standardized to obtain satisfactory survival rate.

More than 50 algal colonies from each treatment, grown in the petri dishes, were randomly selected based on the colour and colony characteristics. These colonies are streaked on the herbicide free BBM slants and allowed to grow under normal growth condition (section 2.2.1). Most of the colonies were found unstable upon subculturing, either bleached or did not show satisfactory growth on slants. The putative mutants which exhibited the stability, visual colony and colour differences were selected for further growth and characterization.

3.14. Growth and astaxanthin production by mutants

The mutants isolated after 15 minutes exposure to UV were designated as U1 and U2 and those after 30 minutes were designated as U3, U4 and U5. Growth profile of these mutants is shown in Figure 3.21A and 3.21B. Out of these five UV mutants characterized for growth, cell number and biomass yield, three of the mutants were comparable with wild type (Figure 3.21A and B). However, their carotenoid content and production was found to be less compared to wild type (Figure 3.21C and D). The mutant U1 showed maximum astaxanthin content of 2.6% (w/w) which is almost 23% higher than that of wild type. However, this increase has not been reflected in astaxanthin production since the biomass yield of this mutant was less (1.95g/L) in comparison to wild type (2.72g/L).

Mutants isolated after treatment with 0.1M EMS were designated as E1, E2 and 0.2M EMS were designated as E3, E4, E5, E6. The growth profile of EMS mutants is shown in Figure 3.22A and B. The cell number of all six mutants tested was comparable with that of wild type. The mutants E5 and E6 produced maximum biomass yield of 3.04 and 2.99 g/L respectively (Fig 3.22B). EMS treatment had significant effect on carotenoid biosynthesis as indicated by lower amounts of total carotenoid and astaxanthin content (Figure 3.22C). Total carotenoid production was in the range of 30-55mg/L which was lower than that of wild type (67.3mg/L).

Mutants isolated after treatment with 0.1mM NTG were designated as N1, N2, N3, N4, N5 and 0.2mM NTG were designated as N6. The growth profile of NTG mutants is shown in Figure 3.23A and B. The cell number of all the 6 mutants tested was comparable with the wild type except the mutant N5 which showed maximum cell number of 64×10^4 cells/ml (Figure 3.23A). The biomass yield was in the range of 2.24-2.51 g/L (Figure 3.23B). Mutation with NTG has resulted in significant increase of 42-59% in the carotenoid content (Figure 3.23C). Out of the six mutants tested for growth and carotenoid production, the mutant N5 produced a maximum of 3.95 % (w/w) total carotenoid content and 89.2mg/L of total carotenoid production (Figure 3.23C and D).

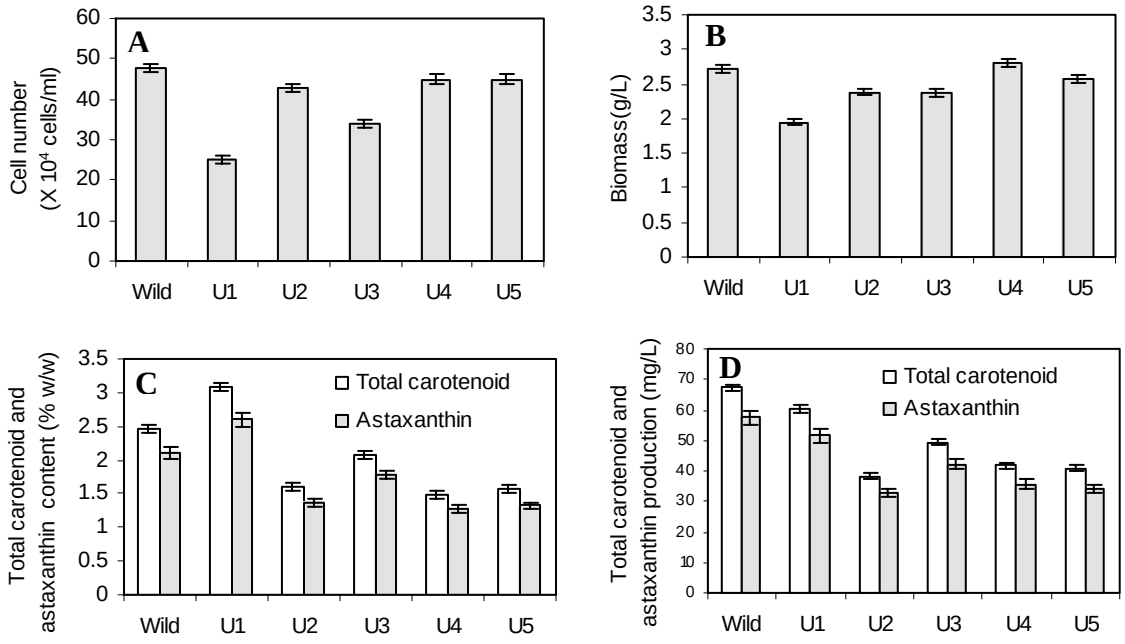


Figure 3.21.Growth and carotenoid production in *H. pluvialis* mutants obtained with UV irradiation

A.Cell number B. Biomass C. Carotenoid content D. Carotenoid production

U1,U2 - UV mutants (15 min) U3,U4,U5 - UV mutants (30 min)

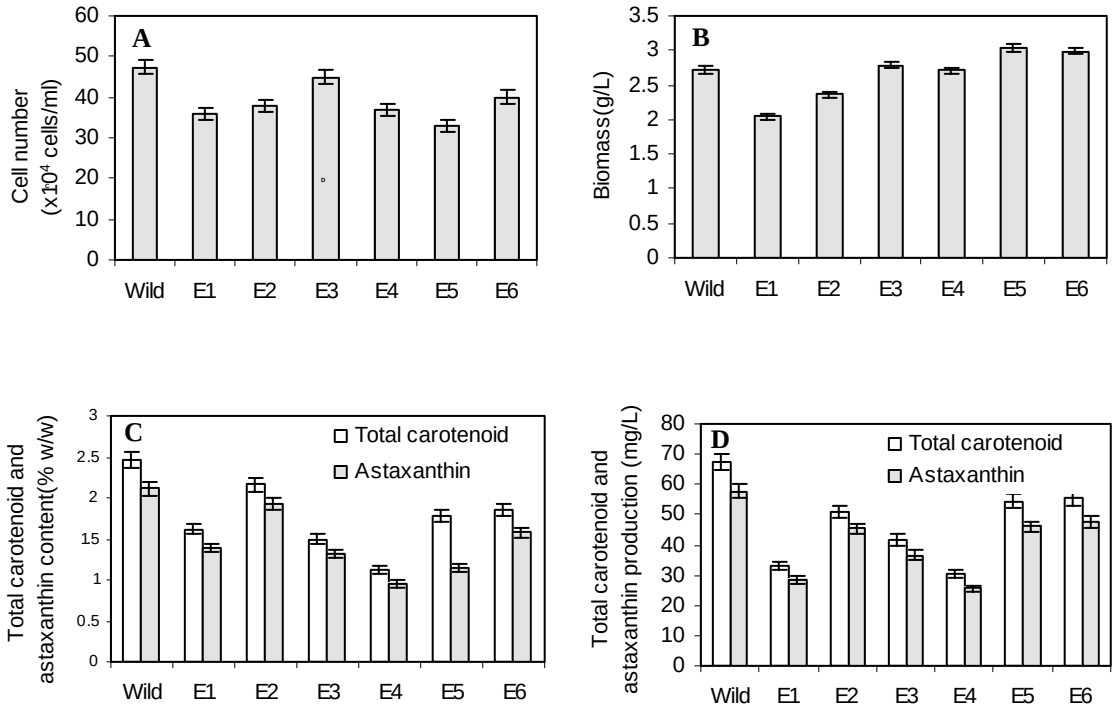


Figure 3.22. Growth and carotenoid production in *H. pluvialis* mutants obtained with EMS treatment

A. Cell number B. Biomass C. Carotenoid content D. Carotenoid production

E1, E2- EMS mutants (0.1M) E3, E4, E5, E6-EMS mutants (0.2M)

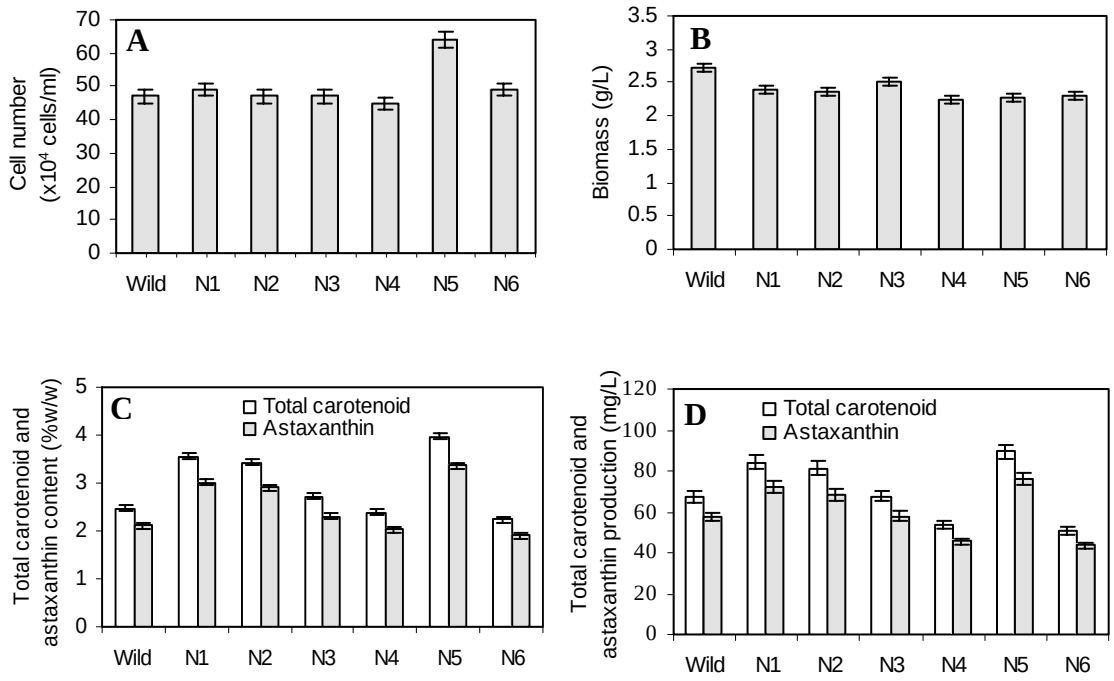


Figure 3.23. Growth and carotenoid production in *H. pluvialis* mutants obtained with NTG treatment.
A. Cell number B. Biomass C. Carotenoid content D. carotenoid production
N1, N2, N3, N4, N5- NTG mutants (0.1mM), N6-NTG mutants (0.2mM)

3.15. Analysis of carotenoid profile

The carotenoid extracts from the mutants were subjected to separation by thin layer chromatograpy. The TLC carotenoid profile is shown in the Figure 3.24. Though there was significant differences in the amount of astaxanthin content among the mutants, the carotenoid profile did not show considerable difference in comparison with wild type. The HPLC profile of carotenoid extract from different mutants as shown in the Figure 3.25 indicated that the carotenoid pattern in the mutant was not altered from the wild type. These results have supported the results of TLC pattern indicating that that the mutagens-UV, EMS and NTG have not affected the carotenoid biosynthetic pathway in a deleterious manner in these mutants.

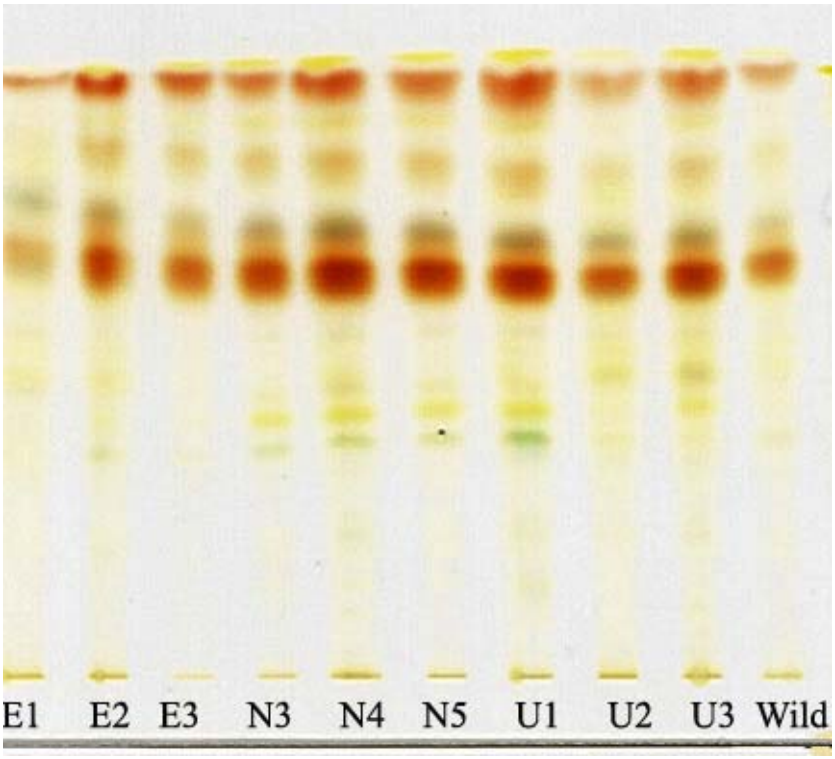


Figure 3.24. TLC profile of carotenoid extract from *H. pluvialis* mutants

E1,E2- EMS mutants (0.1M); E3-EMS mutant (0.2M)
N3,N4,N5- NTG mutants (0.1mM)
U1,U2 - UV mutants (15 min); U3- UV mutants (30 min)

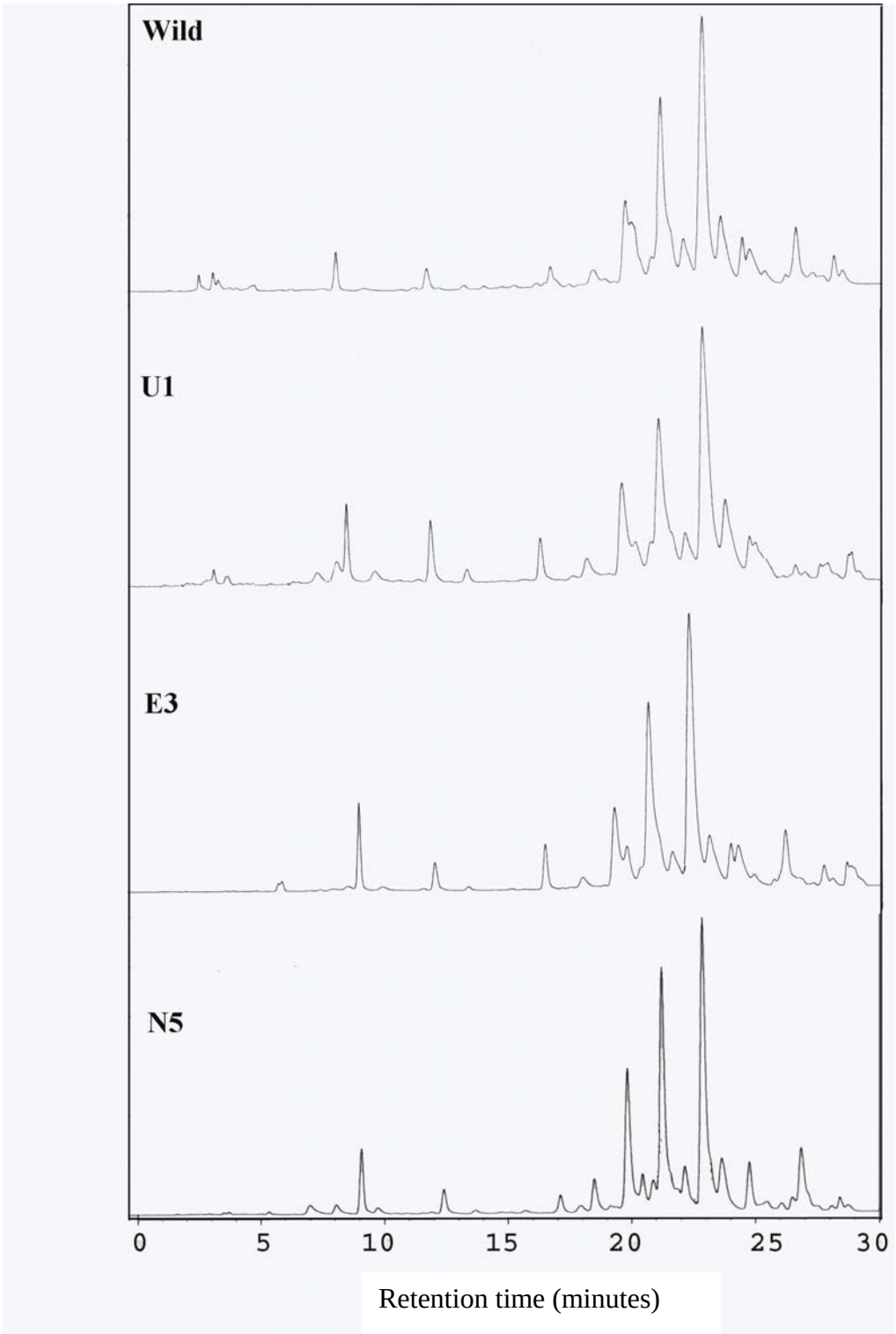


Figure 3.25. HPLC profile of carotenoid extract from *H. pluvialis* mutants

3.16. Analysis of carotenoid profile under normal and stress condition

The effect of salinity stress (42mM) and light stress (3.5Klux) on mutants was studied and compared with the normal growth conditions as explained in the section 2.16.3. The total carotenoid and astaxanthin content in the mutant under normal and stress conditions is shown in the Figure 3.26A and B.

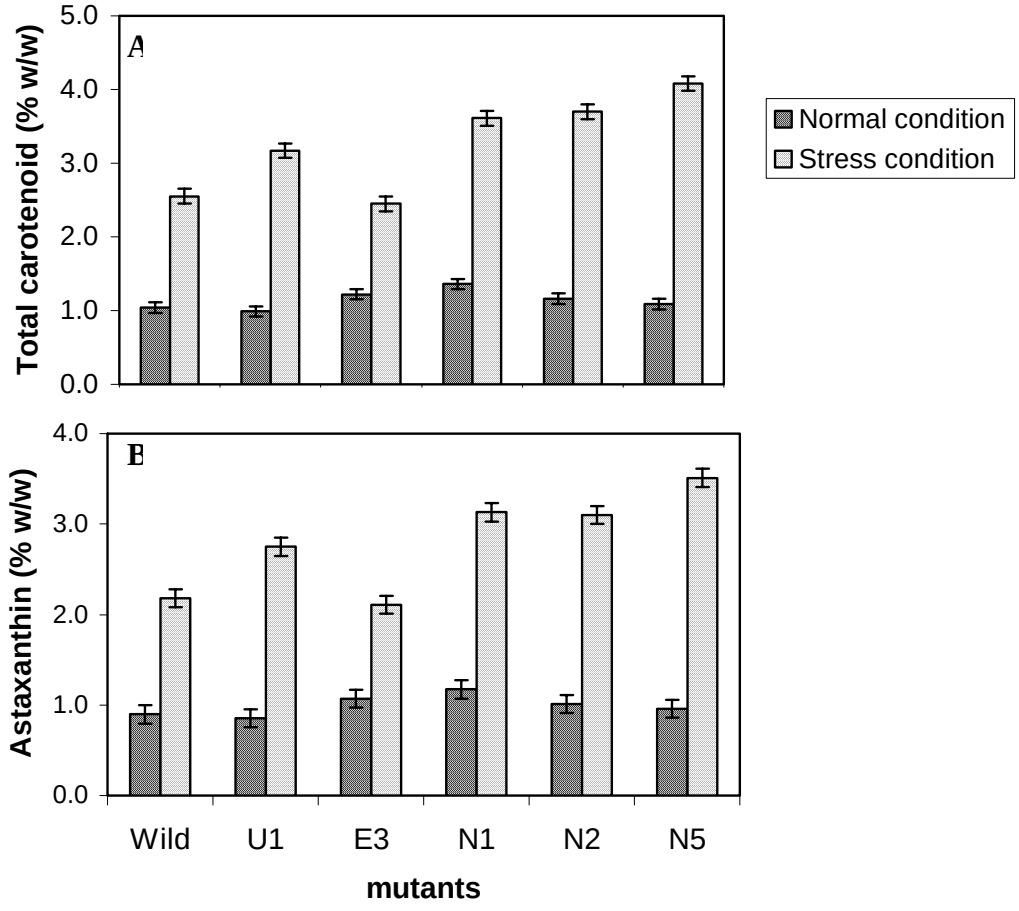


Figure 3.26. Total carotenoid (A) and astaxanthin (B) content in the *H. pluvialis* mutants under normal and stress conditions.

The salinity stress and light stress had a significant effect on the carotenoid content of the mutants. All the mutants have shown an increase of 23-59% in total carotenoid and astaxanthin content in comparison with wild type except the mutant E3 which showed carotenoid content comparable to wild type. The mutant N5 produced a

maximum total carotenoid content of 4.08% (w/w) and astaxanthin content of 3.51%(w/w). Under normal growth conditions, the total carotenoid and astaxanthin content of mutants was comparable with the wild type.

3.17. Effect of herbicide on photosynthetic activity of mutants

The effect of herbicide –Glufosinate (250μM) on actively growing *H. pluvialis* wild type and mutants was evaluated. Photosynthetic activity was measured using the dye 2, 6-dichlorophenol indophenol (DCPIP). As shown in Figure 3.27, the mutants N5 and E3 were resistant to herbicide action and continued active photosynthesis as indicated by the reduction in the dye DCPIP within 5-10min. The photosynthesis was inhibited in the wild type as shown by the absorbance at 620nm. In case of the mutant U1, the extent of photosynthetic inhibition by glufosinate was lesser than that of wild type.

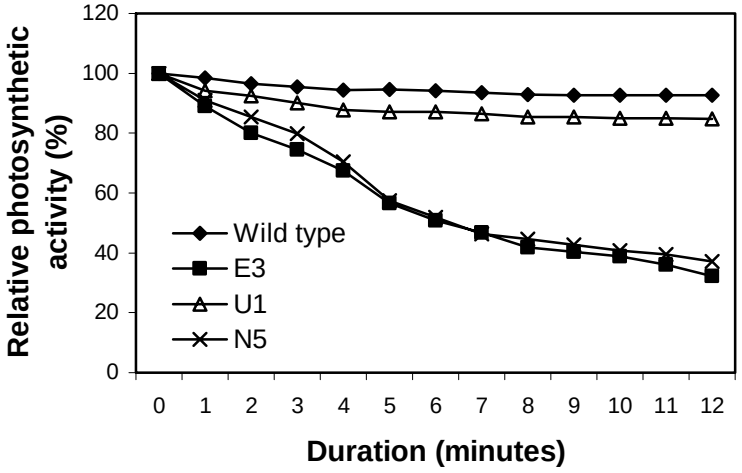


Figure 3.27. Photosynthetic activity in mutants of *H. pluvialis* in presence of herbicide - gulfosinate (250μM).

3.18. Effect of herbicide on chlorophyll fluorescence profile of mutants

The effect of herbicide glufosinate (200μM) on the chlorophyll fluorescence characteristics of the mutants was evaluated. The variable chlorophyll fluorescence (Fv) of the mutants in comparison with the wild type is shown in the Figure 3.28.

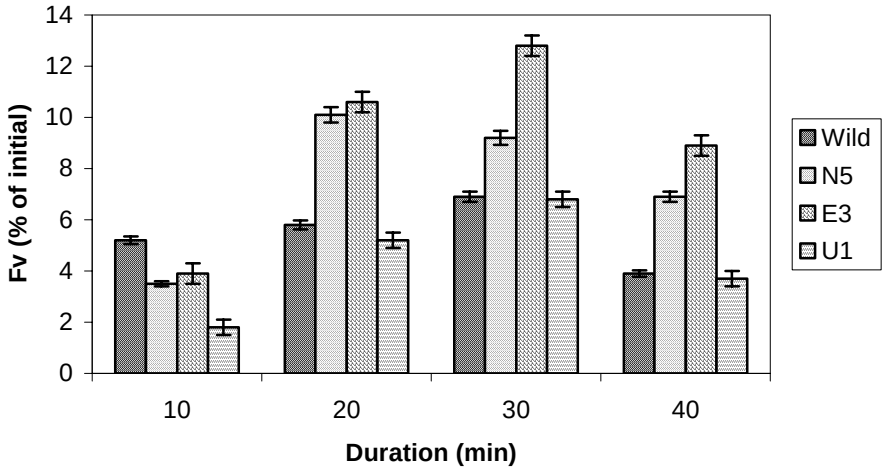


Figure 3.28. Variable fluorescence (Fv) exhibited by mutants in presence of herbicide-glufosinate (200μM)

The *H. pluvialis* mutants N5 an E3 have shown higher Fv (variable fluorescence) in comparison to the wild type. After 20 and 30 minutes of excitation at 400nm, the Fv of the mutants N5 and E3 was almost 1.5 to 2.0 fold higher than that of wild type. The mutant U1 did not show significant difference in the chlorophyll fluorescence emission pattern.

3.19. Lycopene cyclase activity of *H. pluvialis* mutants

Formation of β-carotene from lycopene is a crucial step and it is mediated by the enzyme lycopene cyclase. Hence mutants were evaluated for lycopene cyclase activity. The mutants in the vegetative phase of growth and after stress induction were used to determine the enzyme activity. Lycopene cyclase activity of tested mutants is shown in Table 3.10.

Table 3.10. Lycopene cyclase activity of *H. pluvialis* mutants

Mutant	nmole of β-carotene formed/mg of protein/hr	
	Vegetative cell	Stress induced cells*
Wild type	105.7	91.6
E3	0.794	51.2
U1	458.2	5.8
N2	315.4	75.1
N5	39.7	172.0

* Stress induction by NaCl (42mM) and high light (3 Klux) for 60 hours

In the vegetative phase, the lycopene cyclase activity was highest in the mutant U1 which was 458.2 nmoles of β-carotene formed/mg of protein/hr which is almost 4 fold of that in the wild type. After the stress induction, the mutant N5 showed the maximum enzyme activity of 172.0 nmoles of β-carotene formed/mg of protein/hr.

3.20. Expression analysis of carotenoid biosynthetic genes

The stable mutants from UV, EMS and NTG treatments were selected for carotenogenic genes expression studies. The expression levels of genes associated with general carotenogenesis and specific astaxanthin biosynthesis in mutants and wild strain were quantified by reverse transcription polymerase chain reaction (RT-PCR) and compared. These genes included phytoene synthase (PSY, the first committed step in the carotenoid pathway), phytoene desaturase (PDS, which converts phytoene to lycopene), lycopene cyclase (LCY, which converts lycopene to β-carotene), BKT (specific to astaxanthin biosynthesis, which converts β-carotene to echinenone and to canthaxanthin), and CHY (which convert canthaxanthin to astaxanthin and α- carotene to lutein and other xanthophylls). The transcript levels of these enzymes were analysed after 48hrs of stress induction to wild and mutant strains. The transcript levels of all the genes were found to be higher in mutants than wild strain (Figure 3.29). The mutants differed in the extent of

transcript levels for each carotenogenic genes studied. The transcript levels of PSY, PDS, BKT and CHY were 15-20 fold high in E3 mutant while N5 exhibited 12 fold increase in PSY, PDS and only 3 fold increase in LCY and BKT. Mutant U1 showed only 3 to 5 fold increase in transcripts of LCY, BKT and CHY genes. The lycopene cyclase enzyme activity of the mutants E3 and N5, as shown in the Table 3.10, correlates with lycopene cyclase gene expression.

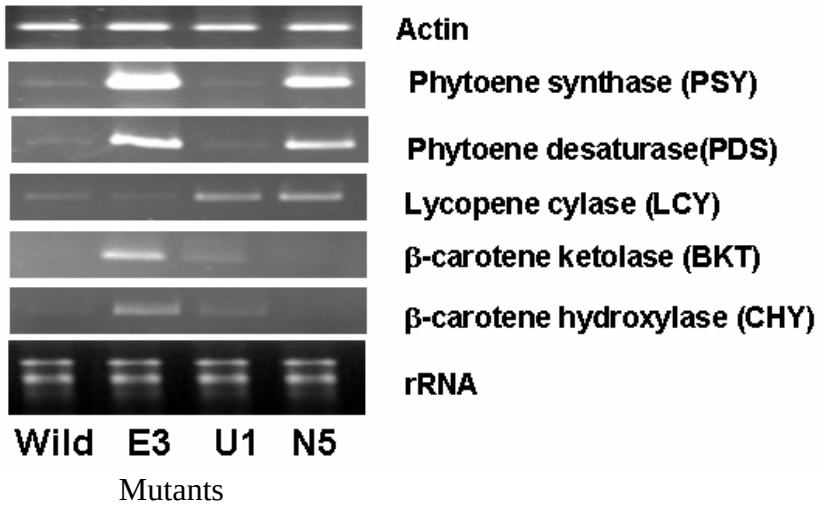


Figure 3.29.Expression of carotenoid biosynthetic genes in *H. pluvialis* mutants

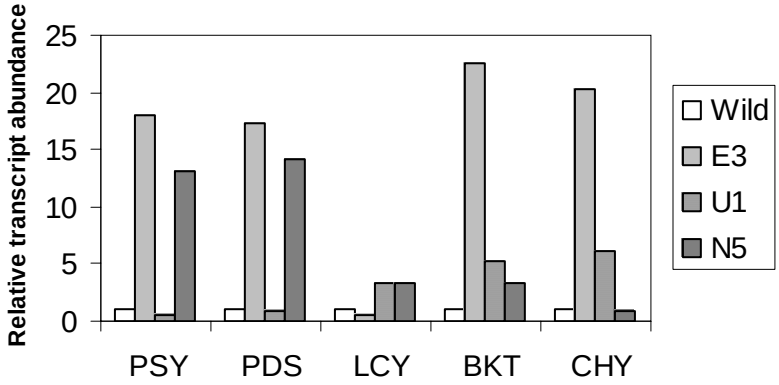


Figure 3.30. The band intensity of each gene in comparison with the band intensity of actin.

3.21. Discussion

The mutagens EMS, NTG and UV have been widely employed for strain improvement in algae (Alonso, 1996; Couderchet et al, 1995; Meireles et al, 2003), fungi (Wang et al, 2004; Yang et al, 1999; Agrawal et al, 1999; Miura et al, 2004) and bacteria (Kim et al, 2004). UV light is known to produce pyrimidine dimers which have slow rates of repair (Ganesan et al, 1983). The mutagens EMS and NTG produces lesions such as alkyl-phosphotriesters which are slowly removed from DNA (Goth-Goldstein, 1980). Growth of algal colony after treatment with mutagen is believed to be the cell's capacity to repair DNA damage produced by that agent (Eckardt et al, 1980). In the current study, these mutagens were used to isolate high yielding *H. pluvialis* mutants and the results reveal the satisfactory outcome of mutagenesis. The isolated mutants exhibited an increase of 23-59% increase in total carotenoid and astaxanthin content (Figure 3.21-3.23) compared to wild type. The mutants also showed distinct differences from wild type in terms of photosynthetic activity, chlorophyll fluorescence profile, lycopene cyclase enzyme activity and expression of carotenoid biosynthetic genes.

The combination of an efficient method for generating random mutation and a strategy that permits high throughput screening is essential for successful mutagenesis approach. In the present study, mutagenesis with chemical and physical agents was attempted to increase the microalgal biomass and carotenoid production in *H. pluvialis*. It was anticipated that the target enzyme would be altered by the action of mutagen. Number of trials was carried out to obtain satisfactory survival rate after treatment with mutagens. The degree of mutagenesis was controlled by changing the parameters such as concentration of EMS or NTG, incubation time or exposure time of UV. Although, on screening, glufosinate containing medium generated acceptable number of colonies, the efforts using the other herbicides like glyphosate, atrazine, diphenylamine and diquat were fruitless. The appropriate concentration of glufosinate required for screening the mutants was determined after checking the minimum inhibitory concentration by growing the wild type *H. pluvialis* strain in wide concentration range.

Herbicides are known for their enzyme inhibitory actions, they disrupt basic metabolic processes essential for plant or algal cells. Resistance of the strain to inhibitor or herbicide has been generally used for screening the mutants with desired properties (Tjahjono et al, 1994b; Erickson et al, 1989; Modi et al, 1991; Tripathi et al, 2001a; Lange et al, 2001). It has been reported that the herbicide –glufosinate efficiently inhibits the cell growth of *H. pluvialis*, induces astaxanthin accumulation by blocking the activity of enzyme glutamine synthetase (GS), a key enzyme in ammonia assimilation (Aflalo et al, 1999). Pigment mutants are unique tools to study the function of photosynthetic complexes and carotenoid pathways (Sun et al, 1998; Chitnis et al, 1997; Hoshino et al, 1994). Shaish et al (1991) have isolated β-carotene rich mutants of *Dunaliella bardawil* and suggested that the mutants are affected in the regulatory path, which controls the β-carotene production. Astaxanthin overproducing mutant of *Phaffia* after treatment with NTG has been reported by Bon et al (1997). Effect of UV irradiation on motility, pigmentation and several metabolic processes of algal system have been reported (Agrawal, 1994; Dohler, 1989). UV light exposure resulted in rapid inactivation of algal cells and consequently yields a low percentage of mutants among the survivors. Alonso et al (1996) have reported the increase in the yield of eicosapentaenoic acid content in microalga *Phaeodactylum tricornutum* by UV-induced mutagenesis. Wachi et al (1995) have reported the effect of UV-A on cyanobacteria- *Oscillatoria* where the chlorophyll biosynthesis was inhibited by the UV irradition. Out of the 5 mutants isolated after UV treatment, 4 have shown less carotenoid content indicating the possible effect of UV irradiation on carotenoid biosynthesis pathway. The herbicide glufosinate, during the screening process, did not influence the photosynthetic ability of the UV induced mutant as demonstrated by the unaltered photosynthetic activity (Figure 3.27) and chlorophyll fluorescence (Figure 3.28).

Numerous herbicide-resistant mutants of *Chlamydomonas* with different patterns of resistance to such herbicides have been reported (Erickson et al, 1989). Green algae can develop resistance to herbicide that block metabolic pathways like photosynthesis by competing with quinines in binding to the chloroplast photosystem II DI polypeptide.

The mutants obtained with different mutagen treatments and subsequent screening over herbicide glufosinate did not show any differences in the carotenoid profile as analysed by HPLC (Figure 3.25). However the mutants differed in growth (biomass yield) and total carotenoid content which may be due to possible changes in photosystem. The differences in the transcript levels of carotenogenic genes in response to stress in different mutants also substantiate the above statement.

The carotenoid production, photosynthetic activity and fluorescence of the mutant N5 was well correlated with the expression of carotenoid genes –PSY and PDS. The relative inhibition of photosynthesis by herbicide in this mutant was less compared to wild type (Figure 3.27). Mutant N5 also showed almost 60% higher variable fluorescence (Fv; Figure 3.28) and 12 fold higher carotenoid gene transcript abundance in comparison with wild type. These results provide insight into the regulation of carotenoid biosynthesis in the mutant N5. It can be contemplated that, by the action of mutagen NTG, the components of the photosynthetic electron transport has been affected, hence the carotenoid expression is dependent on the redox state. This view on photosynthetic regulation is also supported by the investigations by Steinbrenner and Linden (2003), that entire carotenoid biosynthesis in *Haematococcus* is under photosynthetic redox control.

Though light is crucial and governing factor in both photosynthesis and carotenoid formation in *Haematococcus*, maximum astaxanthin biosynthesis is the result of synergistic action by several other factors (Sarada et al, 2002a; Wang et al, 2003; Lababpour et al, 2004). Therefore the above postulation does not hold good for the mutant U1 and E3 and the exact mechanism responsible for their differential expression remains to be elucidated.

From the Indian context, the information on availability of *Haematococcus* is scanty. It is imaginable that the environmental conditions are not well suited for its occurrence and propagation. Hence, as the demand for natural astaxanthin grows, these high yielding mutants hold promise.

The findings of this study reveal that the chemical mutagen and UV irradiation have altered certain biochemical characteristics of the wild type. In concurrence with

the above reports, the altered biochemical properties of *H. pluvialis* mutants can be attributed to the changes at molecular level. This reasoning is also supported by the data on expression of carotenoid biosynthetic genes in mutants. These data provides scope for further studies on molecular aspects of *H. pluvialis* mutants.

Bioactivity of astaxanthin in *in vitro* and *in vivo* models

Background

Gastric hyperacidity and gastric ulcers are reported to be the most common pathological conditions of present days, resulting in uncontrolled acid secretion and pepsin activity (Kaviani et al, 2003). The imbalance between damaging factors within the lumen and protective mechanisms within the gastro duodenal mucosa, reduced mucus-bicarbonate secretion, accumulation of reactive oxygen species, *Helicobacter pylori* infection, enhanced contractibility of the gastric wall, increased H^+,K^+-ATPase activity, reduced gastric mucosal blood flow represents some of the established factors for causing gastric ulcers (Sachs et al, 1995; Galunska et al, 2002; Das et al, 1997; Lai et al, 2003, Rastogi et al, 1998). The continuous use of Non-Steroidal Anti-Inflammatory Drugs by the global population, stressful lifestyle and inadequate intake of nutritious foods/nutraceuticals are adding to the increased incidence of ulcers worldwide (Miller, 1987; Langman et al, 1991). Commercially available drugs for treatment of this disease, when used for long term, are known to cause unpredictable side effects (Debashis et al, 2002) and this warranted identification of safer alternative sources for ulcer management. Free radical scavenging and antioxidant activities play an important role in prevention of free radical –related diseases, including aging and ulcers. Nutraceutical and other beneficial properties of astaxanthin are being explored. In the current study, the effect of astaxanthin fractions from *H. pluvialis* such as total carotenoid extract, astaxanthin esters and saponified astaxanthin were evaluated for their biological potency *in vitro* and *in vivo* models. Effect of astaxanthin esters against ethanol induced ulcers in animals was tested. In addition, the pigmentation efficiency of astaxanthin in poultry and aquaculture was also evaluated.

3.22. Astaxanthin fractions from *H. pluvialis*

In order to determine the antioxidant and ulcer preventive potency, astaxanthin fractions from encysted and freeze dried *H. pluvialis* cells were extracted with acetone and characterized by chromatographic techniques. The total carotenoid content was found to be 2.05% (w/w) on dry weight basis. This total carotenoid extract produced prominent ester bands (astaxanthin esters) on TLC corresponding to Rf value of 0.9 and 0.78. These ester bands from TLC were saponified and characterized by HPLC. The HPLC profile of these fractions and astaxanthin standard is shown in Figure 3.31. The mono and diesters collectively represented around 95% of astaxanthin content in total carotenoid. HPLC profile as shown in Figure 3.31B indicated the abundance of astaxanthin monoester followed by diester. Saponification of astaxanthin esters yielded free astaxanthin which was confirmed by comparison with standard synthethic astaxanthin (Figure 3.31C and D). Percent saponification under the conditions of the experiment described appears to be 73%.

3.23. Assessment of gastric mucosal protection by *H. pluvialis* astaxanthin

Ethanol administration had induced severe lesions including inflammatory patches, bleeding in mucosa and ulcers with different size and degree in ulcerated rats (Figure 3.32) with an ulcer index of 43. No such gastric lesions and bleeding were noticed in healthy controls (Figure 3.32A). Upon pretreatment of animals with total carotenoid, astaxanthin esters at 100 to 500 µg/kg b.w., astaxanthin esters treated rats showed significant reduction (3 fold) in ulcer index as opposed to that of ulcer induced animals (Figure 3.33). No significant protection was observed in total carotenoid treated animal groups at P value < 0.05. Percent ulcers protected after treatment with different doses are provided in Figure 3.34A. No ulcers were found to be induced with only total carotenoid or astaxanthin ester controls suggesting that total carotenoid and astaxanthin esters at the described concentration is safe. Dose dependent protection reaching almost 70 % was observed at 500µg level of astaxanthin esters. At similar concentration, total carotenoid fraction showed ~40% protection suggesting that astaxanthin ester fraction of

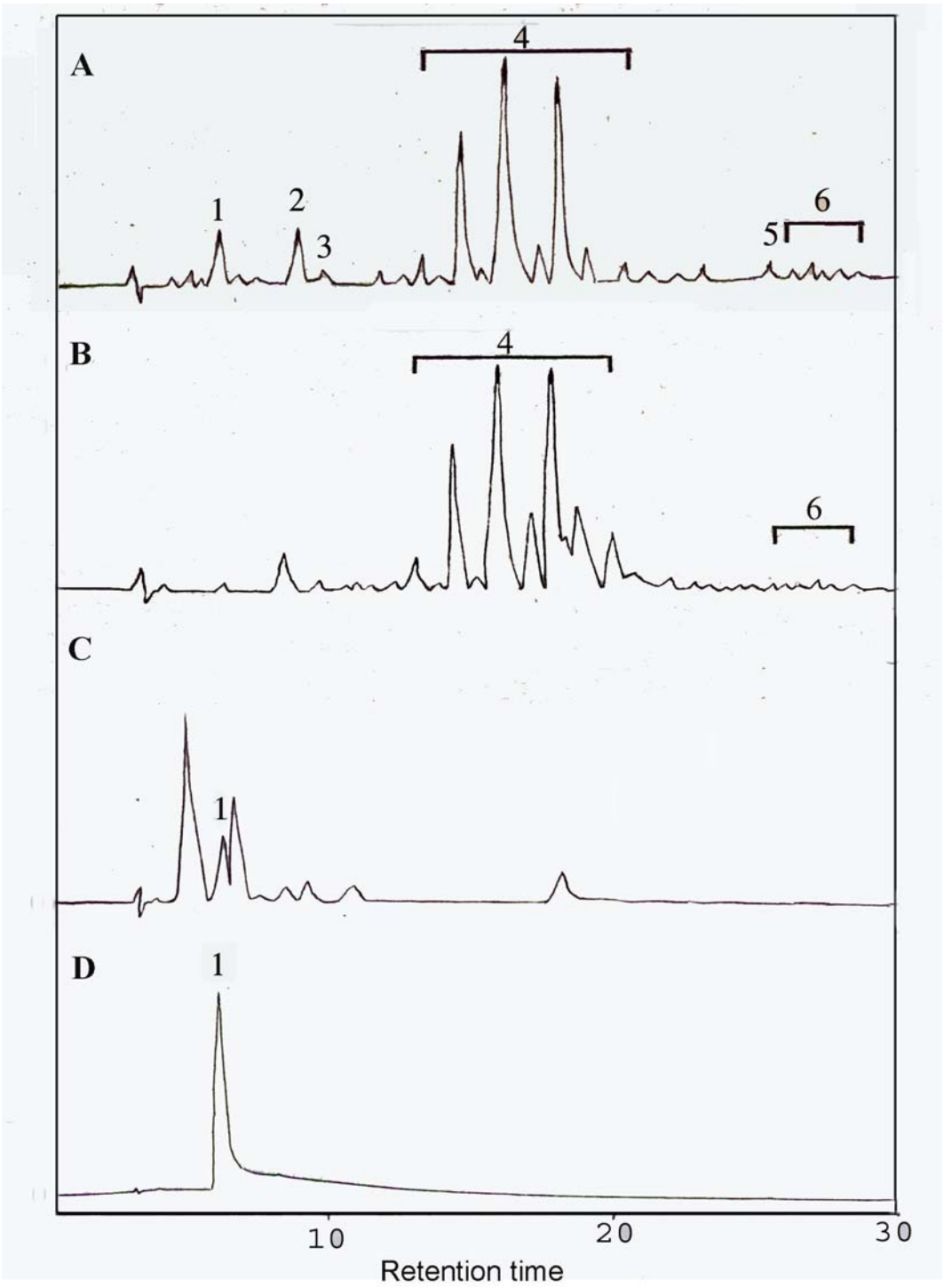


Figure 3.31. HPLC profile of total carotenoid extract (A), esters of astaxanthin (B), saponified astaxanthin (C) and synthetic astaxanthin (D).

1- free astaxanthin, 2-lutein, 3-canthaxanthin, 4-astaxanthin monoesters, 5-β-carotene, 6-astaxanthin diesters

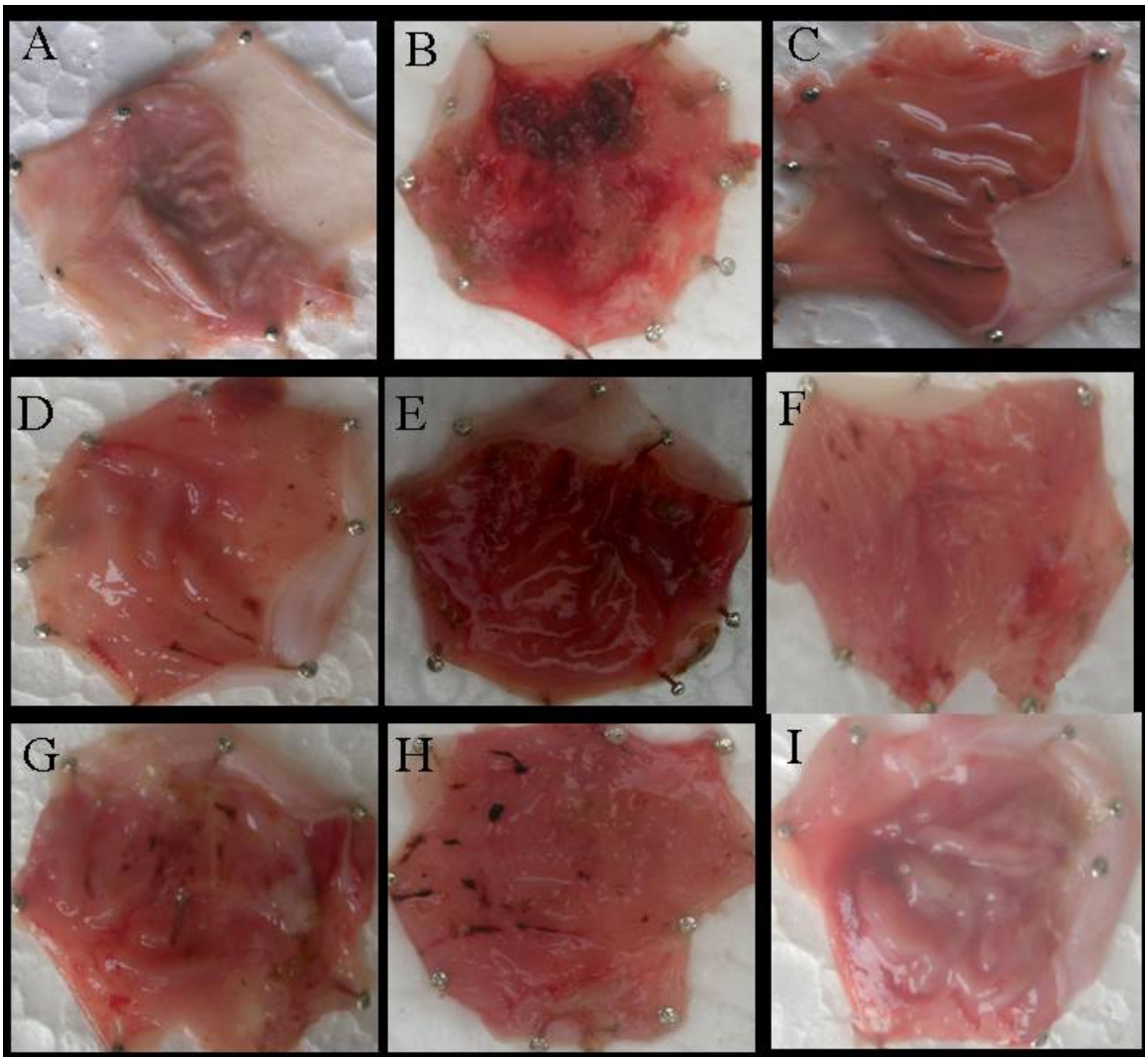


Figure 3.32. Macroscopic observation and ulcer index of stomach from ulcer induced and astaxanthin/omeprazole treated animals.
A-healthy control, B-Ulcerated, C- Omeprazole treated, D- -TC100 μg/kg b.w., E-TC250 μg/kg b.w., F- TC500 μg/kg b.w., G-–EAX 100 μg/kg b.w., H- EAX 250 μg/kg b.w.,I-EAX-500 μg/kg b.w. (TC-Total carotenoid, EAX –Astaxanthin esters).

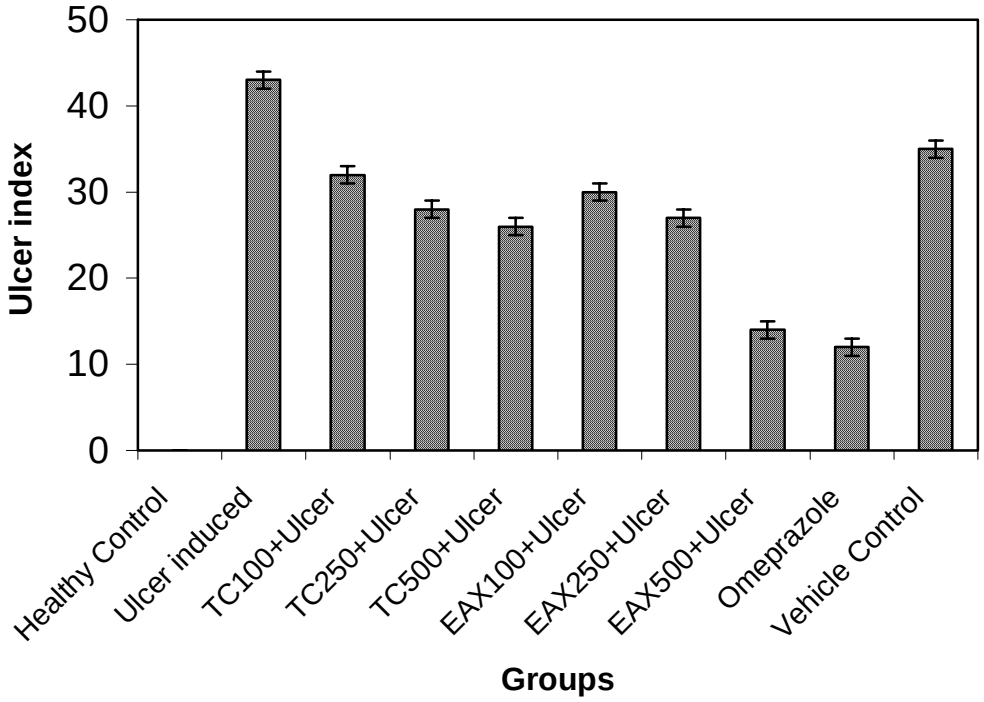


Figure 3.33. Ulcer index of stomach from ulcer induced and astaxanthin/omeprazole treated animals (TC-Total carotenoid, EAX –Astaxanthin esters)

total carotenoid may be responsible for gastroprotection against ulcer. Analogous to this, 61% mucin binding was observed (Figure 3.34B) revealing that protection against ulcer may partly be via inhibiting mucosal damage that are generally caused by free radicals induced by ethanol.

The percent gastro protection offered by astaxanthin samples as shown in Figure 3.34A, were calculated based on inhibition of Ulcer Index. Results showed dose dependent increase in mucosal content, as measured by Alcian blue binding studies. Pre-administration of total carotenoid and astaxanthin esters have shown dose dependent protection of gastric mucosa. Increase in total carotenoid concentration from 100 to 500μg/kg b.w. did not show significant increase in mucosal protection. Astaxanthin esters showed the maximum protection of 67% in rats treated with 500μg/kg b.w. the protective effect of astaxanthin esters was also reflected in mucin content of the ulcerated rats in which 61% mucin binding was observed (Figure 3.34) as evaluated by Alcian blue

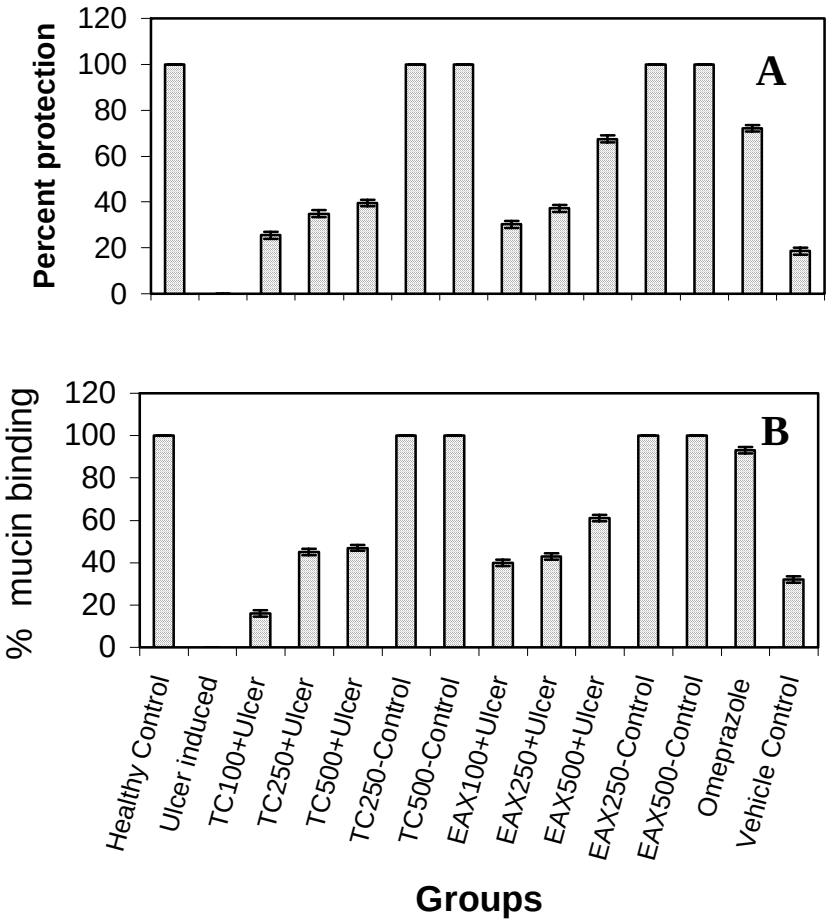


Figure 3.34. Protection offered by total carotenoid and astaxanthin esters against ethanol induced ulcer (A) and mucin binding (B) as measured by alcian blue staining. (TC-Total carotenoid, EAX –Astaxanthin esters)

binding. Almost 1.5 fold enhancement in ulcer preventive effect in astaxanthin esters compared with that of total carotenoid was observed which may be attributed to the purity of astaxanthin esters in the isolated fraction.

3.24. Histopathological analysis

Deep erosions were observed in ulcer induced rats (Figure 3.35B). Rats treated with astaxanthin esters at 500 μg/kg b.w. showed normal histology or very superficial lesions only (Figure 3.35F) similar to those of healthy controls (Figure 3.35A). The microscopic examination clearly indicated the protective effect of astaxanthin esters (Figure 3.35 E and F) and total carotenoid (Figure 3.35C and D). Protective ability was comparable with that of the known anti ulcer drug, Omeprazole (Figure 3.35G). Results were substantiated by measuring mucin content (Figure 3.34 B).

3.25. Changes in the antioxidant enzymes

The stomach superoxide dismutase (SOD) levels in ulcer induced rats were significantly decreased (Table 3.11). The SOD activity was 25.30 ± 0.76 and 23.05 ± 0.75 U/mg protein in ulcerated and vehicle treated rats respectively. Pretreatment of rats with astaxanthin esters at 500 μg/kg b.w. has increased the SOD levels to 89.76 ± 0.98 U/mg protein which is comparable to that of controls (95.20 ± 2.86). Pretreatment at lower concentration of total carotenoid and astaxanthin esters did not exhibit significant increase in SOD levels. Similar effect of astaxanthin esters was also observed in catalase and glutathione peroxidase activity which was 15 and 2 fold higher respectively, when compared to ulcerated rats. The activity of these enzymes in healthy control group was 1.57 and 32.5 U/mg of protein respectively.

The antioxidant enzyme activity in serum and liver homogenates is shown in Table 3.12. A 2-3 fold increase in TBARS in ulcerated animals when compared to healthy animals were significantly normalized with total carotenoid and astaxanthin esters treatment, suggesting the action of total carotenoid and astaxanthin esters against biochemical changes induced by ulceration by ethanol. However, no significant difference was observed between ulcerated and omeprazole treated groups since the mechanism of action is probably via inhibition of H^+,K^+-ATPase and not by antioxidative route.

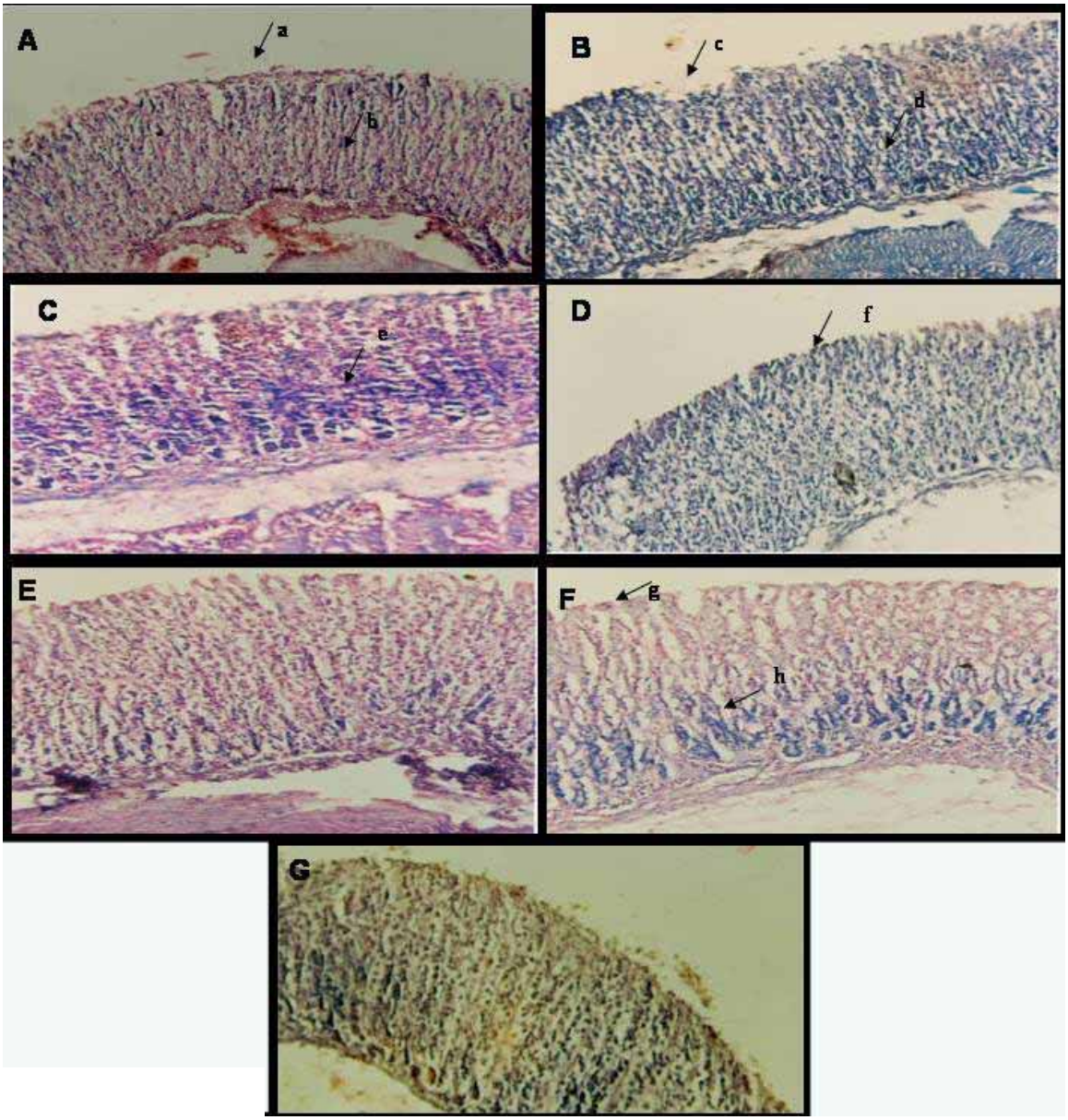


Figure 3.35. Histopathological observation of stomach from ulcer induced and astaxanthin/omeprazole treated animals

Histopathological observation of stomach from ulcer induced/astaxanthin and Omeprazole treated animals; A–D indicates hematoxylin and eosin staining sections (Magnification 10X). Control (A) shows intact mucosal epithelium (a) with organized glandular structure (b). Ulcer induction (B) showed damaged mucosal epithelium (c) and disrupted glandular structure (d). Fig C & D and E & F show a recovery in mucosal epithelium (f, g) and reorganized glandular structure (e, h) by total carotenoid and astaxanthin treatment respectively. Omeprazole (G) also shows mucosal protection.

Table 3.11. Effect of astaxanthin on antioxidant enzymes in stomach homogenate

Groups	SOD (U/mg protein)	TBARS (nM of MDA/mg protein)	Catalase (U/mg protein)	Glutathione Peroxidase (nM of NADPH oxidised/min/mg protein)
Healthy control	$95.20^{a} \pm 2.86$	$2.73^{a} \pm 0.07$	$1.57^{f} \pm 0.03$	$32.50^{d} \pm 1.30$
Ulcer induced	$25.30^{e} \pm 0.76$	$8.20^{h} \pm 0.09$	$0.17^{h} \pm 0.01$	$10.59^{i} \pm 0.42$
TC100*	$21.64^{d} \pm 0.32$	$7.04^{f} \pm 0.08$	$1.34^{g} \pm 0.03$	$35.86^{c} \pm 1.44$
TC 250*	$23.15^{e} \pm 0.69$	$7.53^{g} \pm 0.10$	$2.09^{c} \pm 0.04$	$43.98^{b} \pm 1.76$
TC 500*	$37.83^{c} \pm 1.13$	$5.74^{d} \pm 0.08$	$2.24^{b} \pm 0.04$	$46.45^{a} \pm 1.89$
EAX 100*	$39.23^{c} \pm 1.18$	$5.75^{d} \pm 0.08$	$1.73^{e} \pm 0.04$	$35.67^{c} \pm 1.42$
EAX 250*	$32.67^{d} \pm 0.98$	$5.20^{c} \pm 0.07$	$1.62^{f} \pm 0.03$	$24.69^{f} \pm 0.98$
EAX 500*	$89.76^{b} \pm 0.98$	$5.74^{d} \pm 0.09$	$2.67^{a} \pm 0.05$	$21.60^{g} \pm 0.86$
Omeprazole ⊕	$24.92^{e} \pm 0.75$	$6.84^{e} \pm 0.12$	$1.96^{d} \pm 0.04$	$29.86^{e} \pm 1.19$
Vehicle control	$23.05^{e} \pm 0.69$	$3.45^{b} \pm 0.08$	$0.18^{h} \pm 0.01$	$17.08^{h} \pm 0.68$

TC-Total carotenoid, EAX- astaxanthin esters, * μg/kg b.w. ⊕ 20mg/kg b.w.

Results are expressed as Mean±S.D. Different letters a to i in the column represents that values are significantly different when compared between ulcer induced with healthy control and TC,EAX and omeprazole treated groups. Range was provided by Duncan multiple system at $p < 0.05$

Table 3.12. Effect of astaxanthin on antioxidant enzymes in serum and liver homogenate

Groups	SOD (U/mg protein)	TBARS (nM of MDA/mg protein)	Catalase (U/mg protein)	Glutathione Peroxidase (nM of NADPH oxidised/min/mg protein)
Serum				
Healthy control	$25.30^{a} \pm 0.76$	$1.34^{b} \pm 0.05$	$0.70^{d} \pm 0.04$	$17.72^{a} \pm 0.98$
Ulcer induced	$11.82^{f} \pm 0.35$	$3.76^{g} \pm 0.15$	$0.25^{i} \pm 0.01$	$7.20^{f} \pm 0.40$
TC 100*	$19.40^{cd} \pm 0.58$	$2.97^{e} \pm 0.12$	$0.43^{g} \pm 0.02$	$13.20^{de} \pm 0.73$
TC 250*	$21.28^{b} \pm 0.64$	$3.10^{e} \pm 0.12$	$0.50^{f} \pm 0.02$	$12.20^{e} \pm 0.67$
TC 500*	$19.30^{cd} \pm 0.58$	$0.82^{a} \pm 0.03$	$0.71^{d} \pm 0.03$	$18.80^{a} \pm 1.04$
EAX 100*	$20.16^{c} \pm 0.60$	$2.04^{d} \pm 0.08$	$1.13^{b} \pm 0.05$	$14.10^{d} \pm 0.78$
EAX 250*	$18.91^{d} \pm 0.57$	$1.94^{d} \pm 0.08$	$0.64^{e} \pm 0.03$	$16.90^{bc} \pm 0.93$
EAX 500*	$25.28^{a} \pm 0.76$	$1.56^{c} \pm 0.06$	$0.87^{c} \pm 0.04$	$15.80^{e} \pm 0.87$
Omeprazole 20mg	$16.50^{e} \pm 0.49$	$3.46^{f} \pm 0.14$	$1.37^{a} \pm 0.06$	$12.60^{e} \pm 0.69$
Vehicle control	$8.90^{g} \pm 0.27$	$1.60^{c} \pm 0.06$	$0.31^{h} \pm 0.01$	$5.10^{g} \pm 0.28$
Liver				
Healthy control	$2.10^{e} \pm 0.07$	$3.25^{b} \pm 0.15$	$0.17^{f} \pm 0.01$	$10.64^{a} \pm 0.53$
Ulcer induced	$1.30^{g} \pm 0.05$	$5.97^{f} \pm 0.27$	$0.30^{e} \pm 0.02$	$2.65^{i} \pm 0.13$
TC 100*	$3.27^{b} \pm 0.09$	$5.34^{d} \pm 0.24$	$0.59^{b} \pm 0.04$	$7.15^{d} \pm 0.36$
TC 250*	$3.36^{b} \pm 0.10$	$5.46^{de} \pm 0.25$	$0.38^{d} \pm 0.03$	$6.20^{e} \pm 0.31$
TC 500*	$4.05^{a} \pm 0.11$	$3.89^{c} \pm 0.18$	$0.61^{b} \pm 0.04$	$8.04^{c} \pm 0.40$
EAX 100*	$2.86^{c} \pm 0.08$	$5.57^{de} \pm 0.25$	$0.21^{f} \pm 0.01$	$3.57^{h} \pm 0.18$
EAX 250*	$2.57^{d} \pm 0.08$	$5.77^{ef} \pm 0.26$	$0.44^{c} \pm 0.03$	$4.57^{g} \pm 0.23$
EAX 500*	$2.66^{d} \pm 0.08$	$3.66^{c} \pm 0.17$	$0.70^{a} \pm 0.05$	$5.02^{fg} \pm 0.25$
Omeprazole$^{\oplus}$	$2.13^{e} \pm 0.07$	$6.37^{g} \pm 0.29$	$0.38^{d} \pm 0.03$	$9.65^{b} \pm 0.48$
Vehicle control	$1.54^{f} \pm 0.06$	$2.71^{a} \pm 0.12$	$0.20^{f} \pm 0.01$	$5.20^{f} \pm 0.26$

TC-Total carotenoid, EAX- astaxanthin esters , * μg/kg b.w. $^{\oplus}$ 20mg/kg b.w.

Results are expressed as Mean±S.D. Different letters a to i in the column represents that values are significantly different when compared between ulcer induced with healthy control and TC, EAX and omeprazole treated groups. Range was provided by Duncan multiple system at $p< 0.05$

3.26. *In vitro* antioxidant activity of astaxanthin from *H. pluvialis*

The DPPH radical scavenging activity of total carotenoid, astaxanthin esters and saponified astaxanthin was compared with synthetic astaxanthin and butylated hydroxy anisole (Figure 3.36). Saponified astaxanthin showed the maximum free radical scavenging activity at an IC_{50} of 8.1 μg/ml which is 4.5 fold higher in comparison to standard astaxanthin (IC_{50} 36.5 μg/ml; Table 3.13). Saponified astaxanthin also demonstrated maximum reducing power followed by total carotenoid and astaxanthin esters (Figure 3.36B). Dose dependent increase in activity suggests that activity is increased proportional to the concentration of astaxanthin in the sample. Similarly, with the antioxidant potency, saponified astaxanthin could also inhibit 15-lipoxygenase activity (Figure 3.37) at an IC_{50} of 3.4 μg/ml, which is ~ 6 and 7 fold higher compared total carotenoid and astaxanthin esters respectively (Table 3.13).

3.27. Ability of astaxanthin to inhibit H^+,K^+-ATPase enzyme *in vitro*

H^+,K^+-ATPase inhibitors such as omeprazole, lansoprazole are antiulcerative agents since they block the upregulated activity of H^+,K^+-ATPase. In order to understand the possible mechanism of action of saponified astaxanthin and astaxanthin esters, inhibition of isolated parietal cell plasma membrane H^+,K^+-ATPase activity was studied. Saponified astaxanthin showed maximum H^+,K^+-ATPase activity followed by astaxanthin esters and total carotenoid (Figure 3.38) . Standard astaxanthin exhibited significantly low inhibition while astaxanthin esters showed inhibition at an IC_{50} of 18.2μg/ml which is comparable to that of the known H^+, K^+-ATPase inhibitors like lansoprazole which has IC_{50} of 19.2μg/ml (Table 3.13).

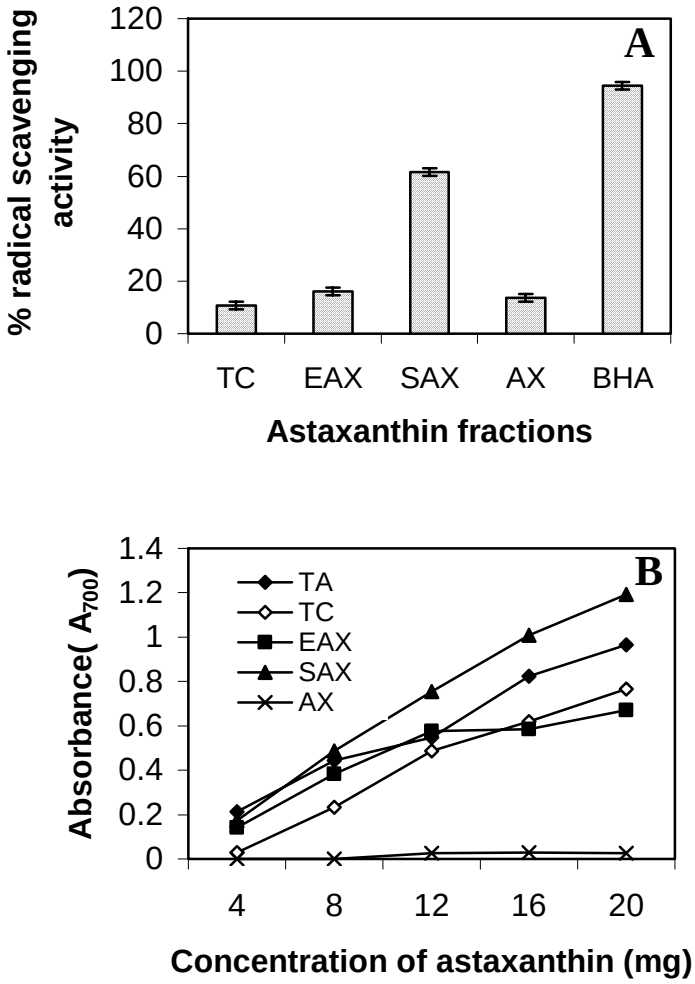


Figure 3.36. *In vitro* antioxidant activity of astaxanthin fractions from *H. pluvialis*.
A. Free radical scavenging activity B. Reducing power activity
TC-Total carotenoid, EAX-Astaxanthin Esters, SAX-Saponified astaxanthin, AX-Synthetic astaxanthin, TA-Tannic acid, BHA-Butylated hydroxy anisole

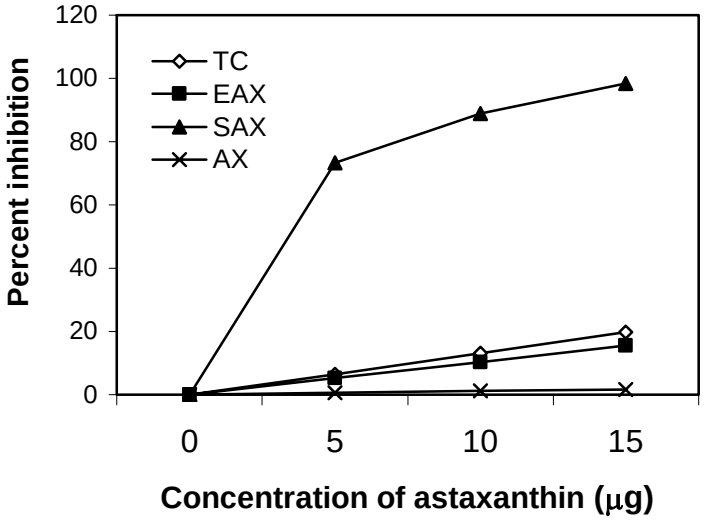


Figure 3.37. Lipoxygenase inhibitory activity of astaxanthin

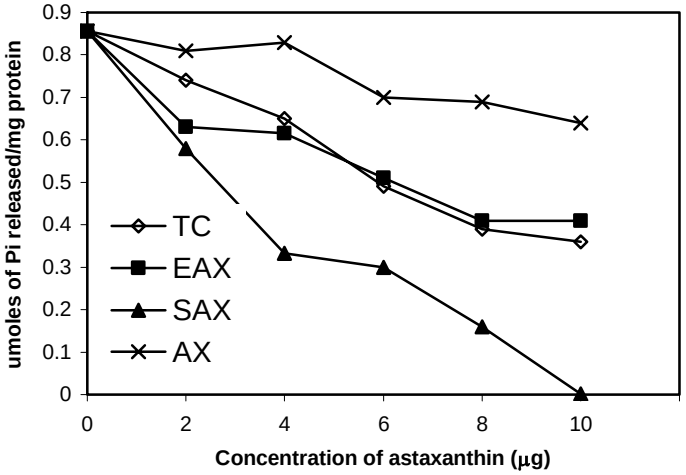


Figure 3.38. H^+, K^+-ATPase inhibition activity of astaxanthin fractions
TC-Total carotenoid, EAX-Astaxanthin Esters,
SAX-Saponified astaxanthin, AX-Synthetic astaxanthin

Table 3.13. *In vitro* antioxidant property of astaxanthin fractions

Astaxanthin sample	**Free redical scavenging activity - IC_{50}-(μg/ml)**	**Reducing power activity (unit/g)**	**H^+,K^+-ATPase inhibition activity -IC_{50}-(μg/ml)**	**Lipoxygenase inhibition activity IC_{50}-(μg/ml)**
Total carotenoid	46.7	38.35	14.4	19.1
Astaxanthin ester	31.2	33.55	18.2	24.4
Saponified astaxanthin	8.1	59.60	6.2	3.4
Synthetic astaxanthin	36.5	1.30	36.0	568
Butylated Hydroxy anisole	8.5	-	-	-
Lansoprazole	-	-	19.2	-
Tannic acid	-	48.25	-	-

3.28. Pigmentation efficiency of *H. pluvialis* in egg yolk

White leg horn layers of 20 weeks old were fed with the experimental feed at 0.5, 2, 4 mg astaxanthin per kg diet for a period of 4 weeks. Egg carotenoid content and quality parameters were monitored after two weeks of feeding. First day of 3rd week was considered as 'day 1' for recording the carotenoid content and colour measurement of egg yolk. The carotenoid in the cell-free extract form (diet 4) at 0.5 mg/kg reached to saturation level in egg yolk by two weeks and the yolks contained same level of carotenoid till the four weeks period which was found to be always higher than the control (Table 3.14). Carotenoid at 2 and 4 mg/kg level (diet 2 and 3) increased with time but the absorption of carotenoid into egg yolks at 2 and 4 mg/kg was similar without significant difference. A maximum of 44 μg of carotenoid/g of egg yolk was observed in experimental birds, which is 2 fold higher compared to control (Table 3.14).

The internal quality parameter of the egg indicates improvement in all the tested parameters in *H. pluvialis* supplemented feed at 2 mg/kg carotenoid level. Haugh Unit score was found to be 76 and USDA grade AA in the eggs of layers fed with feed containing *H. pluvialis* at 2mg/kg carotenoid level (Table 3.15). Subjective colour

evaluation showed that egg yolk colour was constant from 2nd to 4th week of feeding experimental diet. Intense colour of fresh egg yolk was observed as caused by the *H. pluvialis* supplemented diet (Figure 3.39). Yolk colour score was found to be 11.00 in the experimental eggs (2 mg/kg level) whereas the control eggs showed a colour score of 10.0 (Table 3.15).

Egg yolk colour was measured by reflectance colorimetry. Colour parameters were recorded on alternate days starting from day of egg collection. The colour of egg yolk showed significant differences for all the colour parameter (L a b) as an effect of *H. pluvialis* supplemented feed (Table 3.16). The egg yolk lightness (L) showed little variation as a result of *H. pluvialis* supplemented feed. Egg yolk colour showed highest tendencies towards red tone for diet 3 as indicated by redness parameter (a).

Table 3.14. Carotenoid (mg/g of yolk) content in the egg yolk fed with experimental diet*.

	Diet 1	**Diet 2**	**Diet 3**	**Diet 4**
Day 1	0.016^{c}	0.035^{ab}	0.04^{a}	0.038^{a}
Day 3	0.014^{c}	0.029^{a}	0.03^{a}	0.029^{a}
Day 5	0.017^{c}	0.031^{a}	0.031^{a}	0.03^{a}
Day 7	0.022^{c}	0.037^{a}	0.037^{a}	0.032^{b}
Day 9	0.024^{cd}	0.042^{b}	0.049^{a}	0.038^{b}
Day 11	0.027^{cd}	0.04^{ab}	0.044^{a}	0.038^{b}
Day 13	0.025^{c}	0.044^{a}	0.046^{a}	0.029^{a}

Data recorded after 2 weeks of feeding.
Means within a column followed by the same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).
Diet 1- control (without *H. pluvialis* supplementation);
Diet 2 and 3- *H. pluvialis* biomass (carotenoid equivalent) 2 mg/kg and 4mg/kg respectively. Diet 4- *H. pluvialis* extract (carotenoid equivalent) 0.5mg/kg.

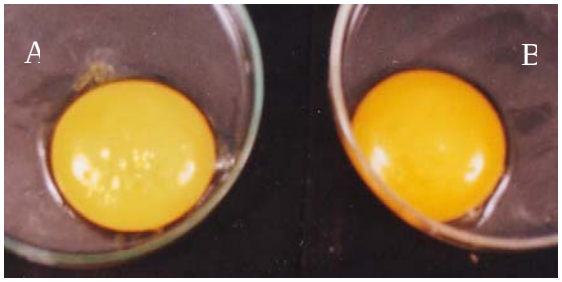


Figure 3.39. Pigmentation in egg yolk by feeding astaxanthin rich *H. pluvialis* biomass.
A- Fed with diet 1 (without *H. pluvialis* supplemenatation)
B- Fed with diet 2 (supplemented with 2mg/kg *H. pluvialis* biomass)

Table 3.15. Internal quality of eggs from experimental layers

Group	Egg weight (g)	Albumin index	Haugh units score and USDA Grade	thick albumin (g/100g)	Shell thickness (mm)	Yolk index	Yolk colour
Diet 1	46.06^{c}	0.057^{b}	67 A	61.53^{c}	0.014^{c}	0.66^{ab}	10.0^{bc}
Diet 2	47.68^{c}	0.068^{a}	**76 AA**	72.68^{a}	0.015^{b}	0.72^{a}	11.0^{a}
Diet 3	48.18^{c}	0.048^{c}	59 A	63.97^{c}	0.015^{a}	0.61^{bc}	10.3^{cd}
Diet 4	49.97^{a}	0.055^{bc}	66 A	66.31^{b}	0.015^{a}	0.57^{c}	10.3^{bc}

Data recorded after 4 weeks of feeding.
Means within a column followed by the same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

Table 3.16. Colour values of egg yolk fed with experimental diet*.

		L	a	b
After 3 weeks	Diet 1	55.5^{bc}	5.14^{c}	32.57^{bd}
	Diet 2	56.2^{bc}	5.91^{c}	35.05^{ab}
	Diet 3	55.62^{bc}	6.05^{bc}	33.05^{bc}
	Diet 4	57.06^{b}	4.41^{cd}	34.11^{b}
After 4 weeks	Diet 1	55.55^{bc}	4.16^{d}	34.0^{b}
	Diet 2	57.19^{b}	5.99^{bc}	33.11^{bc}
	Diet 3	57.81^{ab}	6.4^{bc}	34.59^{ab}
	Diet 4	54.61^{c}	4.6^{c}	34.34^{b}

Means within a column followed by the same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

3.29. Pigmentation efficiency of *H. pluvialis* in ornamental fish

Diet containing *H. pluvialis* cells were fed to Koi carp (*C. carpio*) fishes in order to impart attractive skin colouration. As shown in Figure 3.40, *H. pluvialis* supplemented diet did not affect the growth of fishes. The diet containing *H. pluvialis* (25 mg/kg) exhibited improved yellowness in fish skin as indicated by Hunter 'b' colour values (Table 3.17 and Figure 3.41).

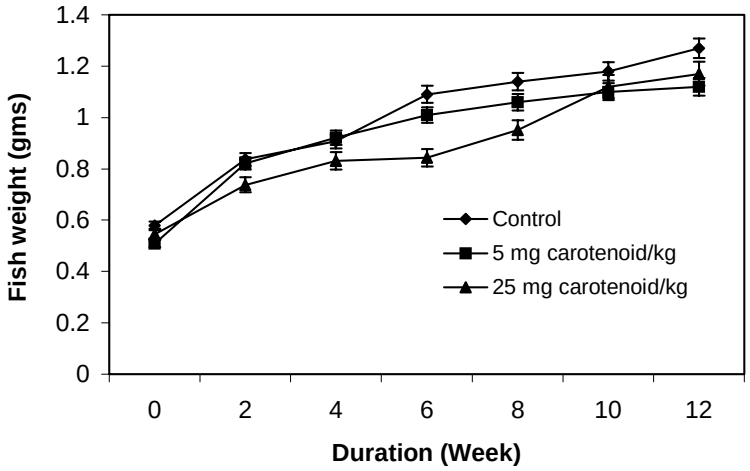


Figure 3.40. Growth profile of fish fed with *H. pluvialis* supplemented diet.

Table 3.17. Colour values of fishes fed with *H. pluvialis* supplemented diet.

Group	**L**	**a**	**b**
Control	46.65 ± 1.09	-0.06 ± 0.08	9.42 ± 0.96
H. pluvialis supplemented diet (equivalent to 25mg carotenoid/kg)	54.46 ± 0.82	0.96 ± 0.32	16.89 ± 0.79

*values are mean ±SD (n=6)

Control *H. pluvialis* supplementation

Figure 3.41. Koi carp fishes fed with *H. pluvialis* supplemented diet

3.30. Discussion

The present study demonstrates for the first time that orally administered total carotenoid and astaxanthin esters exerts a dose-dependent gastroprotective effect on acute, ethanol-induced gastric lesions in the rat. Ethanol consumption, leading to health complication in humans reportedly has become a serious problem throughout the world (Tapiero, 2004). Ulcerous bleeding till death, liver dysfunction etc are complications arises from ethanol. Throughout the world, 14.5 million people are known to be suffering from gastric ulcer (http://digestive.nidk.nih.gov/statistics/statistics.htm/peptic ulcer prevalence). The percent incidence is much more in the developing countries since alcohol consumption

together with lack of healthy diet adds to the seriousness of the disease. The release of oxygen-derived free radicals has drawn attention as a possible pathogenic factor of gastric mucosal injury associated with ethanol (Szabo et al, 1992; Smith et al, 1996). Ethanol has been known to penetrate rapidly into the gastric mucosa and this causes membrane damage, erosion of gastric cells, impairment in H^+ pumping into the gastric lumen and hence gastric ulceration. Investigations of Terano et al (1986) and Szabo et al (1992) have revealed the ethanol induced gastric damage is mediated by the generation of free radicals.

Besides preventing the extreme reactivity of ROS, the control of acid secretion is essential for the treatment of these diseases. While acid secretion by parietal cells is regulated through several stimulatory receptors, such as histamine H_2, muscarinic M_3 and gastrin, the final step is mediated by gastric pump, also called as proton pump (Hersey et al, 1995). Thus the effective therapeutic control of acid secretion involves both the blockade of these receptors and the inhibition of the proton pump. Free saponified astaxanthin from *H. pluvialis* has shown maximum H^+, K^+- ATPase inhibitory activity (Table 3.13) which implies its ulcer preventive effect.

Recently, Kim et al (2005) documented that astaxanthin from yeast-*Xanthophyllomyces* exhibited its ability to inhibit ethanol induced gastric ulceration and they proposed that the inhibition of gastric ulceration is via activation of antioxidant enzyme. As detailed in section 1.9, *H. pluvialis,* synthesize the (3S, 3′S)-isomer, whereas yeast *Xanthophyllomyces* produces the opposite isomer having the (3R,3′R)-configuration (Visser et al, 2003). Standard astaxanthin consists of a mixture 1:2:1 of isomers (3S, 3′S), (3R, 3′S) and (3R, 3′R) respectively (Higuera-Ciapara, 2006). In the current study, the efficacy of astaxanthin ester was addressed in comparison with total carotenoid extract from *H. pluvialis* against ethanol induced ulceration at lower doses. Determining the ability of astaxanthin esters and saponified astaxanthin was aimed at inhibiting H^+,K^+-ATPase, a key enzyme responsible for gastric acidity and the gastric mucin- a gastroprotectant. It is well known that astaxanthin is highly lipophilic compound; therefore the function of astaxanthin as a free radical scavenger and antioxidant is likely assisted by the ease with which it crosses morphophysiological barriers. The study by Tso and Lam (1996) has demonstrated that astaxanthin can cross blood retinal barrier in

mammals and can extend its antioxidant benefits beyond that barrier. In addition to the fact that astaxanthin can readily enter into subcellular compartments, where free radicals may be generated, it has no known toxic effects (Guerin et al, 2003).

Presence of astaxanthin esters in *H. pluvialis* has an added advantage that, generally carotenoids, although are potential antioxidants, many a times in *in vivo*, they lack such properties because of pro-oxidant effect. Esterified astaxanthin shows comparatively better stability than free astaxanthin, and hence they may pose more health beneficial effects than free astaxanthin. *H. pluvialis* may be a potential natural source for the isolation of esterified astaxanthin and to deploy them for health beneficial effects against several disorders. Further, carotenoid esterification does not pose impediment for bioavailability in humans (Bowen et al, 2002), hence astaxanthin esters can play a role in ulcer prevention.

Inhibition of 15-lipoxygenase enzyme by saponified astaxanthin and total carotenoid fractions of *H. pluvialis* (Figure 3.37) has been demonstrated in the current study. Both total carotenoid and astaxanthin esters from *H. pluvialis* showed potent inhibition with an IC_{50} of 19.1 and 24.4 µg/ml which is ~ 24-29 fold higher than the standard astaxanthin (Table 3.13). Results may imply their beneficial role in the potential management of ulcers. The process of oxidation of low-density lipoprotein is mediated by 15-lipoxygenase, and is believed to play a key role in mediating inflammatory reactions in ulcerous conditions and atherosclerosis (Steinberg, 1999; Gundersen et al, 2003; Cornicelli and Trivedi, 1999). Ulcerogens such as alcohol and Nonsteroid-antiinflammatory drugs have been known to inhibit leukotrienes and prostaglandins that are important for proliferation of mucin synthesizing - mucosal cells. Inhibitors of lipoxygenases hence would potentially contribute towards the regulation of inflammatory reactions towards the synthesis of gastric mucin and hence mucosal protection during ulcerous condition.

George et al (2001) have reported a significant contribution of lipoxygenase enzyme towards atherogenesis in animals. 15-lipoxygenase has also been implicated in prostate cancer, and in spontaneous abortions (Kelavkar et al, 2001; Dar et al, 2001). Hence development of new and selective 15-lipoxygenase inhibitors appears to be an important task. There is good correlation for inhibitory activity for the soybean and

mammalian 15-lipoxygenase enzyme from rabbit or human reticulocytes (Whitman et al, 2002). In this view, astaxanthin esters and saponified astaxanthin were evaluated for their lipoxygenase inhibitory activity. Saponified astaxanthin has shown 7 fold higher inhibition activity in comparison with astaxanthin esters (Table 3.13) and its *in vivo* potency needs to be established.

The present data on *in vivo* antiulcer properties of total carotenoid and astaxanthin esters, thus suggest that astaxanthin esters may be a major antiulcer component present in the *H. pluvialis* extract. Further evaluation of biochemical changes like catalase, superoxide dismutase, glutathione peroxidase in control, ulcer induced and treated animal groups revealed that the antiulcerogenic potency is due to a) inhibition of H^+,K^+-ATPase which suppresses the acid secretion, b) upregulating mucin content partially which protects the gastric mucus layer against oxidative damage leading to ulceration and; c) by increasing antioxidant status which would eliminate the oxidative stress condition during ulceration.

Egg yolk colour is an important characteristic when evaluating the quality of egg. Odunsi (2003) reported feeding of lablab leaf meal as a feed ingredient and yolk coloring agent in the diet of layers and found increase in yolk coloration. Other sources of carotenoids have been tested and the results showed them to be good for pigmentation of egg yolks, such as the alga *Chlorella vulgaris*. Maize is a usual ingredient of chicken feed and is the major source of carotenoids, pigmenting egg-yolk and meat. Poultry accumulate carotenoids in liver, skin, and shank (Allen, 1988). Since poultry do not produce carotenoids, they must be supplied in feed for proper pigmentation (Bortolotti et al., 2003). Currently efforts are continuing to improve the nutritional quality of eggs. In this context, the present study focused on feeding of poultry with algal (*H. pluvialis*) cells containing carotenoid to enrich the eggs with carotenoid content and also to impart colour. Oxycarotenoids were reported to be accumulated at various sites, particularly in the skin, plumage, fatty tissue and egg yolk (Gouveia et al, 1996b). Waldenstedt et al, (2003) reported increase in tissue astaxanthin and carotenoid concentrations with increasing levels of algal meal (*H. pluvialis* cells) inclusion in the diet and study confined to distribution of astaxanthin in different tissues but not in egg yolk. The algal meal

mixed with oil and sprayed onto the pellet resulted in higher tissue concentrations than the algal meal added prior to pelleting. In the present study also carotenoid extract (astaxanthin) at 0.5 mg/kg level showed similar carotenoid content as that of algal cells at 2mg/kg level in the egg yolk.

Williams et al (1963) reported that absorbed carotenoids by laying hens were transported to egg yolk within 48 h and carotenoids in egg yolk reached the maximum concentration at day 8–10. The carotenoid levels in the egg yolks supplemented with *H. pluvialis* also showed 2 fold increases in carotenoid content by 2 weeks period.

It was demonstrated that laying hens will transfer part of the carotenoids consumed to the egg yolk and various feed ingredients were found inevitably to affect the colour of the yolk. The carotenoid content reported by Gonzalez et al (1999) was 30mg/kg yolk. In the present study, maize in the diet was the major source for carotenoids (such as lutein and zeaxanthin) present (Sommerburg et al, 1998). The *H. pluvialis* supplemented diet was fed to the layers through colour less capsules, so that whatever the colour of the egg yolk in the control and experimental birds must be due to diet ingredients. The observed increase in carotenoid content in the egg yolks of algae supplemented diet is attributed to dietary carotenoids. Absence of further increase in the carotenoid content in the egg yolks supplemented with 4 mg/kg astaxanthin is in accordance with the observations of Waldenstedt et al (2003) that the high concentration of carotenoids in feed did not increase the efficiency of absorption proportionately. The lower the concentration of carotenoids in the feed, the higher the absorption rates from feed to blood and from blood to skin. Therefore astaxanthin feeding through dietary supplementation of *H. pluvialis* at 2mg/kg would be sufficient to elevate the carotenoid level in egg yolks to 44μg/g.

An appealing skin colouration is the crucial factor determining the premium price in freshwater ornamental fish industry. Dietary carotenoids play a major role in the regulation of skin and muscle colour. Efficient deposition and pigmentation by particular carotenoid source is species specific (Ha et al, 1993). Further, there seems to be no correlation of carotenoid absorption with growth. As shown in Figure 3.40, the growth of fish upon feeding with *H. pluvialis* supplemented diet remained unaffected. This result is

in agreement with the earlier studies on rainbow trout by White et al (2003) and on red porgy by Chatzifotis et al (2005) who have reported that carotenoids do not cause any notable increase in growth.

Effective red colouratin in red porgy (*Pagrus pagrus*) was obtained by Chatzifotis et al (2005) by feeding natural astaxanthin-Naturose®. Bowen et al (2002b) have reported the efficient pigmentation in rainbow trout (*Oncorhynchus mykiss*) using astaxanthin esters and synthetic unesterified astaxanthin. Gouveia et al (2003) have attributed the poor performance of *Haematococcus* biomass in *C. carpio* to lower digestibility as a result of thickness of its cyst walls and to the esterified forms of carotenoid which predominate in its biomass. This obstacle was overcome in the current study by pretreatment of cells followed by homogenization of biomass prior to its use in diet. The colour values as given in Table 3.17 indicate efficient pigmentation in fish skin.

Chapter 4
Summary and Conclusion

The green alga *Haematococcus* is one of the potent natural sources for astaxanthin which accumulates 2-3% on dry weight basis under stress conditions. The present investigation was aimed at developing an autotrophic cultivation method which involved understanding of critical factors during growth and carotenogenesis, processing conditions effect on carotenoid content, enhancement of growth and carotenoid contents through mutation, characterization of mutants and illustration of pigmentation, antioxidant and antiulcer properties of astaxanthin.

The experimental design consisted of autotrophic cultivation in different designs of bioreactors of closed and open mode for growth and carotenogenesis under the influence of CO_2 and stress conditions, influence of drying and storage temperatures on carotenoid profile in *Haematococcus pluvialis* cells, mutants selection after UV and chemical mutagens treatment and characterization of mutants, bioactivity of astaxanthin in terms of pigmentation in egg yolk of poultry birds and skin colouration in fishes and antioxidant activity in *in vitro* models and antiulcer property in experimental animals.

The results have provided important information on the autotrophic growth of *H. pluvialis* and the critical factors involved in both growth phase and carotenogenesis phase. Influence of various ammonia salts including commercial salts, such as nitrogen-phosphorous- potash (NPK) mixture, diammonium phosphate (DAP) as source of nitrogen, was studied on *H. pluvialis* growth and astaxanthin production. The data indicated that *H. pluvialis* could utilize ammonia salts in the range of 3mM – 4.7mM concentration and at higher concentration, growth was inhibited. Modified autotrophic medium with ammonium salts replacing sodium nitrate facilitated consistent and extended growth phase. Continuous cultivation in commercial salts resulted in reduced growth. Modified autotrophic medium was found to be suitable for maintenance of culture and batch cultivation using commercial salts has resulted in significant increase in biomass and astaxanthin yields. The study using inhibitors L-methionine DL- sulfoximine (MSX) and azaserine has shown that the assimilation of ammonia is through glutamine synthetase (GS) /glutamate synthase (GOGAT) pathway.

Various prototypes like open rectangular glass type, closed tubular polyethylene sleeves, open raceway type were evaluated for their suitability for *H. pluvialis* growth and carotenogenesis. *H. pluvialis* was grown in these prototypes under controlled light, temperature, CO_2 and salinity stress. Maintenance of the constant carbon dioxide level in the headspace of the tubular bioreactor resulted in effective gas-liquid mass transfer as indicated by high biomass yield. Maximum biomass yield of 0.89 g/L with a specific growth rate of 0.13 d^{-1} and astaxanthin content of 1.8% (w/w) was obtained in closed tubular bioreactor.

A two stage cultivation method like growth in closed photobioreactors for 10 to 12 days followed by carotenogenesis in outdoor open raceway ponds for 5-7 days has been shown as an ideal method for production of astaxanthin. Among the stress conditions, sunlight and sodium chloride (42mM) was effective for maximum astaxanthin accumulation.

Harvesting by sedimentation followed by low speed centrifugation found suitable as the encysted cells of *H. pluvialis* tend to settle at the bottom of culture vessel. Of the drying methods tested, oven drying was found suitable and relatively low cost method to obtain dry *H. pluvialis* biomass without significant loss of carotenoid content. Storage of *H. pluvialis* cells at lower temperature in dark conditions has shown better stability of cells without significant change in carotenoid content and profile.

The image processing method developed for estimation of carotenoid content in *H. pluvialis* cells has shown correlation (R^2=0.967) with the analytical method. Since this method exploits the colour characteristics of the organism for estimation of pigment, it can also be adopted for analysis of other red, green and brown algal forms.

H. pluvialis cells were treated with chemical mutagen 1-methyl 3-nitro 1-nitrosoguanidine (NTG), Ethyl methane sulfonate (EMS) and UV irradiation followed by plating on media containing herbicide glufosinate. The survival rate was found to be concentration dependent. The mutants obtained have shown significant increase in carotenoid content (23-59%) compared to wild type without significant increase in

growth rates. The mutants did not exhibit significant variation in carotenoid profile on qualitative basis as analyzed by TLC and HPLC. The putative mutants were also characterized by their photosynthetic activity, fluorescence profile and lycopene cyclase activity. The photosynthetic activity in wild type was inhibited by herbicide glufosinate at 250μM level, where as the mutants could over come the effect of the herbicide. The fluorescence profile in mutant obtained after treatment with EMS has shown altered emission profile with 2 fold increase in chlorophyll fluorescence when compared to wild type.

The mutants obtained were evaluated for lycopene cyclase activity, a key enzyme in biosynthetic pathway of carotenoids. *H. pluvialis* cells in the vegetative and intermediate stage were harvested and cell extract was used in the reaction mixture for enzyme assay. Reaction products were analysed by HPLC and the mutant obtained by UV irradiation showed the highest enzyme activity (458 nmole of β-carotene formed/mg of protein/hr) followed by NTG mutant (315 nmole of β-carotene formed/mg of protein/hr) compared to the wild strain (105 nmole of β-carotene formed/mg of protein/hr).

The mutants were found to be stable for more than two years and have shown 38% higher carotenoid accumulation in response to stress conditions. Expression analysis of carotenoid biosynthetic genes such as Phytoene synthase, Phytoene desaturase, Lycopene cyclase, β-carotene ketolase and β-carotene hydroxylase in the mutants exhibited increase in transcript levels compared to wild type when tested after stress induction. Lycopene cyclase enzyme activity of mutants E3 and N5 was well correlated with its gene expression.

The results obtained for *in vitro* studies on astaxanthin fractions from *H. pluvialis* indicated a dose dependant radical scavenging, lipoxygenase inhibitor activity, reducing power and H^+,K^+ ATPase inhibition activities and among the fractions saponified free astaxanthin exhibited high activity. Saponified astaxanthin from *H. pluvialis* showed 4.4 fold higher free radical scavenging activity (IC_{50} value of 8.1μg/ml) when compared to

that of astaxanthin esters. Saponified astaxanthin also showed maximum reducing power of 59 U/g equivalent to that of tannic acid (48.25 U/g). Astaxanthin esters showed 1.6 fold lesser (33.5U/g) reducing power activity. Saponified astaxanthin also exhibited 5 fold higher soybean lipoxygen inhibitory activity (IC_{50} ~3.4µg/ml) when compared to total carotenoid fraction. Moreover, saponified and astaxanthin esters effectively inhibited the gastric proton potassium ATPase enzyme that is involved in the acid secretion during gastric conditions. Saponified astaxanthin was found to be the potent inhibitor of gastric H^+ K^+ ATPase with IC_{50} -6.2µg/ml than astaxanthin esters (IC_{50} – 18.2µg/ml).

The *in vivo* studies have demonstrated the gastroprotective effect of *H. pluvialis* astaxanthin against ethanol induced ulcer, which is reported for the first time. Results revealed that the astaxanthin esters, at 500µg/kg b.w., protected ulcerous condition by ~67% equivalent to that of known antiulcer drug- omeprazole which offered ~72% protection at 20 mg kg^{-1} b.w. Astaxanthin ester has been shown to be the major antiulcer component present in the *H. pluvialis* extract. The possible mechanism of antiulcerogenic potency of astaxanthin ester has also been proposed based on its antioxidative and H^+,K^+ATPase inhibitory activity.

Evaluation of biochemical changes like catalase, superoxide dismutase, glutathione peroxidase *in vivo* in control, ulcer induced and treated animal groups revealed that the antiulcerogenic potency is due to a) inhibition of H^+K^+ ATPase which suppresses the acid secretion, b) upregulating mucin content partially which protects the gastric mucus layer against oxidative damage leading to ulceration and; c) by increasing antioxidant status which would eliminate the oxidative stress condition during ulceration. Thus this study provides sound scientific basis for antiulcer property of astaxanthin.

The pigmentation efficiency of *H. pluvialis* cells rich in astaxanthin has been shown in egg yolk of layers fed with *H. pluvialis* cells. Poultry birds fed with astaxanthin rich *H. pluvialis* showed an increase in yolk colour intensity as indicated by Roche Yolk colour fan (Yolk colour score-11.00) and improved egg quality as per FAO standards

(Haugh unit score -76 and USDA grade AA). A maximum of 44μg of carotenoid content per gram of yolk was observed in experimental birds, which is 2-3 fold higher compared to control (15μg/g of egg yolk). The skin colouration in ornamental fish koi carp is increased considerably when fed with *H. pluvialis* cells incorporated at 25mg/kg in the feed.

The results indicate the potential of *Haematococcus pluvialis* cultivation in autotrophic conditions which will make its commercial cultivation economical. Stable mutants will further enhance the astaxanthin content and overall yields. The biological activity of astaxanthin such as antioxidant, pigmentation efficiency and antiulcer properties shows its potential for applications in food and nutraceutical industry.

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Digital image processing—an alternate tool for monitoring of pigment levels in cultured cells with special reference to green alga *Haematococcus pluvialis*

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Abstract

A method for analyzing carotenoid content in *Haematococcus pluvialis,* a green alga was developed using digital image processing (DIP) and an artificial neural network (ANN) model. About 90 images of algal cells in various phases of growth were processed with the tools of DIP. A good correlation of $R^2 = 0.967$ was observed between carotenoid content as estimated by analytical method and DIP. Similar correlation was also observed in case of chlorophyll. Since the conventional methods of carotenoid estimation are time consuming and result in loss of pigments during analysis, DIP method was found to be an effective online monitoring method. This method will be useful in measurement of pigments in cultured cells.

Keywords: *Haematococcus*; Chlorophyll; Carotenoid; Astaxanthin; Image processing; Neural network

1. Introduction

Haematococcus pluvialis (Chlorophyte) is one of the potent natural sources for the production of high value ketocarotenoid, astaxanthin. Carotenoids from natural sources have gained importance due to their high antioxidant activity (Miki, 1991). This implied their application in many degenerative diseases in humans and animals besides their use as colours. Astaxanthin has nutraceutical and pharmacological applications besides being used as pigmentation source in farmed salmon, trout and poultry (Lorenz and Cysewski, 2000). *Haematococcus* has two distinct phases in its life cycle, viz.—green flagellated motile phase and non-motile non-flagellated cyst phase formed due to stress conditions. The stress conditions such as nutrient stress, salinity stress and/ or high light induces astaxanthin accumulation (Boussiba et al., 1999; Sarada et al., 2002; Tjahjono et al., 1994). The cyst cell with carotenoid accumulation appears red. It consists of thick hard cell wall made of sporopollenin like material (Hagen and Braune, 2002), which hinders solvent extraction and cracking of the cell requires high-pressure homogenization at low temperature. A conventional method like homogenization results in the loss of pigment. All the reported methods suggest cell disruption (Zlotnik and Sukenik, 1993) or extract with dimethyl sulfoxide (Boussiba and Vonshak, 1991) at high temperature which involve loss of carotenoid. Therefore the present study was envisaged to develop a digital image processing (DIP) system to quantify the redness of the cell and to estimate the carotenoid content without disrupting the cell wall.

DIP, which involved image acquisition, preprocessing, segmentation, feature extraction and the final recognition and interpretation was done using a knowledge base specifically created for the analysis of the problem domain. Also, a supervised artificial neural network (ANN) was used to correlate colour information to carotenoid and chlorophyll content in the alga.

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2. Materials and methods

2.1. Culture conditions

H. pluvialis (SAG 19-a) was obtained from Sammlung von Kulturen, Pflanzen Physiologisches Institut, Universitat Gottingen, Gottingen Germany. Stock cultures were maintained in autotrophic bold basal medium (BBM) as described by Tripathi et al. (1999). *Haematococcus* culture grown in autotrophic medium was used.

The two-tier vessel consisting of two 250 ml narrow-neck Erlenmeyer flasks was used for enriching carbon dioxide in the culture environment. The lower compartment of the flask contained 100 ml of 3 M buffer mixture ($KHCO_3/K_2CO_3$) at specific ratio, which generated a partial pressure of CO_2 at 2% in the two-tier flask (Tripathi et al., 2001). The upper chamber contained 40 ml of medium with 10 ml of inoculum so as to obtain an initial cell count of 13×10^4 cells per ml. The cultures were incubated at $25 \pm 1\,^{\circ}C$ under cool white fluorescent light source of an intensity of 2.99 W/m^2. After 15 days of growth phase, the cultures were exposed to 5.24 W/m^2 light intensity for encystment and carotenoid accumulation.

2.2. Extraction and analysis of pigments

Known volume of culture was centrifuged and the lyophilized biomass was taken for extraction. The cells were homogenized and carotenoids were extracted with acetone. Total carotenoid and chlorophyll contents were analyzed by the method of Lichtenthaler (1987) by measuring the absorbance at 470 nm for carotenoid and 645 and 661.5 nm (Shimadzu UV–vis spectrophotometer UV 160-A) for chlorophyll. The content of total carotenoid and astaxanthin were expressed in terms of percent dry weight. Astaxanthin content was determined at 480 nm by using an extinction coefficient of 2500 at 1% level (Davies, 1976). *Haematococcus* cells at various stages of carotenoid formation ranging from green vegetative phase to red encysted phase (10 different stages) were analyzed for carotenoid content and expressed in terms of % (w/w) on dry weight.

2.3. Digital image processing—methodology

Digital image processing adopted encompassed a broad range of hardware, software, and theoretical underpinnings. This involves image acquisition and a series of image processing steps as shown in Fig. 1 (Gonzalez and Woods, 1992). The problem domain referred is the images of *H. pluvialis* containing different amount of carotenoids.

2.4. Image acquisition

Image acquisition involves capturing the image by means of a Camera-monochrome or colour. Charge couple device (CCD) cameras are usually employed. These cameras have discrete imaging elements called 'photosites', which give out a voltage proportional to the light intensity. A frame grabber card (FlashBus FBG 4.2, 1996, Integral Tech, Inc.) was used to convert the analog image signal into the digital form.

The analysis of carotenoid content was achieved by exploiting the colour-based method. In this method the sample images were captured using CCD camera (Watec, WAT202D version) and the captured images were processed and analyzed by making use of DIP tools.

Fundamental algorithms for colour to gray conversion, threshold, filtering, segmentation, were implemented using the C programming language (Lindley, 1990). These steps were aimed at extracting the colour and intensity information from the images.

The image of algal cells was grabbed by the CCD camera and the same was first converted to the gray scale. Threshold was carried out for convenient processing and to get a uniform background and shape information of the image. The boundary of the object was detected and the region within the boundary was filled to achieve clear distinction between the object and the boundary. Hue being

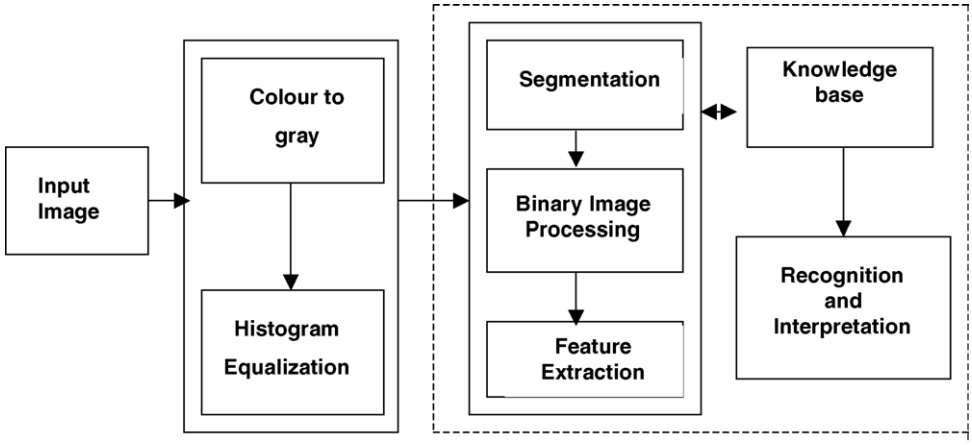

Fig. 1. Steps involved in image processing.

a colour attribute, describes the pureness of the colour and is expressed as an angle with reference to the colour triangle. Based on the detected boundary information, the Hue values for each of the original colour image were computed by converting them from red green blue (RGB) model to Hue saturation intensity (HSI) model.

Hue (H) is calculated using the equation:

$$H = \cos^{-1}\left(\frac{(1/2)[(R-G)+(R-B)]}{[(R-G)^2+(R-B)(G-B)]^{1/2}}\right)$$

where R, G, B are red, green and blue values at each pixel of the image (Gonzalez and Woods, 1992).

The concept of artificial neural networks (ANN) was used (Schalkoff, 1997) to relate hue values to carotenoid/chlorophyll content. An artificial neural network is an information-processing paradigm that is inspired by the way biological nervous systems, such as the brain, process information. The key element of this paradigm is the novel structure of the information processing system. It is composed of a large number of highly interconnected processing elements (neurons) working in unison to solve specific problems.

The Hue value so obtained was categorized to 28 classes depending on its distribution in the various stages and fed as input values to the neural network. The topology of the back propagation neural network model used was:

- 28 input Hue units (0–360°)
  - A1–A6: 0–30° in the intervals of 5°,
  - A7: 30–105°,
  - A8: 105–150°,
  - A9–A17: 150–195° in the intervals of 5°,
  - A18: 195–240°,
  - A19–A21: 240–255°in the intervals of 5°,
  - A22: 255–330°,
  - A23–A28: 330–360° in the intervals of 5°;
- 1 hidden layer with 12 units;
- 2 output units representing % carotenoid and % chlorophyll (target).

The network devised to achieve the desired output had an output threshold of 0.5, learning rate of 0.6, momentum of 0.9 and an error margin of 0.0001.

The neural network was accomplished on a computer with Pentium 2 processor, 550 MHz. The network was trained to obtain the target values utilizing 27 learning sets. Neural network software, Neuroshell Utility™ (Rel 4.01, Ward System Group Inc. USA) was used for the purpose. Fig. 2 depicts the neural network model devised for the purpose. The network devised to achieve the desired output had an output threshold of 0.45, learning rate of 0.6, momentum of 0.9 and an error margin of 0.0001.

The weight matrix W_{ij} between the 28 units of input layer (i) and 12 units of hidden layer (j) was:

$$W_{ij} = \begin{pmatrix}
-0.39 & -0.32 & -2.97 & -0.35 & -0.7 & -0.21 & -0.48 & -0.42 & 0.39 & -0.47 & -20.1 & -1.06 \\
-0.21 & 0.2 & -0.7 & 0.25 & 0.61 & -0.61 & 0.81 & -0.39 & -0.27 & 0.15 & -13.4 & 1.37 \\
-0.18 & 0.18 & 0.1 & -0.14 & 0.27 & -0.01 & 0.02 & 0.17 & 0.12 & 0.12 & -0.19 & 0.07 \\
-0.03 & -0.32 & -0.67 & 0.12 & -0.17 & -0.08 & -0.36 & 0.01 & 0.3 & -0.14 & 1.01 & 0.1 \\
-0.23 & -0.03 & 0.06 & -0.16 & -0.48 & -0.01 & -1.12 & 0.03 & 0.3 & -0.35 & 4.27 & -0.62 \\
-0.18 & -0.13 & -0.87 & -0.2 & -0.52 & -0.71 & -0.9 & -0.58 & -0.47 & -0.23 & -8.14 & -0.27 \\
-0.1 & -0.15 & -2.95 & -0.19 & -0.08 & -1.04 & 0.39 & -0.71 & -0.19 & 0.27 & 11 & -0.36 \\
-0.59 & -0.07 & 0.29 & -0.09 & -0.63 & -0.08 & 0.12 & -0.32 & -0.11 & -0.49 & -1.28 & -0.28 \\
0.26 & 0.28 & 0.25 & 0.25 & -0.06 & 0.11 & -0.12 & 0.02 & 0.12 & 0.14 & 0.25 & 0.19 \\
-0.2 & -0.05 & -0.75 & -0.5 & -0.73 & -0.24 & -0.7 & 0.05 & 0.12 & -0.6 & -0.2 & -0.27 \\
0.57 & 1.19 & -3 & 0.5 & -1.34 & -0.89 & -0.66 & 0.44 & -0.96 & -0.31 & -1.03 & -0.49 \\
-0.39 & 0 & -2.16 & -0.49 & -0.26 & -0.56 & -0.21 & -0.13 & -0.62 & -0.32 & 17.5 & 0.7 \\
-0.48 & -0.76 & 0.04 & -1.06 & 1.43 & -0.06 & -0.58 & -0.92 & 0.58 & -0.33 & 43.3 & -0.03 \\
0.51 & -0.22 & 1.29 & 0.83 & -0.03 & -0.45 & 2.44 & -0.23 & -0.02 & 0.02 & -22.4 & 0.35 \\
-0.57 & -0.37 & 1.04 & -0.34 & 0.48 & -0.07 & 0.88 & -0.42 & -0.1 & 1.23 & 0.75 & 1.14 \\
-0.19 & -0.02 & -0.11 & 0.34 & 0.48 & -0.34 & 0.68 & 0.04 & -0.55 & 0.27 & 1.03 & 0.45 \\
0.57 & -0.01 & -2.5 & 0.33 & -0.29 & 0.37 & 0.57 & 0 & -1.17 & -0.13 & 0.74 & -0.99 \\
1.58 & -0.88 & 3.93 & -1.28 & -2.7 & -0.6 & -1.8 & -0.59 & 1.14 & -1.37 & 5.15 & -4.56 \\
0.55 & 0.11 & 0.17 & -0.47 & -1.83 & -0.38 & -1.53 & 0.28 & 0.96 & -1.17 & -4.49 & -3.2 \\
-0.95 & -1.22 & 6.97 & -1.08 & -1.19 & -0.42 & -1.03 & -0.59 & -1.04 & -1.52 & -34.7 & 1.95 \\
-1.5 & -0.58 & 2.9 & -0.22 & 1.64 & -0.35 & 1.33 & -1.06 & -1.78 & -0.35 & -16.1 & 4.03 \\
-0.55 & -0.17 & -0.21 & -0.06 & 0.55 & 0.72 & -0.01 & 0.25 & -0.23 & -0.31 & 31.7 & 0.88 \\
-0.01 & 0.02 & -0.05 & 0.11 & -0.09 & -0.04 & -0.33 & 0.14 & 0.22 & -0.12 & 0.21 & -0.14 \\
-0.07 & -0.07 & -0.24 & 0.09 & -0.06 & -0.06 & 0.18 & 0.27 & -0.1 & 0.32 & -0.05 & 0.06 \\
0.31 & 0.1 & -0.82 & 0.4 & 0.82 & -0.4 & 0.9 & 0.12 & -0.18 & 0.94 & -0.32 & 1.74 \\
0 & 0.02 & 0.03 & 0.25 & -0.09 & -0.08 & 0.11 & -0.26 & 0.55 & 0.32 & -2 & 0.86 \\
-0.02 & -0.11 & -0.1 & -0.03 & 0.81 & -0.08 & 1.26 & 0.22 & -0.14 & 0.57 & -1.49 & -0.72 \\
-0.56 & 0 & -2.65 & -0.34 & -0.72 & -0.06 & -0.02 & 0.12 & -0.23 & -0.59 & -2.21 & 1.11
\end{pmatrix}$$

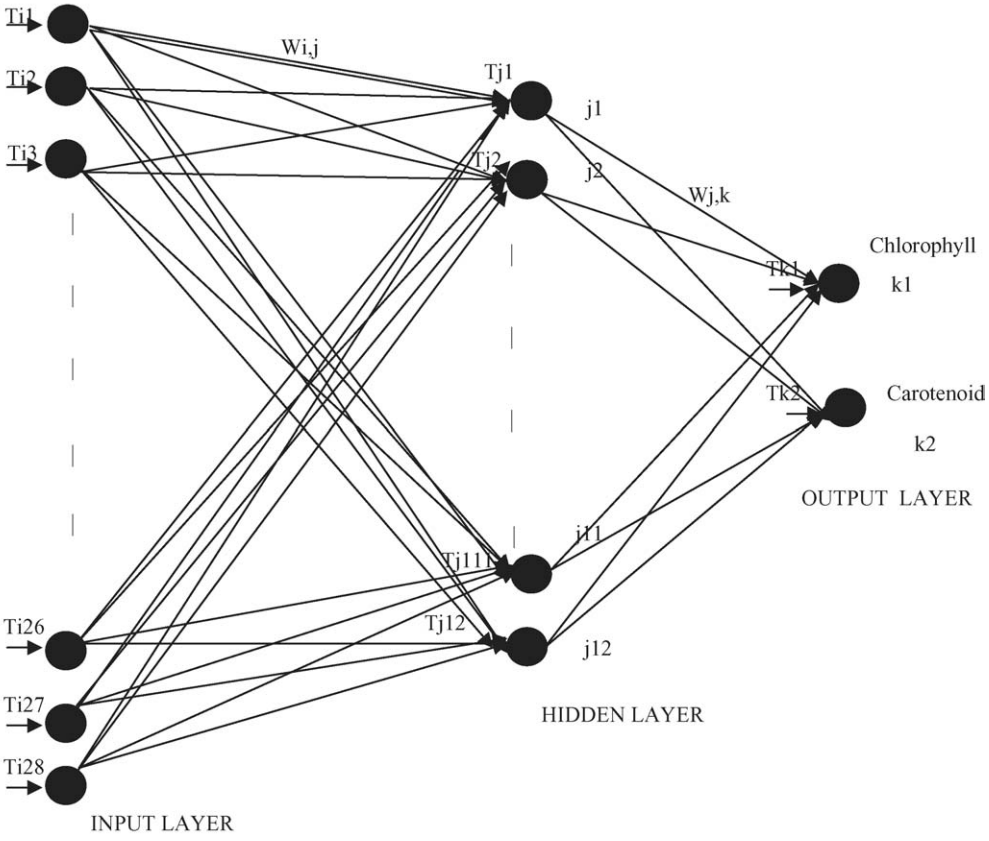

Fig. 2. Back-propagation neural network model.

The weight matrix W_{jk} between the 12 units of hidden layer (j) and 2 units of output layer (k) was:

$$W_{jk} = \begin{pmatrix} 2.03 & 0.19 \\ 0.85 & 0.31 \\ -3.33 & -0.3 \\ 0.76 & -0.38 \\ 0.81 & -1.46 \\ -0.79 & -0.61 \\ 0.99 & -1.68 \\ 0.55 & -0.34 \\ 1.2 & 1.99 \\ 0.9 & -0.57 \\ -7.96 & -0.01 \\ 2.41 & 0.51 \end{pmatrix}$$

The threshold values for the three layers of the neural network model were:

- Input layer: {27.8, 19, 1.6, 3.3, 7.7, 13.2, 17.4, 4.4, 2.1, 4.4, 11.4, 23.4, 49.6, 28.8, 7.4, 4.5, 7.7, 25.6, 15.1, 52.7, 31.9, 35.6, 1.5, 1.6, 7.1, 4.6, 5.5, 8.6}.
- Hidden layer: {−2.3, −3.6, −3.48, −3.57, −3.3, −2.83, −3.42, −3.18, −2.62, −3.63, 10.1, −2.99}.
- Output layer: {5.42, −2.08}.

3. Results and discussion

Astaxanthin a red coloured ketocarotenoid is accumulated in green alga *Haematococcus* (2–3% on dry weight basis). The green vegetative cell (Fig. 3A) contained more chlorophyll and less carotenoid. On exposure to high light and nutrient deficient conditions, the organism accumulated carotenoid (Fig. 3B and C) which could be seen as pockets of red colour in the cytoplasm. The whole cell appeared red when carotenoid accumulated completely (Fig. 3D). Astaxanthin constitutes 85–88% of total carotenoid in *Haematococcus*.

Haematococcus cells in different growth phases were selected for carotenoid and chlorophyll estimation and the cells were photographed, processed by digital image processing. The images were captured by a CCD camera and processed using image processing techniques. As the culture grows, there will be limitation for nutrients which induces cyst formation and the stress condition enhances the accumulation of carotenoids. The Hue values for the green motile phase 53.24° and for the carotenoid accumulated phase were in the range 293.4°. The neural network model developed (Fig. 1) was applied to compute the carotenoid and chlorophyll content in the algal cells.

The analytically estimated values were correlated with predicted value. A good correlation of $R^2 = 0.967$ was ob-

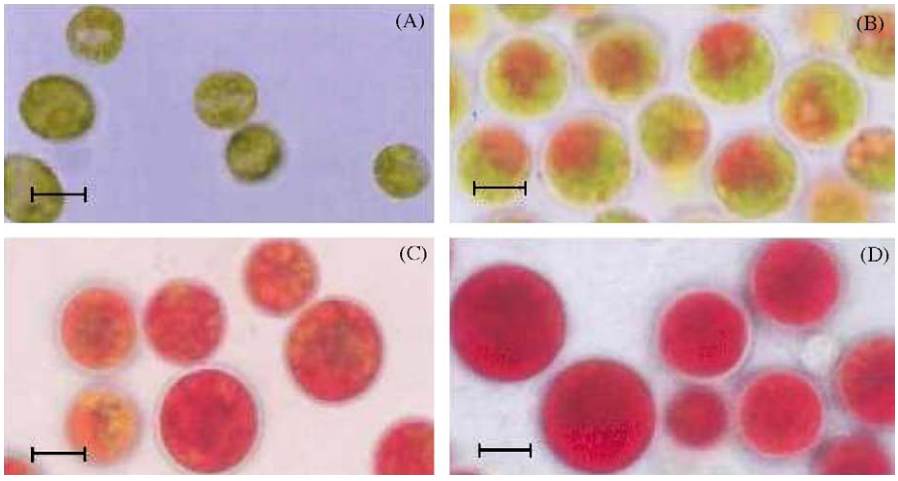

Fig. 3. *H. pluvialis* cells in different phases of growth in autotrophic medium. (A) Green motile phase. (B) Initiation of carotenoid accumulation. (C) Encysted cells. (D) Complete accumulation of carotenoid. *Note*: the cells in the photograph represent a portion of images processed for DIP (scale bar 20 μm).

served in case of carotenoid (Fig. 4A). A similar correlation of $R^2 = 0.997$ was observed for chlorophyll (Fig. 4B). These results clearly showed that digital image processing method could be applied to estimate carotenoid pigment content.

During carotenogenesis, the chlorophyll content significantly decreases (Sarada et al., 2002) and the decrease in green colour relating to chlorophyll is seen clearly in the DIP also. Image processing technique has been applied for quantifying adulteration in roast coffee powder by Sano et al. (2002). Coupled with neural network model this technique could be used for online monitoring of the carotenoid content just by observing the cells under microscope, capturing the image by CCD Camera, for further processing by DIP.

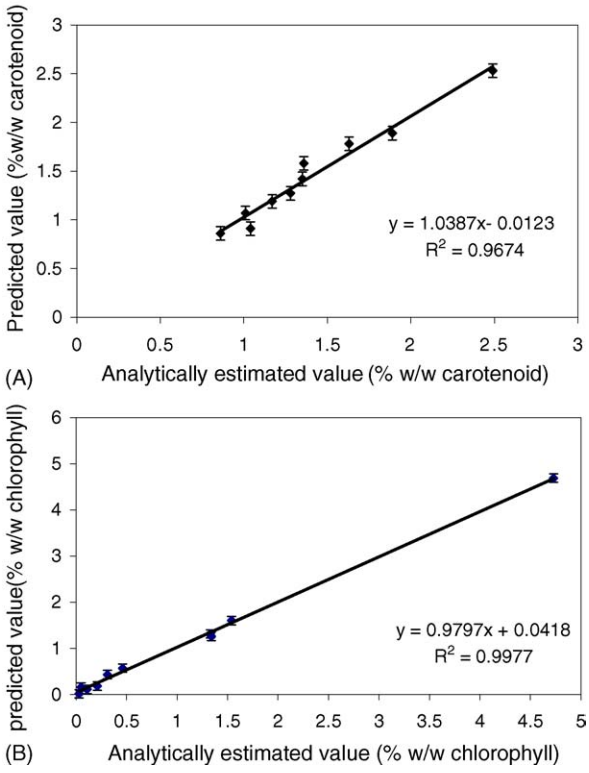

Fig. 4. Correlation of analytically estimated carotenoid (A), chlorophyll (B) and predicted content.

Estimation of pigment content in microalgal cells is an integral part of algal cultivation process. The method explained is useful in analyzing the carotenoid content of more number of algal samples in short span of time. Requirement of very small quantity of sample for analysis is the advantage of this method. Since this method exploits the colour characteristics of the organism for estimation of pigment, it can also be adopted for analysis of other red, green and brown algal forms.

4. Conclusion

The work aims at demonstrating the applicability of digital image processing technique as a tool for quality control of biotechnological processes. It was established that digital image processing method helped in analyzing the carotenoid content from microalgal cells such as *Haematococcus* eliminating the conventional homogenization of cells and extraction with solvents. It also helped in manipulating the culture conditions to enhance carotenoid content and thereby facilitating easy and immediate analysis of carotenoid and chlorophyll contents in the cells. The technique could be used for online monitoring of pigment contents in a variety of cultured cells.

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Differential expression of carotenogenic genes and associated changes in pigment profile during regeneration of *Haematococcus pluvialis* cysts

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Abstract *Haematococcus pluvialis* is a green alga known to accumulate astaxanthin in extra-plastidic lipid vesicles under stress conditions. The present study revealed the influence of few cultural parameters and temperature treatments on regeneration efficiency of red cysts along with changes in pigment profile and expression of carotenogenic genes during regeneration. Regeneration efficiency has been improved by incubating less aged cyst cells in a medium containing ammonium carbonate, 16:8 light–dark cycle with a light intensity of 30 μmol m^{-2} s^{-1}. During regeneration, there was a decrease in total astaxanthin, total carotenoids, and carotenoid to chlorophyll ratio, and increase in β-carotene, lutein, total chlorophyll, and chlorophyll a to b ratio. Expression analysis revealed the presence of transcripts of carotenogenic genes, phytoene synthase (PSY), phytoene desaturase (PDS), lycopene cyclase (LCY), β-carotene ketolase (BKT), and β-carotene hydroxylase (CHY) in cyst cells, and these transcripts were up regulated transiently upon transfer to favorable conditions. As the culture growth progressed, carotenogenic gene expressions were decreased and reached basal expression levels of green motile vegetative cells. In addition, this is the first report of detection of carotenogenic gene transcripts in red cysts, and their differential expression during regeneration. The present study suggests the use of red cysts as alternate inoculum for mass cultivation to combat protozoan predation.

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Keywords Astaxanthin · Carotenoid biosynthesis genes · Carotenoids · Extractability · *Haematococcus pluvialis*

Introduction

Astaxanthin (3,3′-dihydroxy-β,β-carotene-4,4′-dione), a high value ketocarotenoid is not only used as pigmentation source in aquaculture, but also has potential applications in pharmaceutical, nutraceutical, and cosmetic industries due to its higher antioxidant activity (Guerin et al. 2003; Jin et al. 2006). Among the astaxanthin producing organisms, *Haematococcus pluvialis* is found to be a promising source because of its ability to accumulate astaxanthin up to 4% (w/w) of dry weight (Boussiba 2000).

The life cycle of *H. pluvialis* contains three main stages viz. green motile vegetative cells, nonmotile vegetative cells (palmella), and nonmotile cysts (aplanospore). Green motile cells dominate under optimal growth conditions, and their growth and multiplication are limited to few divisions followed by palmella. Under stress conditions, such as nutrient deprivation, high light intensity, salinity, and acetate addition, both motile and nonmotile vegetative cells transform into cysts (Margalith 1999; Jin et al. 2006). During the transformation, a trilaminar sheath and acetolysis-resistant material formed and thickened, coinciding with massive accumulation of astaxanthin in extra-plastidic lipid vesicles and expansion of cell volume (Montsant et al. 2001). The cyst wall is composed of an outer primary wall, a trilaminar sheath, secondary wall, and tertiary wall. After maturation, transfer of cysts to non-stressed conditions released many flagellated cells by germination (Triki et al. 1997; Hagen et al. 2001), and astaxanthin in these cells are degraded slowly (Fabregas et al. 2001). The major problem

in outdoor cultivation is the susceptibility of vegetative cells to protozoan predation, while cyst cells are resistant to predation. Therefore, regeneration of cyst cells to large number of flagellated cells has been explored in the present study under different conditions. The possibility of using germinated flagellated cells as starting material for cultivation scheme has been suggested by Hagen et al. (2001).

Astaxanthin biosynthesis in this alga follows a general carotenoid biosynthesis pathway up to β-carotene, and from β-carotene, astaxanthin is produced by the action of β-carotene ketolase (BKT) and β-carotene hydroxylase (CHY; Jin et al. 2006). For a better understanding of astaxanthin biosynthesis, knowledge on accumulation and degradation of carotenoids and their relation with the expression of carotenogenic genes are necessary. Information on carotenogenesis during stress-induced accumulation of astaxanthin is well documented (Steinbrenner and Linden 2001, 2003; Grunewald et al. 2000; Sun et al. 1998), whereas information on changes during cyst germination is limited to astaxanthin, chlorophyll, and protein contents (Kobayashi et al. 1997; Fabregas et al. 2003). Information regarding transcriptional changes is completely lacking. Therefore, the present study has been taken up to understand the changes occurring at pigment level and expression of carotenoid genes during germination of cysts, along with the influence of cultural parameters and temperature treatments on the regeneration of cyst cells.

Materials and methods

Algal strains and culture conditions

H. pluvialis (SAG 19-a) culture was obtained from Sammlung von Algenkulturen, Pflanzen Physiologisches Institüt, Universität Göttingen, Göttingen, Germany, and grown in autotrophic medium (Usha et al. 1999). The cultures were incubated at 25±1°C under 30 μmol m^{-2} s^{-1} light intensity with 16:8 h light–dark cycle for a period of 1 week. Later, the cultures were incubated at 60 μmol m^{-2} s^{-1} with an addition of 0.2% NaCl and 4.4 mM sodium acetate for secondary carotenoid induction. Encysted red cyst cells were harvested by centrifugation, and these cells were taken for regeneration studies.

Effect of N source, light cycle, age of cyst cells, and temperature treatment on regeneration

The effect of culture parameters on regeneration was tested as indicated in Tables 1 and 2. For regeneration, freshly harvested red cysts were inoculated into autotrophic media to a cell density of 15×10^4 cells ml^{-1} and exposed to the light intensity of 30 μmol m^{-2} s^{-1} at 25±1°C, and observed for their regeneration efficiency under the influence of N source (sodium nitrate 0.24 g l^{-1}; ammonium carbonate 0.16 g l^{-1}; and potassium nitrate 0.41 g l^{-1}, in autotrophic bold basal medium), light cycle (alternate light and dark for 18:6 h or continuous light), age of cyst cells (3, 5, and 7 months old), and temperature treatment of cyst cells (0 and 4°C for varying periods of time). The initial cyst cell count and number of cyst cells after 3 days of incubation were counted using haemacytometer to calculate the regeneration efficiency. Regeneration efficiency was calculated using the formula (initial cell count−final cell count)/initial cell count×100.

Growth and pigment changes during regeneration

A time course study on changes in growth and pigment profile was carried out. Three-month-old cyst cells were exposed to favorable conditions. Aliquots of culture were harvested at different intervals, lyophilized, and weight was estimated gravimetrically. The lyophilized cells were extracted with 90% acetone repeatedly until the pellet becomes colorless. The pooled extracts absorbance was read at 470, 645, and 661.5 nm, and chlorophyll and total

Table 1 Influence of the N source, light–dark cycle, and age of the cyst cells on regeneration

Treatment	Conditions			Percent of cysts germinated
	N source	Light–dark cycle (h)	Age of cysts (months)	
A	Ammonium carbonate	16:8	3	83.05±0.78(a)
B	Potassium nitrate	16:8	3	61.22±2.31(b)
C	Sodium nitrate	16:8	3	64.82±2.62(b)
D	Ammonium carbonate	16:8	3	83.67±0.47(a)
E	Ammonium carbonate	Continuous light	3	65.48±1.68(b)
F	Ammonium carbonate	16:8	3	83.3±0.42(a)
G	Ammonium carbonate	16:8	5	41.36±1.32(b)
H	Ammonium carbonate	16:8	7	30.00±2.83(c)

Each value under "percent of cysts germinated" represents the mean of two separate experiments, each with three replicates. Means within a column followed by the same letter (inside parentheses) are not significantly different as indicated by Duncan's multiple range test ($p\leq0.05$).

Table 2 Influence of temperature treatments on regeneration

Treatment	Percent of cysts germinated
Control	83.60±1.27[ab]
0°C for 5 min	67.55±1.24[e]
0°C for 10 min	71.15±2.24b[e]
0°C for 5 min followed by 10-min incubation at 30°C for three cycles	87.75±0.35[a]
0°C for 10 min followed by 10-min incubation at 30°C for three cycles	44.44±1.41[g]
0°C for 1 h	Cells bleached
4°C for 5 min	77.23±2.15[d]
4°C for 10 min	61.77±2.75[f]
4°C for 30 min	80.16±1.80b[cd]
4°C for 5 min followed by 10-min incubation at 30°C for three cycles	81.67±2.35[bc]
4°C for 1 h	78.98±1.45[cd]
4°C for 5 h	57.95±1.49[f]

Each value under "percent of cysts germinated" represents the mean of two separate experiments, each with three replicates. Means within a column followed by the same letter are not significantly different as indicated by Duncan's multiple range test ($p \leq 0.05$).

carotenoid contents were calculated (Lichtenthaler 1987). Carotenoid extracts were subjected to high-performance liquid chromatography (HPLC) analysis in Shimadzu LC-10AT liquid chromatograph instrument using reversed phase C18 column (Supelco, 25 cm×4.6 mm). Acetone and 90% methanol were used at a flow rate of 1.25 ml min^{-1} (Sarada et al. 2006). The separated carotenoids and astaxanthin esters were identified using a photodiode array detector (SPD-M10AVP, Shimadzu) and by comparing with authentic standards. The peaks were integrated at 476 nm to quantify ketocarotenoids and 445 nm to quantify other carotenoids. Standard β-carotene, lutein, and astaxanthin were purchased from Sigma-Aldrich (St. Louis, MO, USA), and canthaxanthin was obtained from ChromaDex (Santa Ana, CA, USA). Neoxanthin and violaxanthin were gift from Dr. Akhihiko Nagao of the National Food Research Institute, Tsukuba, Japan.

Extractability of carotenoids

The extractability of carotenoids from regenerating cells under favorable conditions at different intervals was studied to evaluate the fragility of the cell wall. The extractable carotenoid content was estimated by treating the lyophilized cells with 90% acetone for 1 h without any homogenization. For each sample, extraction with 90% acetone by homogenization was served as total carotenoid and carotenoid content was calculated as per Lichtenthaler (1987). Extractability was calculated by using the formula modified from Kobayashi et al. (1997): extractability %=extractable carotenoids % (w/w)/total carotenoids % (w/w)×100.

RNA isolation and reverse transcription–polymerase chain reaction

Cyst cells were exposed to favorable conditions for regeneration. At different intervals, 1×10^8 cells were harvested, frozen under liquid nitrogen, and subsequently powdered using a mortar and pestle. Then, total RNA was extracted using RNAqueous® kit according to the instruction manual (Ambion, Austin, TX, USA). Possible contaminant genomic DNA in RNA extract was removed using turbo DNA-free™ kit (Ambion). The concentration of total RNA was determined spectrophotometrically at 260 nm. The integrity of RNA was checked by electrophoresis in formaldehyde denaturing gels stained with ethidium bromide. The gene-specific primers for the genes PSY, PDS, LCY, BKT, and CHY, were designed using Primer3 software (Table 3) and synthesized (Sigma–Genosys, Bangalore, India). First-strand complementary DNAs were synthesized from 1.5 μg of total RNA in 20-μl final volume, using M-MuLV reverse transcriptase and oligo-dT (18 mer) primer (Fermentas GmbH, Germany).

PCR amplifications were performed using PCR mixture (15 μl) that contained 1 μl of RT reaction product as template, 1× PCR buffer, 200-μM dNTPs (Fermentas GmbH), 1 U of Taq DNA polymerase (Bangalore Genei,

Table 3 Gene-specific primers and annealing temperatures used for RT–PCR

Primer	Primer sequence (5′–3′)	Annealing temperature (°C)	GenBank ID	Amplified fragment size (bp)
PSY forward	ATGTACCATCCCAAGGCAAG	60	AY835634	402
PSY reverse	CTGGACCAGGCCTACGAC			
PDS forward	TCCATGATCTTTGCCATGC	60	AY768691	462
PDS reverse	CGGGAGTTGAACATGAGGTC			
LCY forward	CTTCTTCTCCGCCTTCTTCA	60	AY182008	565
LCY reverse	GCATCCTACCGCTCAAAGAA			
BKT forward	CATCTCCTTGTACGCCTGGT	55	X86782	423
BKT reverse	CAGTGCAGGTCGAAGTGGTA			
CHY forward	CTACACCACAGCGGCAAGTA	55	AF162276	521
CHY reverse	GCCTCACCTGATCCTACCAA			

Bangalore, India), and 0.1 μM of each primer depending on the gene. PCR was performed at initial denaturation at 94°C for 4 min, 30 or 22 cycles (1 min at 94°C; 1 min at 55 or 60°C; 1 min at 94°C), and final elongation (10 min at 72°C) using a thermal cycler (Eppendorf Thermal cycler, Germany). The PCR products obtained were separated on 1.8% agarose gel, stained with ethidium bromide (0.001%), and documented in a gel documentation system (Herolab GmbH Laborgerate, Germany). The size of the amplification products was estimated from the 100-bp DNA ladder (Fermentas GmbH). The band intensity of each gel was checked using the Herolab E.A.S.Y Win 32 software (Herolab GmbH Laborgerate). The transcript levels of each gene in green motile cells were taken for comparison in calculating the transcript abundance of respective genes during regeneration.

Experimental design and data analysis

Each experiment was repeated twice with three replications. All the observations and calculations were made separately for each set of experiments and were expressed as mean ± standard deviation. The significance ($p<0.05$) of the variables studied was assessed by simple student t test using Microsoft® Excel 2002. The mean separations were performed by Duncan's multiple range test for segregating means where the level of significance was set at $p\leq0.05$ (Duncan 1955).

Results

Regeneration efficiency of *Haematococcus* cysts under the influence of cultural parameters and temperature treatments

The regeneration rate of encysted (aplanospore) *Haematococcus* cells varied based on nitrogen source, light–dark cycle, age of cells, and treatment temperature (Tables 1 and 2). Autotrophic media differing in N source were compared for regeneration. Regeneration efficiency was found to be more in medium with ammonium carbonate when compared to that in media with sodium nitrate and potassium nitrate as N source (Table 1). A maximum regeneration efficiency of 83% was observed in ammonium carbonate medium when compared to 60–65% in nitrate as N source. The regeneration efficiency of cyst cells decreased with the increase in the age of cyst cells. A maximum regeneration of 83% was observed in 3-month-old cyst cells. Under high light intensity (60 μmol m^{-2} s^{-1}) combined with other favorable conditions, the cyst cells started to regenerate but soon they were bleached. The exposure of cyst cells to low light intensity with alternate light–dark cycle favored faster and higher regeneration (84%) than continuous light. The data obtained on the regeneration of encysted cells after pretreatment at 4 and 0°C had shown that the regeneration was more in cells exposed to 0°C than to 4°C. Short intervals of freezing and thawing enhanced the regeneration efficiency (Table 2), while the exposure of cells to 0°C for a longer duration (1 h) injured the cells and affected the regeneration ability of the cells. However, regeneration was not affected when the cells were exposed to 4°C for longer duration (1 h). Complete regeneration of cyst cells was observed over a period of time in all treatments except in cells exposed to 0°C for a long time, where cells were bleached partially or completely (Table 2).

Growth and pigment changes during regeneration

Growth of regenerated cultures were estimated as dry biomass per liter and showed initial slight decrease and further constant increase during regeneration (Fig. 1a). In the first day itself, very few cells that were fast moving and flagellated were observed microscopically. In the encysted cells, total carotenoid content was 1.9 to 2.0% on dry weight basis. Astaxanthin constituted 85–90% of total carotenoids of which monoester constituted 71.8%, diester 27.7%, and around 0.5% free astaxanthin. During regeneration in the autotrophic medium, although the astaxanthin content decreased significantly, the components of astaxanthin, i.e., astaxanthin monoester, diester, and free astaxanthin ratio, did not show much variation. Their contents ranged from 73.8–63.5% for monoesters, 32.8–25.9% for diesters, and 3.6–0.3% for free astaxanthin. In the first few days, the total carotenoid content (w/w) increased marginally followed by a decrease, while chlorophyll pigments showed a continuous increase (Fig. 1b). The chlorophyll a to b ratio (chl a/b) increased with a concomitant decrease in the carotenoid to chlorophyll ratio (car/chl). As the cyst cells started regeneration, there was significant decrease in astaxanthin content with a corresponding increase in lutein (major component) and β-carotene, and very low quantities of canthaxanthin, echinenone (intermediates in the formation of astaxanthin from β-carotene), neoxanthin, and violaxanthin were also detected (Fig. 2a,b). As regeneration progressed, the chlorophyll and carotenoid content in the germinated cells reached to that in green motile cells. After complete regeneration, traces of astaxanthin were also detected up to 2–3 sub-culturing in a nutrient medium.

Extractability of carotenoids

As shown in Fig. 3, the extractability increased from almost nil in the cysts on the day of inoculation to 65–70% by 5–6 days of regeneration. After the sixth day, the extractability of the carotenoids decreased to 40%. In

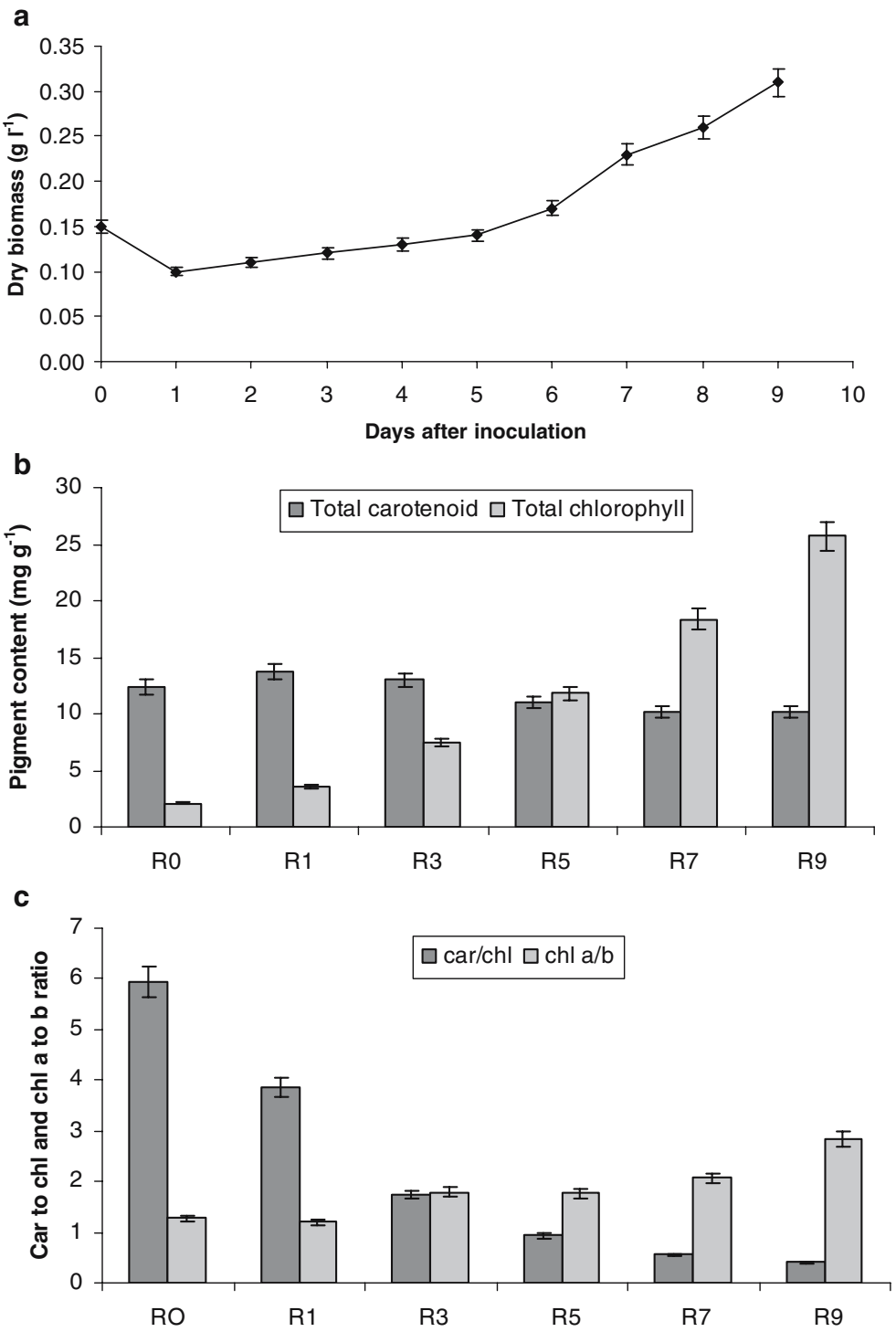

Fig. 1 Growth and pigment changes during regeneration of *Haematococcus pluvialis*.**a** Biomass (g/l), **b** changes in total carotenoid and total chlorophyll contents, and **c** carotenoid to chlorophyll ratio (car/chl), and chlorophyll a to b (chl a/b) ratio. Three-month-old cysts (*R0*) were exposed to favorable conditions (autotrophic medium with ammonium carbonate as N source incubated at 25±1°C, 30 μmol m^{-2} s^{-1} light intensity with 16:8 h light–dark cycle) and harvested 1 day (*R1*), 3 days (*R3*), 5 days (*R5*), 7 days (*R7*), and 9 days (*R9*) after inoculation. The harvested cells were lyophilized, and the pigments were analyzed

addition, 3-day-old cells had 36% extractability, and 67% of the total carotenoid was astaxanthin.

Changes in the transcripts of carotenogenic genes during regeneration

The expression levels of genes associated with general carotenogenesis and specific astaxanthin biosynthesis during regeneration of *Haematococcus* cysts were quantified by reverse transcription–polymerase chain reaction (RT–PCR) and compared with the expressions of respective genes in green motile cells. These genes included phytoene synthase (PSY, the first committed step in the carotenoid pathway), phytoene desaturase (PDS, which converts phytoene to lycopene), lycopene cyclase (LCY, which converts lycopene to β-carotene), BKT (specific to astax-

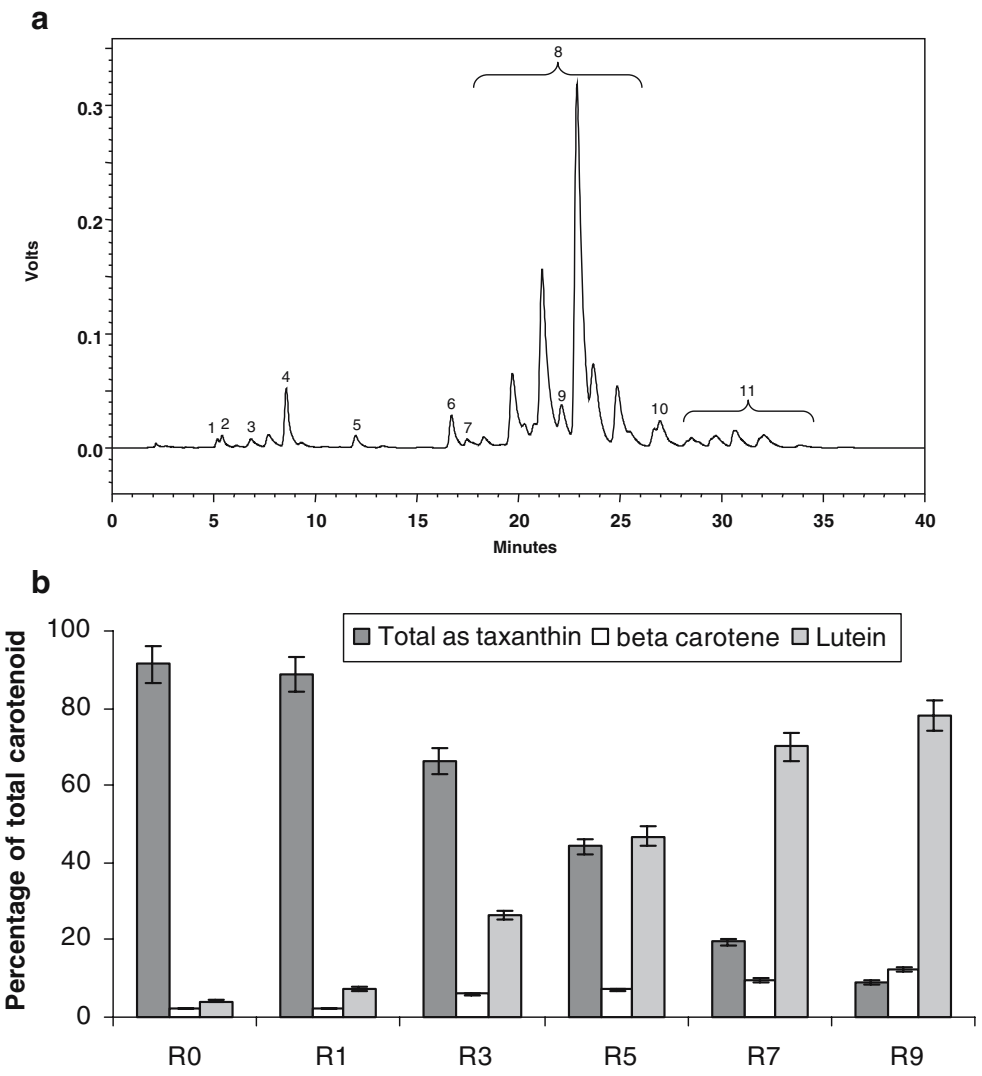

Fig. 2 Changes in carotenoid composition during regeneration of *H. pluvialis* cells. **a** HPLC analysis of the carotenoids extracted from 3-month-old red cyst cells of *H. pluvialis*: *1* Neoxanthin, *2* violaxanthin, *3* free astaxanthin, *4* lutein, *5* canthaxanthin, *6* chlorophyll b, *7* chlorophyll b', *8* astaxanthin monoesters, *9* echinenone and chlorophyll a', *10* β-carotene, and *11* astaxanthin diesters. **b** Changes in concentration of total astaxanthin, β-carotene, and lutein during the regeneration of the 3-month-old cyst cells (*R0*) that were harvested 0 day (*R0*), 1 day (*R1*), 3 days (*R3*), 5 days (*R5*), 7 days (*R7*), and 9 days (*R9*) after inoculation

anthin biosynthesis, which converts β-carotene to echinenone and to canthaxanthin), and CHY (which convert canthaxanthin to astaxanthin and α-carotene to lutein and other xanthophylls). Transcripts of PSY, PDS, LCY, BKT, and CHY were detected in 3-month-old red cyst cells of *Haematococcus* (Fig. 4a). In addition, this is the first report of carotenoid gene expression in red cysts and their differential regulation during regeneration. The exposure

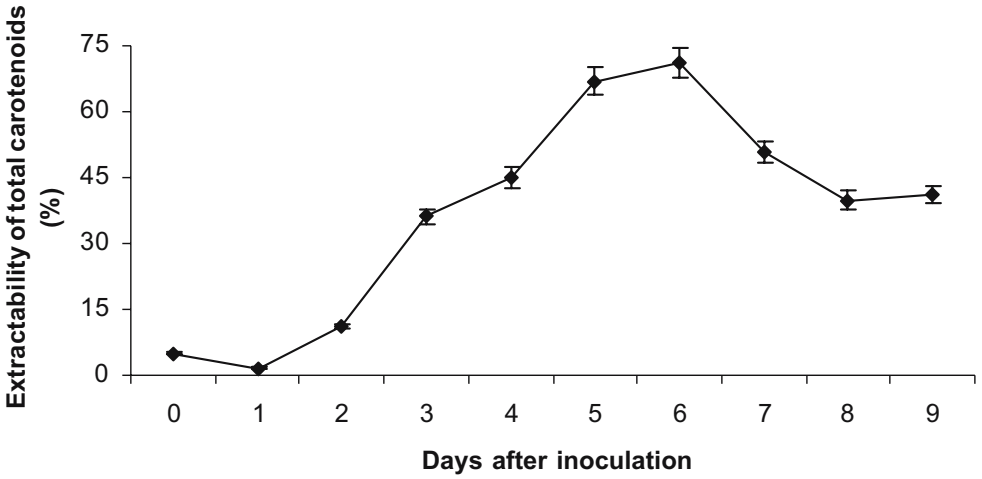

Fig. 3 Extractability of carotenoids during the regeneration of the *H. pluvialis* cells. The three-month-old cysts were exposed to favorable conditions. The cells at different intervals were harvested, lyophilized, and the extractability was estimated

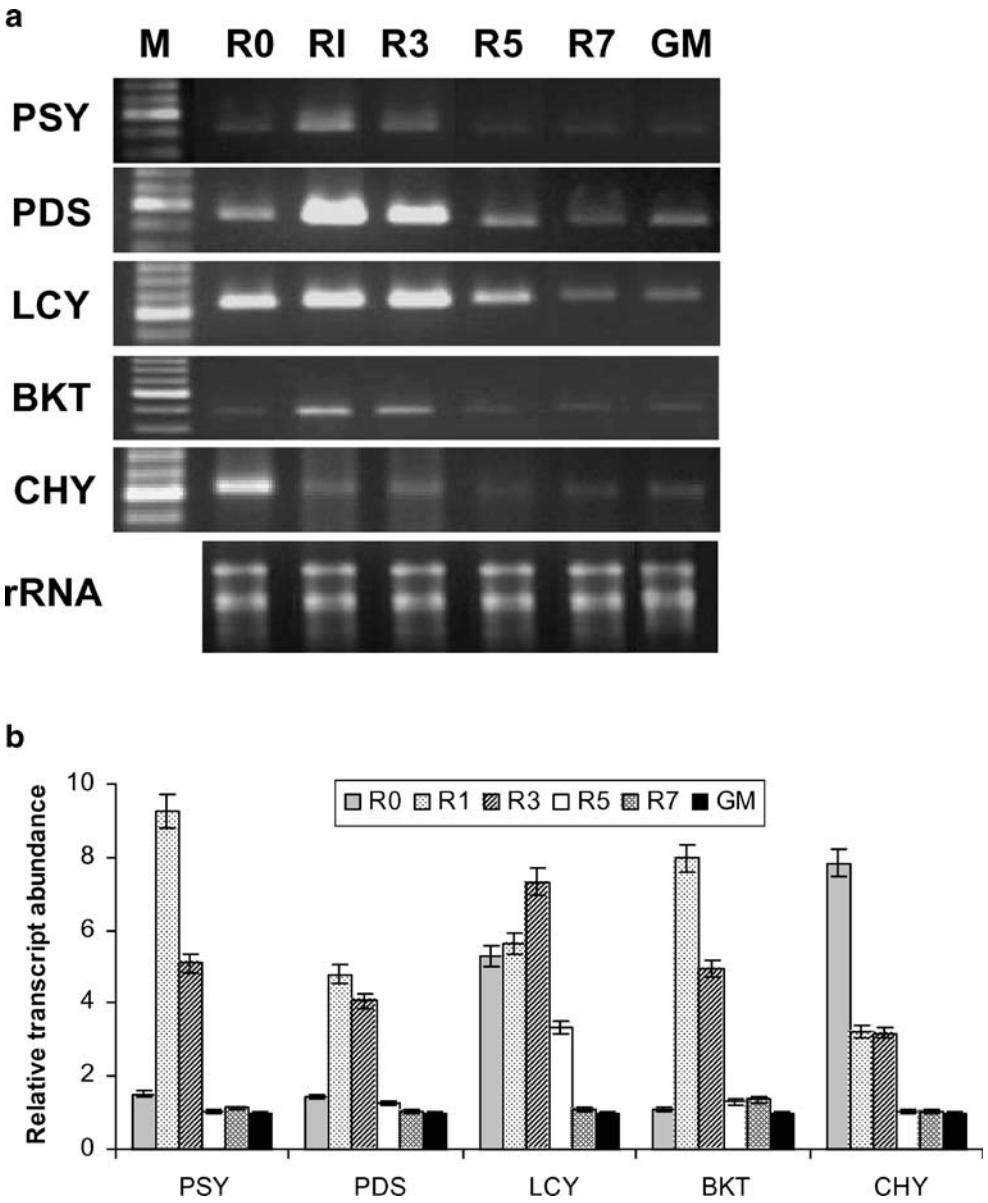

Fig. 4 The transcripts of the carotenogenic genes were up regulated transiently during the regeneration of the *H. pluvialis* cysts. The regenerating *H. pluvialis* cells were harvested at 0 day (*R0*), 1 day (*R1*), 3 days (*R3*), 5 days (*R5*), and 7 days (*R7*) after exposure to favorable conditions, and RNA was isolated. RT–PCR was performed as described in Materials and methods. **a** The PCR products were analysed by agarose gel electrophoresis. For comparison, total RNA was stained with ethidium bromide (*lower panel*). *M* 100-bp DNA ladder plus (Fermentas). **b** Data shown are the mean±SD of the three independent experiments expressed as the fold increase in PSY, PDS, LCY, BKT, and CHY expression levels compared with the value for green motile cells (*GM*)

of the cysts to favorable conditions (autotrophic medium with ammonium carbonate as N source, 25±1°C under 30 μmol m^{-2} s^{-1} light intensity with 16:8 h light–dark cycle) induced the PSY expression transiently (from 1.5- to 9.3-fold) on the first day of exposure to favorable conditions and considerably decreased on the fifth day and remained low later on. The expression of PDS showed a transient increase from 1.4- to 4.8-fold on the first day of regeneration, thereafter showing a decreasing trend. The transcripts of LCY were up regulated and reached an eightfold increase from 5.3-fold on the third day, and then decreased. The BKT transcripts have shown an eightfold increase on the first day. The expression of CHY was reduced immediately upon exposure to favorable conditions (from 7.8- to 3.2-fold compared to green motile cells). On the seventh day of regeneration, the expressions of all carotenoid genes studied reached basal expression levels of green motile cells.

Discussion

The present study was undertaken to evaluate the methods for achieving the maximum regeneration rate of cysts and to study changes in carotenogenesis during regeneration. The results showed the influence of nitrogen source, light–dark cycle, age of cells, and treatment temperature on the regeneration efficiency of cyst cells. The bleaching of the cells observed during the regeneration of the cyst cells exposed to 0°C for a long time may be due to the internal ice-crystal formation in the absence of the added cryoprotectants, thereby affecting the regenerating ability of the

cells. The complete regeneration obtained in the cells treated at 4°C for different durations indicates that cells can withstand that temperature without losing the viability. Earlier reports had shown the germination of aplanospores in dark (Fabregas et al. 2003; Hagen et al. 2001), urea-enriched medium (Lee and Ding 1994), and N-sufficient medium, and in N-free cultures, aplanospore germination was not induced (Fabregas et al. 2003). During regeneration, there was a decrease in total astaxanthin content; however, the relative proportion of free, mono, and diester forms of astaxanthin did not show much variation. This indicated active degradation of astaxanthin esters. Fabregas et al. (2003) reported that nutrient availability was a main factor triggering the degradation of astaxanthin, while light intensity has no effect on the loss of astaxanthin during germination.

The cyst cells contain a thick cell wall made up of algaenan that hinders carotenoid extraction by solvents. This also reduces carotenoid bioavailability when whole, intact cells were used in nutraceutical preparations. Therefore, the cells require homogenization under high pressure at cryogenic conditions or cracking before usage. However, during regeneration, the extractability of carotenoids increased significantly. This could be due to the breakage of algaenan containing trilaminar sheath and of the secondary and tertiary wall during germination, as reported by Damiani et al. (2006). This characteristic feature is having biotechnological importance because pigment extraction from flagellated cells becomes easier (Hagen et al. 2001).

As the growth progresses, the transcripts of carotenogenic genes studied—PSY, PDS, LCY, BKT, and CHY—were decreased and their levels reached the basal expression level of green motile cells (Fig. 4a,b). This is in concordance with the report of Huang et al. (2006) for the basal expression of BKT in green flagellated cells. Although transcripts of BKT were detected in green motile cells in our study, the decrease in astaxanthin during regeneration could be due to the reduced level of the BKT transcripts that might have been below a threshold amount necessary for astaxanthin biosynthesis (Huang et al. 2006).

During astaxanthin accumulation in *Haematococcus*, it has been reported that high light reduced the plastoquinone pool that seems to function as redox sensor for the transcriptional activation of carotenogenic genes (Steinbrenner and Linden 2003). Although red cyst cells are photosynthetically competent, they operate at photosynthetically reduced level, which may be due to impaired linear electron flow from PS II to PS I (Tan et al. 1995). An increase in chlorophyll, decrease in carotenoid, and transient induction of carotenogenic genes observed during regeneration indicates the possible function of the plastoquinone pool as an electron crossover point between photosynthesis and carotenoid synthesis, as suggested by Kobayashi et al. (1997).

The slight increase in total carotenoid and total astaxanthin content observed immediately after exposure of cyst cells to favorable conditions correlated with the transient increase in the transcript levels of the carotenogenic genes. This shows the organism's adaptability to a new environment. Because, immediately upon exposure to favorable conditions, extractability decreases, the slight increase in the total carotenoid and total astaxanthin may not be due to the change in fragility or permeability of the cell wall, and it may be the result of the transient induction of the carotenogenic genes. In the present study, we observed an increase in primary carotenoids and chlorophyll and a decrease in secondary carotenoids and car/chl ratio. It is reported that induction of carotenogenic genes expression and increase in total carotenoids and secondary carotenoid occur under stress conditions (Jin et al. 2006). This suggested that induction of carotenogenic gene expression occurs when cells are exposed to new conditions (temporary increase in expression under favorable conditions and higher expression under stress conditions); otherwise, they are maintained at basal expression level.

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