Screening of some microalgae for astaxanthin

DISSERTATION

Submitted by

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In partial fulfilment for the award of the degree of

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Under the Guidance of

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Thapar University, Patiala

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CERTIFICATE

Certified that the thesis entitled **"Screening of some microalgae for astaxanthin"** submitted by Ms. **Aakriti Bhat** (601504001) in partial fulfilment of the requirement for the award of the degree of **Master of Technology** in the Department of Biotechnology, Thapar University, Patiala, Punjab,India is the record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree.

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DECLARATION

I hereby declare that the work which is being presented in this thesis "**Screening of some microalgae for astaxanthin**" submitted by me for the award of the degree of **Master of Technology** in Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, Thapar University, Patiala, Punjab, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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Dedicated
To
My parents

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BBM	Basal Bold medium
BG11	Blue Green medium
$CaCl_2.2H_2O$	Calcium chloride dehydrate
$CaCl_2.H_2O$	Calcium chloride heptahydrate
$Co(NO_3)_2$	Cobalt(II) nitrate
CO_2	Carbon dioxide
$CuSO_4.5H_2O$	Copper(II) sulfate pentahydrate
DHA	Docosahexaenoic acid
DMBA	Dimethylbenz(a)anthracene
DMSO	Dimethylsufoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
$FeSO_4.7H_2O$	Iron(II) sulfate heptahydrate
H_3BO_3	Boric acid
K_2HPO_4	Dipotassium phosphate
K_2HPO_4	Potassium hydrogen phosphate
KH_2PO_4	Potassium dihydrogen phosphate
KOH	Potassium hydroxide
$MgSO_4.7H_2O$	Magnesium sulphate heptahydrate
$MgSO_4.7H_2O$	Magnesium sulfate heptahydrate
$MnCl_2.4H_2O$	Manganese(II) Chloride tetrahydrate

MoO_3	Molybdenum(VI) oxide
$Na_2MoO_4.2H_2O$	Sodium molybdate
NaCl	Sodium chloride
$NaNO_3$	Sodium nitrate
OH	Hydroxyl
PER	Protein Efficiency Ratio
pH	power of Hydrogen
UV	Ultra violet
$ZnSO_4.4H_2O$	Zinc sulfate tetrahydrate

LIST OF SYMBOLS

%	Percent
mg	Milligram
ml	Millilitre
g	Gram
lts	Litres
nm	Nanometer
m	Meter
^{o}C	Degree Celsius
α	Alpha
β	Beta
Ω	Omega

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ABSTRACT

Present work was focused on screening of astaxanthin content in three different algal species *Haematococcus pluvialis, Chlorella vulgaris* and *Anabaena variabilis*. Growth was studied in terms of biomass for all species and cell count in *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11. *Haematococcus pluvialis* produced highest biomass of 0.867 mg/ml with 0.015% astaxanthin content. *Haematococcus pluvialis* and *Chlorella vulgaris* producing higher amounts of astaxanthin were grown in two different media BG11 and BBM under indoor and outdoor conditions. *Haematococcus pluvialis* in BG11 gave maximum biomass yield of 0.6 mg/ml and astaxanthin content of 0.006% under indoor cultivation whereas 4.276 mg/ml biomass and 0.096% astaxanthin under outdoor cultivation. Among three different microalgae, astaxanthin content was higher in *Haematococcus pluvialis* followed by *Chlorella vulgaris* and *Anabaena variabilis*.

Chapter-1 Introduction

Microalgal biotechnology has gained importance due to its potential to produce bioactive compounds like β-carotene, astaxanthin, lutein etc. Ketocarotenoid astaxanthin is a pigment and member of the xanthophyll family of carotenoids that constitutes the highest value product derived from microalgae with a vast range of applications in food, feed and pharmaceutical sector. Natural astaxanthin from microalgae accounts for <1% of the global market, since the synthetic alternative derived from petrochemicals involves lower production costs.

Nutraceuticals market has expanded into a multibillion euro industry with the forecast of continued growth within the emerging global markets (Frost and Sullivan, 2011). Nutraceuticals is a term that combines "nutrition" and "pharmaceutical", and is used to describe a food that functions in maintaining the wellbeing and health enhancement of an individual thus preventing and treating specific disease (Ramaa *et al*., 2006). There are numerous reasons for the continued growth in this sector but in essence the industry is catering for the consumer concern regarding traditional synthetic additives and the trend has been towards incorporating more natural substitutes which are viewed as a healthier alternative to traditional additives into food products. One particular area which has gained considerable attention is the role played by antioxidants within food products (Shahidi, 2000) and also the preventive role these additives may play as free radical scavengers within the human body post consumption (Capelli and Cysewki, 2007). While numerous synthetic compounds have been used to achieve these goals in the past, the white paper by Frost and Sullivan (2011) predicts a push towards the development of natural nutraceuticals for the consumer market. The logic behind this driving force is somewhat perceived notion that there is a lower risk of side effects associated with natural products, while at the same time being potentially more bioavailable. Within this group of naturally occurring antioxidants, the carotenoids represent a commercially important class of molecules.

Microalgae, under specific stress conditions accumulate carbohydrates, lipids and secondary metabolites. These metabolites have an enormous industrial applications that

strongly enhance a bio-based economy (Markou and Nerantzis, 2013). 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, which play important role in human and animal health. 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. Among these metabolites, astaxanthin is one of the most valuable compounds with a enmorous range of applications in the food, feed, cosmetics and pharmaceutical industry (Cardozo *et al*., 2007; Borowitzka, 2013; Koller *et al*., 2014; Pérez-López *et al*., 2014).

Some species of microalgae have been commercially exploited to produce carotenoids like β-carotene, astaxanthin, lutein etc. 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* is one of the natural sources known for its ability to accumulate high amount of astaxanthin (2-3% w/w). With this background, *Haematococcus pluvialis, Chlorella vulgaris and Anabaena variabilis* were screened as a suitable source for the production of astaxanthin in indoor and outdoor cultivation.

Chapter-2 Review of Literature

Microalgae are prokaryotic or eukaryotic photosynthetic aquatic microorganisms, which are naturally found in freshwater and marine environments, inhabiting a vast range of ecosystems (Guschina and Harwood, 2006). They are one of the oldest living organisms on earth representing large number of species (Alam *et al*., 2012). Nevertheless, only a small fraction of around 30.000 species has been studied and analyzed (Mata *et al*., 2010). Unlike normal plants, microalgae lack roots, stems and leaves, while their basic composition involves the elements: carbon, oxygen, hydrogen, nitrogen, phosphorus and sometimes iron and silicon (Brennan and Owende, 2010; Chisti, 2007). These elements constitute the building blocks of the different products that can be extracted from microalgae. The present system of classification of microalgae are based on (i) type of pigments, (ii) chemical nature of the stored products, and (iii) cell wall components, which define the different groups of species of microalgae (Dragone *et al*., 2010 and Alam *et al*., 2012). Table 1 portrays some major groups of microalgae in terms of color (Alam *et al*., 2012).

Group	Colour
Chrysophyceae	Golden algae
Rhodophyceae	Red algae
Phaeophyceae	Brown algae
Chlorophyceae	Green algae
Xanthophyceae	Yellow-green algae
Cyanophyceae	Cyanobacteria

Table 1: Major groups of microalgae in terms of colour

The biodiversity of microalgae is enormous and represents an almost untapped resource. Microalgae have so far been a rather under explored resource despite being potential producers of a wide spectrum of natural substances which are useful for humans (Goyal and Goyal, 1998). In recent years, microalgal biotechnology has gained attention due to advancements in production technology.

2.1 Nutritional modes of microalgae

Microalgae may have four types of metabolism and metabolic shift occurs as a response to changes in the environmental conditions. These modes are discussed in detail in the following sections.

2.1.1 Photoautotrophic metabolism

Photoautotrophic metabolism is mostly used to cultivate microalgae in open ponds and photobioreactors. Sunlight or artificial light is used as energy source and inorganic carbon as the carbon source for the formation of biochemical energy through photosynthesis. This mode has three major advantages: First, autotrophic cultivation enhances sustainable development, since it requires large quantities of CO_2 as a carbon source, which could be derived from factories and power plants (Chen *et al*., 2011). Secondly, chances of contamination are very less as compared to other modes (Chiu *et al*., 2008). Third, the simplicity of growing microalgae photoautotrophically makes this option the most cost-efficient among the different nutritional modes (Mohan *et al*., 2014). In photoautotrophic cultivation the algal cells are mostly subjected to stress conditions to force the cells to accumulate the desirable metabolite (Chen *et al*., 2011).

2.1.2 Heterotrophic metabolism

In this mode of nutrition, microalgae utilize solely organic carbon as primary energy source for their growth (Mata *et al*., 2010; Chen *et al*., 2011). Heterotrophic metabolism takes place in conventional fermentors in absence of light (Chen *et al*., 2011; Perez-Garcia *et al*., 2011). The two major advantages of this mode are- cell density (the highest among the different modes and nearly 20 times higher than that obtained under photoautotrophic cultivation and the facilitation of wastewater as a base environment for cultivation (Chen *et al*., 2011; Perez-Garcia *et al*., 2011). However, a heterotrophic system frequently suffers from contamination problems (Olguin *et al*., 2012). Furthermore, the costs are higher than in photoautotrophic metabolism, mainly due to the high cost of organic carbon used for algal cultivation (Chen *et al*., 2011).

2.1.3 Mixotrophic metabolism

Mixotrophic metabolism constitutes both photoautotrophic and heterotrophic metabolism and photosynthesis is the main energy source, however, in the absence of light, organic carbon could serve the role of energy carrier. Regarding carbon assimilation, both organic compounds as well as inorganic carbon (CO_2) facilitate the process (Chang *et al*., 2011). The advantage of this technique is that microalgae possess flexibility to switch their nutritional mode (either photoautotrophic or heterotrophic conditions, or both) based on substrate availability and light conditions available (Mohan *et al*., 2014). Highly controlled environmental conditions are essential for successful mixotrophic cultivation. Therefore, photobioreactors (PBRs) are deemed to be the most suitable cultivation systems (Chen *et al*., 2011).

2.1.4 Photoheterotrophic metabolism

In this mode, the microalgae uses light as energy source and organic compounds as the carbon source (Chen *et al*., 2011). The difference between mixotrophic and photoheterotrophic cultivation is that the latter requires only light as the energy source, while mixotrophic cultivation can use either light or organic compounds to serve this purpose. Thus, photoheterotrophic cultivation needs both sugars and light simultaneously (Chojnacka and Marquez-Rocha, 2004). This mode is suitable only for those algal strains, which accumulate high quality of specific metabolites only under photoheterotrophic conditions (Mata *et al*., 2010). Like mixotrophic culture systems, photoheterotrophic systems are highly sophisticated, which leads to high operational costs (Chen *et al*., 2011).

Nutritional mode	Energy source	Carbon source	Biomass productivity	Culture system	Advantages	Disadvantages
Photoautotrophic	Light	Inorganic	Low	Open pond or PBR	Industrial CO_2 uptake; Less contamination; cost-efficient;	Low Cell density; Site limitation
Heterotrophic	Organic	Organic	High	Conventional fermentor	High biomass productivity; wastewater facilitation	Contamination; High substrate cost
Mixotrophic	Light and Organic	Inorganic and organic	Medium	Sophisticated PBR	Growth parameters independence	Contamination; High operational costs;
Photoheterotrophic	Light	Organic	Medium	Sophisticated PBR	High quality metabolites	High operational costs;

Table 2: Findings on the different nutritional modes

2.2 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). The blue-green microalga *Spirulina* has had a long history in human nutrition. In 1964, *spirulina* had been used as health food in Japan. Spray-dried biomass is mostly used for health foods, food additives and feed supplements (Venkataraman *et al*, 1995; Yamaguchi, 1997). *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). 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* is present in market in form of tablets or capsules as health (Metting, 1996). Microalgal oils have been used in infant milk formulations, as dietary supplements and as food additives (Kyle and Gladue, 1996).

2.3 Bioactive compounds from microalgae

Microalgae have already been used as cheap and effective biocatalysts to obtain highly value added 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 functioniong of brain and eye in infants and has been shown to support cardiovascular health in adults (Kroes *et al*, 2003).

2.4 Pigments

Among these secondary metabolites, pigments are considered as the algal products of highest potential for commercial success in the near future (Spolaore *et al*., 2006). Algal pigments serve very important for order microalgae in order to thrive: They are responsible for light harvesting, CO_2 fixation, protection of algal cells against damage by excessive illumination, and, macroscopically, the coloration of the algal culture (Koller *et al*., 2014). major groups of pigments which are found in microalgae are i) carotenoids (among them, carotenes provide an orange coloration, ii) xanthophylls are responsible for yellowish shade), iii) phycobilins (red or blue coloration), and iv)chlorophylls (green coloration) (Spolaore *et al*., 2006).

2.5 Carotenoids from microalgae

The carotenoids represent a large class of naturally occurring fat soluble molecules. These coloured pigments are found in both the plant and animal kingdoms and can vary from blue to red in colour. The carotenoid family includes over 800 individual molecules, which is divided into two major groups: Carotenes and Xanthophylls.

Various different carotenoids have been shown to be extremely effective nutraceuticals in numerous studies, e.g. lycopene has been associated with the decreased risk of cardiovascular diseases and chronic cancer (Rao and Agarwal, 2000) and lutein can reduce the risk of lung and breast cancer (Ribaya-Mercado and Blumberg, 2004). 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. 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 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).

Microalgae	Bioactive compounds	Reference
Spirulina sp. *Spirulina platensis*	Polysaccharides, Phycocyanin, C-phycocyanin, Phenolic acids, tocopherols, neophytadiene, phytol, PUFAs (n-3) fatty acids, oleic acid, linolenic acid, palmitoleic acid	Ibanez and Cifuente, 2013; Plaza *et al*., 2009; Singh and Dhar, 2011
Spirulina fusiformis	Diacylglycerols	Singh and Dhar, 2011
Haematococcus pluvialis	Astaxanthin, lutein, zeaxanthin, canthaxanthin, lutein, β-carotene, oleic acid	Markou and Nerantzis, 2013; Plaza *et al*., 2009; Singh and Dhar, 2011
Chlorella sp. *Chlorella vulgaris*	Carotenoids, sulfated polysaccharides, sterols, PUFAs (n-3) fatty acids, Canthaxanthin, astaxanthin, peptide	Priyadarshani and Rath, 2012; Plaza *et al*., 2009; Amaro *et al*., 2013
Chlorella minutissima	Eicosapentaenoic acid (EPA)	Singh and Dhar, 2011
Dunaliella salina	β-carotene,oleic acid, linolenic acid, palmitic acid	Palavra *et al*., 2011
Dunaliella	Diacylglycerols	Singh and Dhar, 2011
Botryococcus braunii	Linear alkadienes (C25, C27, C29, and C31), triene (C29)	Palavra *et al*., 2011
Chlorella zofingiensis	Astaxanthin	Markou and Nerantzis, 2013 Raposo *et al*., 2013
Chlorella protothecoides	Lutein, zeaxanthin, canthaxanthin	
Chlorella pyrenoidosa	Lutein, sulfated polysaccharide	Plaza *et al*., 2009
Nostoc spongiaeforme *Nostoc sp.*	Borophycin, Cryptophycin	Singh and Dhar, 2011

Table 3: Principal bioactive compounds extracted from microalgae

2.6 Astaxanthin

Astaxanthin ($C_{40}H_{52}O_4$, 3,3'-dihydroxy-β,β'-carotene-4,4'-dione) is a member of the xanthophyll family of carotenoids and constitutes a high value product with high commercial potential that is ubiquitous in the watery nature (Markou and Nerantzis, 2013; Zhang *et al*., 2014). It is a substance best known for giving the pinkish-red hue to the flesh of salmonids (salmons and trouts), as well as shrimps, lobsters and crayfishes (Koller *et al*., 2014). These animals cannot synthesize astaxanthin *de novo*, since only plants, algae and some fungal and bacterial species synthesize astaxanthin among other carotenoids. Therefore, they have to introduce this pigment in their diet in order to absorb it (Goodwin, 1984). In the watery environment, astaxanthin is introduced in the food chain via biosynthesis in the microalgae (phytoplankton). This is the primary production level. Zooplankton consumes these microalgae and, in turn, is ingested by the above mentioned aquatic animals (Lorenz and Cysewski, 2000). Besides the colorant properties, astaxanthin displays a central role for the immune-system of these fishes and positively impacts their fertility. Furthermore, astaxanthin is considered as the most powerful antioxidant in the nature. It is claimed to possess as much as 10 times the antioxidant potential of other carotenoids such as β-carotene, canthaxantin, zeaxanthin and lutein; and 100 times more that α-tocopherol. In other words it serves the role of a highly efficient scavenger of free radicals build up within the human body (Koller *et al*., 2014). These antioxidant properties are of great significance in the human metabolism, since it is believed to play a key role in the amelioration/prevention of several human pathological processes: Astaxanthin is a substance that protects the skin against UV-induced photo-oxidation and that is used for anti-tumor therapies, inflammation and age-related diseases (Cardozo *et al*., 2007).

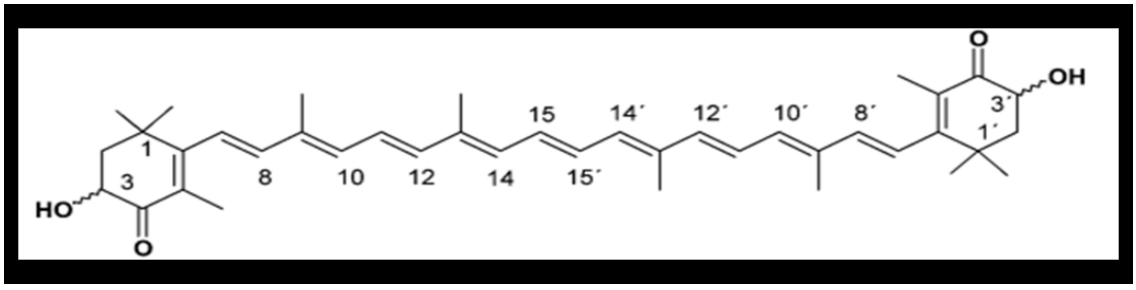

Fig. 1: Chemical structure of astaxanthin with a numbering scheme (Zhang *et al*., 2014).

Astaxanthin has a great potential in the market. It is estimated that the market value of astaxanthin depending on product purity varies usually from $2500-7000/kg and may reach in special occasions $100000/kg, while its global market potential was estimated at 280 metric tons and was valued at $447 million in 2014 for synthhetic and natural astaxanthin (Milledge, 2010; Borowitzka, 2013; Koller *et al*., 2014; Pérez-López *et al*., 2014; Industry Experts, 2015). Of this market, more than 95% consumes synthetically derived astaxanthin, while astaxanthin derived from microalgae amounts to less than 1% of the commercialized quantity (Koller *et al*., 2014; Pérez-López *et al*., 2014). Synthetic astaxanthin is derived from petrochemical sources. This attribute raises the issues of food safety, pollution, and sustainability (Milledge, 2010). Furthermore, the production costs of the synthetic version are considerably high ($1000/kg) (Olaizola, 2003; Li *et al*., 2011). Therefore, as society, nowadays, stimulates a transition towards less expensive 'green' solutions, synthetic astaxanthin seems to be much less desirable (Pérez-López *et al*., 2014). In this realm, Li *et al* (2011) estimated a production cost of $718/kg astaxanthin, by conceptually scaling up a pilot plant, which resulted in an annual astaxanthin yield of 900 kg. These production costs are significantly lower than the ones from the synthetic version, posing clear market opportunities for natural astaxanthin derived from microalgae. In fact, studies have predicted the potential market value to be over 1.5 billion dollars by 2020 (Nguyen, 2013).

2.6.1 Chemistry of Astaxanthin

Astaxanthin can exist in three configurational isomers: two enantiomers (3S, 3′S and 3R, 3′R) and a meso form (3R, 3′S) (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). 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.

2.6.2 Astaxanthin chemical structure

The molecular formula for astaxanthin is $C_{40}H_{52}O_4$ and the molecule consists if two carbon hexagon cages connected by an alternating series of nine carbon- carbon single and double bonds. The IUPAC name for astaxanthin is (3S,3'S)-3,3'dihydroxy-β, β-carotene-4, 4'-dione) and it consists of a long hydrocarbon chain terminated in two cyclohexane rings, both of which contain hydroxyl (OH) and carbonyl functional groups (C=O). The presence of these two groups with their electronegative oxygen atoms gives astaxanthin its unique functional abilities. A few of these abilities include bonding to muscle tissue, antioxidant activity and colouration (Naguib, 2000). These functions have been the object of interest in an increasing number of studies as they have been found to help in the treatment and prevention of certain ailments such as cancer (Khanafari *et al*., 2007). The structure of astaxanthin is similar to lycopene except that it is cyclized at both ends. The alternating double bonds along the backbone of the molecule form a polyene chain which gives this and other similar like molecules their unique properties. The alternating sequence of single and double bonds allow for the possibility of a number of geometrical isomers to exist, with the number of possible steric forms exceeding over a thousand possible structures. However this number is greatly reduced when steric hindrance is taken into account (Britton *et al.*, 1996). In nature most carotenoids exist in the all trans configuration and in this regard astaxanthin is no exception. Exposure to light is known to isomerise parent carotenoid molecules, which can affect the shape of the molecule with the trans form being structurally more rigid. This change in confirmation can affect the solubility and absorbability of the molecule. The trans forms of the carotenoids also tend to crystalize or aggregate more easily and thus are less easily absorbed. The end groups and the degree of oxygenation of these groups also effect the carotenoid properties. Most carotenoids are hydrophilic and are only soluble in organic non polar solvents (Yuan *et al*., 2008). Astaxanthin however is

	Organism	Content (% w/w dry weight)	Reference
Green algae	*Chlorella zofingiensis*	<0.01	Ip and Chen, 2005
	Haematococcus pluvialis	2.0-3.0	Lorenz and Cysewski, 2000
	Neochloris wimmeri	0.6	Orosa *et al*., 2000
	Nannochloropsis gaditana	<0.3	Lubian *et al*.,2000
	Scenedesmus vacuolatus	0.01	Orosa *et al*., 2000
	Chlorococcum	< 0.2	Zhang *et al*., 1997a
	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	*Agrobacterium aurantiacum*	0.01	Yokoyama *et al*., 1995
	Brevibacterium sp	0.003	Neils and Leenheer, 1991
	Mycobacterium lacticola	0.003	Simpson *et al*.,1981
Animals	Shrimp-*Pandalus borealis*	0.014	Shahidi and Synowieck,1991
	Backs snow crab *Chinoecetes opilio*	0.011	Shahidi and Synowiecki,1991
	Shrimp-*Pandalus clarkia*	0.015	Meyers and Bligh, 1981

Table 4: Natural sources of astaxanthin

an exception and it possess the ability to solubilise itself in both polar and non-polar solvents. The presence of the conjugated double bond system with its associated delocalised π electrons provide carotenoid molecules like astaxanthin their unique electrochemical properties. These highly delocalised electrons only require a small amount of energy to promote them into an excited state. As a result visible light is sufficiently energetic enough to bring about an electronic transition with the molecule. Carotenoids like astaxanthin are susceptible to oxidative dehydration during storage and processing and as such care has been taken when handling to prevent photobleaching and oxidative damage (Miao *et al*., 2013, Ahmed *et al*., 2015).

2.6.3 Health benefits of Astaxanthin

The most important and widely used xanthopyhlls is astaxanthin; it has many applications within the pharmaceutical, food and cosmetic industries. This pinkish-red pigment can be found in a variety of foods and some common examples of the natural occurrence of astaxanthin include salmon, seaweeds and crustaceans. The majority of these organisms with the notable exceptions of algae and bacteria cannot produce astaxanthin directly and it is thus introduced through their diet. Once it is absorbed it can have different effects on the organism's body, a few include: pigmentation, the ability to oxidise essential fatty acids and immunological reactions. The consumer demand for commercial astaxanthin has resulted in a dramatic increase in the market in recent years. Natural astaxanthin is in great demand because of its benefits including antioxidant activity, antimicrobial activity and anti-inflammatory activity (Capelli and Cysewski, 2007). Until recently the main application for astaxanthin usage had been in fish farming, where astaxanthin was added to the salmon's diet in order for the fish flesh to develop its popular pink/red pigmentation. It also had the additional benefit of conferring protection (cellular) from oxidation (Baker and Günther, 2004). The wide variety of benefits associated with astaxanthin gives it the potential to be a powerful nutraceutical. 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. 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, α and β-carotene, and α-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.

Studies have shown that ingestion of astaxanthin may confirm benefits such as antioxidant and anti-inflammatory activity (Bagchi *et al*., 2010). Further studies by Nagendraprabhu and Sudhandiran (2011) on rat colon carcinogenesis showed that astaxanthin exhibits anti-cancer and anti-inflammatory effects. In 7,12-Dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis

astaxanthin has been found to have a chemo-preventative effect as it disrupts the NF-κ B and Wnt/ß-catenin signalling pathways through inhibiting phosphorylation of kinases and transcription factors (Kavitha *et al*., 2013). However these antioxidant and anti inflammatory functions can be disrupted through the formation of complexes with other atoms within the digestive system, e.g. transition metals (cobalt, copper, etc.). Recent research has shown the that the cyclohexane ring of a astaxanthin molecule can chelate metal to from complexes with Ca^{2+} and Zn^{2+} ions at low salt concentrations (Polyakov *et al*., 2010).

2.6.4 Sources of Astaxanthin

While astaxanthin can be obtained from numerous sources each with its own benefits the majority of commercially available astaxanthin is extracted from *Haematococcus pluvialis* flakes; an algae which is known to have the largest concentration of the pigment in nature. While a synthetic form of the molecule is available, the natural form of the molecule has been found to have a greater bio-availability and thus more research has been done to explore improved methods of its extraction from natural sources. Other sources of astaxanthin which have been exploited include red yeast (*Xanthophyllomyces dendrorhous*), green algae (*Haematococcus pluvialis*) and oil extracted from krill (Dore and Cysewski, 2003).

2.6.5 Astaxanthin extraction techniques

Irrespective of the source material removing the astaxanthin from its bound matrix is intrinsically important commercially and the choice of purification strategy is an essential step in the recovery of a usable astaxanthin source. There are a number of astaxanthin extraction methods available, these include various acid treatments, organic solvent extraction and microbial extraction. Finding a suitable solvent system for an extraction is of key commercial importance. Like most carotenoids the backbone of astaxanthin is hydrophobic in nature but unlike most other carotenoids both of the cyclohexane ends (heaxatomic) of the molecule possess hydrophilic functional groups. This solubility character duality confers some unusual characteristics upon the molecule compared to other carotenoids and consequently astaxanthin exhibits an ability to

solubilise in both aqueous (polar) and non-aqueous (non-polar) systems alike (Yuan *et al*., 2008). One of the most popular solvents used in astaxanthin extraction is acetone primarily due to its higher solubility in the organic solvents. Various extractions have been employed together for optimisation and production of the largest astaxanthin yield,. Khanafari *et al* (2007) compared both chemical and microbial extraction of astaxanthin from shrimp (*Penaeus semisulcatus*) waste. In wet chemical extractions both hexane and DMSO were used in combination whereas in the microbial extraction *Lactobacillus sp.* culture was used for fermentation and the collected culture was then subjected to chemical extraction using a 3:1 ratio of hexane to acetone. The results showed the microbial extraction proved to be more effective than the chemical extraction yielding 23 mg more astaxanthin per gram of starting material (Khanafari *et al*., 2007). Chunhua *et al* (2013) employed an additional acid wash step in the organic solvent extraction from *Phaffia rhodozyma*. This step was employed to eliminate the cell wall and membrane to improve the efficiency of the solvent extraction increasing the astaxanthin yield by threefold. The use of different organic solvents has also been examined to determine the best ratio of polar and nonpolar extracting solvent, which were found to be acetone and hexane respectively (Sachindra *et al*., 2006). A binary mixture of both a polar and non-polar solvent was found to be most effective as it resulted in a 60% increase in yield when compared to the use of a single solvent. Given the nature of the raw material to be utilised in the initial stages of this work the astaxanthin extraction from brown crab (*Cancer pagurus*) shell, starfish (*Asterias rubens*) and algae may prove difficult due to the presence of matrix effects and an acid washing step to free the astaxanthin may be added as an extra step to help improve the recoverable yields.

2.7 Algal sources

The algae are a primitive class of organism which inhabits both freshwater and saltwater aquatic ecosystems (Graham and Wilcox, 2000). They are photosynthetic organisms which are less complex in structure when compared to land plants. Even so the algae represent the one of the largest marine sources for astaxanthin (Graham and Wilcox, 2000). Within certain algae cells large accumulations of astaxanthin can occur after the cell has been subjected to high levels of sunlight. A similar effect can also be induced by varying the nutrient supply levels to the cell e.g. by altering the amount of available nitrogen. The accumulation of the astaxanthin occurs within the extra-chloroplastic lipid droplets where astaxanthin is considered to reduce cellular damage and photoinhibition. An example of this protective effect in nature can be found in snow algae (*Chlamydomonas nivalis*), where in once the snow melts the algae cells are exposed to sunlight, which results in the algae producing astaxanthin as a protective agent. This is observed as a red coloured snow which can be found usually in open exposures on the snow (Graham and Wilcox, 2000). The commercial production of astaxanthin exploits this defensive mechanism for manufacturing purposes whereby cultures of algae are grown and then submitted to high levels of irradiation and once astaxanthin has been accumulated within the cell then the algae is harvested and the pigment is extracted from the cell and purified (Dore and Cysewski, 2003).

2.8 Seaweed sources

The seaweeds are a group of algae but unlike simpler algae the seaweeds are autotrophic and multicellular in nature. They are similar in appearance to terrestrial plants and exhibit stems, roots and leaf analogues. Worldwide about 8 million tonnes of wet seaweed is harvested annually and recently its nutritional benefits have gained worldwide interest, due to the presence of proteins, vitamins, minerals and carbohydrates (Banerjee *et al*., 2009). Astaxanthin is also found in various red seaweeds (e.g. *Catenella repens*) and studies have demonstrated the effect of seasonal variations on the biomass of the seaweed along with ecological factors, population structure, water salinity and nutrient availability. Some of these seasonal variations can also effect the

production of astaxanthin within the seaweed e.g. high temperature and lack of nutrients etc. (Banerjee *et al*., 2009). It was found that astaxanthin concentrations varied in India in pre-monsoon, monsoon and post-monsoon conditions with the highest concentration been found in pre-monsoon seaweed samples (which was probably attributed to the higher temperature) and the lowest in monsoon samples (Banerjee *et al*., 2009). This information can help in developing the conditions in which seaweed produces optimum levels of astaxanthin which can then be applied and used in astaxanthin manufacture.

2.9 Echinoderms sources

Echinoderms intake various carotenoid precursors through their diet, they feed on the phytodetritus material deposited on the sea bed and play an important role in recycling the nutrients within this material. The phytodetritus has been linked to reproduction and growth seasonal patterns in the benthic biomass. Several marine animals such as echinoderms accumulate the carotenoids within their integuments assisting in camouflage, protection and signalling (e.g. colour during breeding) (Maoka, 2011). Research has shown that the carotenoid content of echinoids from different sampling sites is larger than that of holothurians (sea cucumbers), this can be due to the difference in diet intake as holothurians feed lower in the sediment rather than the phyto detrital layer (Wigham *et al*., 2008). Also numerous starfish species were also found to primarily contain the carotenoids astaxanthin, 7,8,7',8'-didehydroastaxanthin and 7,8-didehydroastaxanthin (Maoka, 2011).

2.10 Crustacean sources

The crustaceans are part of the arthropods phylum and all the species of this phylum have an exoskeleton made up of protein, carotenoids and chitin. With over 40,000 individual species the malacostraca are the largest of the six crustacean classes (Bernie and Wislon, 2001). Many species within the class are highly coloured due to the presence of various proteins bound to the carotenoid molecules within the exoskeleton. Indeed the actual amount of rare and unusual carotenoids within some of these species can result in them acting as a viable source of carotenoids for commercial usage once freed from the shell (Khanafari *et al*., 2007, Sachindra *et al*., 2005). Numerous

astaxanthin extraction methods have been developed, which can vary from a straight forward microbial digestion to numerous complex wet chemical extraction methods.

Indeed both types of extraction procedure have been applied to the recovery of astaxanthin from Persian Gulf shrimp waste (*Penaeus semisulcatus*) (Khanafari *et al*., 2007). Various studies have focused on optimising the extraction conditions by varying the choice of extraction solvents and extraction conditions (Khanafari *et al*., 2007, Sachindra *et al*., 2005, Vilasoa-Martínez *et al*., 2008). The major pigments found in this species of shrimp were found to be dominantly astaxanthin and its esters. Astaxanthin recovery from waste shrimp shell has also been performed using microbial extraction methods with even greater effectiveness when compared to solvent extraction techniques (Khanafari *et al*., 2007). In a study on a marine crab (*Charybdis cruciata*) astaxanthin and its esters were also found to be predominant at concentrations of 65.5 g/100g of the total carotenoids extracted (Sachindra *et al*., 2005). The same results were found when testing the snow crab (*Chionoecetes Opilio*) shell from the North Atlantic as astaxanthin was the primary carotenoid along with smaller concentration of its esters (Vilasoa-Martínez *et al*., 2008).

2.11 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 microalgae. 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).

2.12 Autotrophic and heterotrophic system for growth

Many microalgae, including *Haematococcus*, are capable of autotrophic as well as heterotrophic growth (Sarada *et al*., 2002b). Heterotrophic cultivation has a potential for achieving high cell concentration and it has been demonstrated for the production of

Chlamydomonas biomass (Chen and Johns, 1996). Due to the problem 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) reported glucose uptake and photosynthetic and respiratory performance in light by *Chlorella vulgaris* UAM 101 and concluded CO_2 as the major carbon species taken from the medium.

2.13 *Haematococcus pluvialis*

Haematococcus pluvialis is a freshwater strain of green microalga with a very unique life, which is divided in two stages (Boussiba, 2000): The first refers to a green, motile vegetative stage, in which the microalgal cells continuously divide and proliferate (following specific growth kinetics), synthesizing chlorophyll. The second refers to a red, non-motile resting stage, in which cell division stops and chlorophyll levels do not fluctuate, resulting in a continuous increase of astaxanthin content and cellular dry weight. In order *Haematococcus pluvialis* to transit from stage one ('green stage') to stage two ('red stage'), specific stress conditions during cultivation are needed. This means that the accumulation of astaxanthin can be induced by any factor that inhibits cell proliferation.

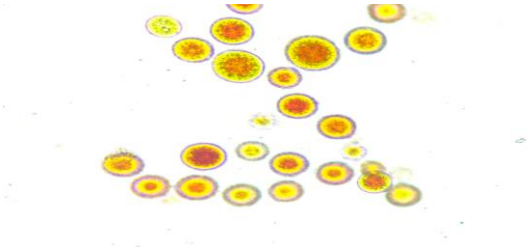

Fig. 2: Microscopic image of *Haematococcus pluvialis*

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). *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). During the morphological transformation, a trilaminar sheath and acetolysisresistant 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).

2.14 *Chlorella vulgaris*

Chlorella vulgaris is a eukaryotic, non- motile freshwater unicellular green alga. Most of the important features deals with its ability to rapidly grow. Common practice normally involves growing populations in photobioreactors. These chambers are consistently shaken and used to control certain aspects of metabolism in *C. vulgaris*. Variables such

as media, carbonation, and light have been researched heavily to understand the best means of optimal growth. Several uses of *C. vulgaris* have been researched. First, due to its high mineral and protein levels, it is used in vitamins and has even thought to be a viable food when dehydrated. It has powerful effects in boosting human health. Secondly, many algae produce lipids through photosynthesis. This makes these organisms a viable source for biofuel. Research shows that *C. vulgaris* reduced the color dye by 41.8%, ammonium by 44%, phosphate by 33%, and Carbon dioxide by 33-62%. *C. vulgaris* ability has also been considered for reducing emissions from power plant. Despite the range of benefits, a negative aspect is the cost to grow *C. vulgaris*. CO_2 is a limiting resource for large quantities of *C. vulgaris* to grow rapidly, in exception to a coal burning power plant. *C. vulgaris* is somewhat versatile with fixing carbon. *C. vulgaris* is a photolithoautotroph. Depending on the environment (media) it exists in, changes the byproducts resulting from metabolic processes. Chlorellan also can have properties of antitumor, antiviral, and even antifungal benefits. Patients in need of detoxification can sometimes be treated with *Chlorella*, making it a valuable tool for healthcare. *C. vulgaris* is known to survive under certain stressors such as viruses, bacteria, fungi, and many types of pollutants. The reason for these attributes stems from its ability to rapidly repair its DNA. When a break occurs, *C. vulgaris* is able to mutate and assimilate rapidly. Because of this, researchers are trying to further understand this process to benefit human health. Furthermore, when consumed it acts similar to an antibiotic. *C. vulgaris* is similar to most phototrophs because light is absorbed *via* the chloroplast. Green algae then fixes the CO_2 into fatty acids within the cell. *C. vulgaris* fatty acid biomass changes with different amounts of carbohydrates present in the media. The presence of carbohydrate causes the formation of intercellular fatty acids to have long chains. In situations with little or no carbohydrates, this green algae forms linolenic acid. Similar to most green and red algae, *C. vulgaris* doesn't make unsaturated fatty acids. This is important for the high lipid amounts found in green algae biomass. Also rather important, when *C. vulgaris* is grown on inorganic media, it contains more linolenic acid. This relates to the vast research about using *Chlorella* as a food source.

Some green algae can be high in protein and is believed to be a healthy substance for human consumption. Once the appropriate fatty acids are formed oxygen is then respirated and the CO_2 is stored.

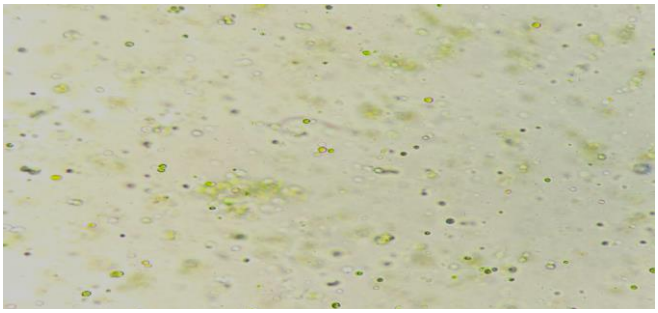

Fig. 3: Microscopic image of *Chlorella vulgaris*

2.15 *Anabaena variabilis*

Anabaena is a genus of filamentous heterocystous cyanobacteria- a prokaryote that exist as plankton. They are known for nitrogen-fixing abilities, and they form symbiotic relationships with certain plants, such as the water fern (Azolla). They are one of four genera of cyanobacteria that produce neurotoxins, which are harmful to local wildlife, as well as farm animals and pets. Production of these neurotoxins is assumed to be an input into its symbiotic relationships, protecting the plant from grazing pressure. A DNA sequencing project was undertaken in 1999, which mapped the complete genome of *Anabaena*, which is 7.2 million base pairs long. The study focused on heterocysts, which convert nitrogen into ammonia. Certain species of *Anabaena* have been used on ricepaddy fields, proving to be an effective natural fertilizer. Under nitrogen-limiting conditions, vegetative cells differentiate into heterocysts at semiregular intervals along the filaments. Heterocyst cells are terminally specialized for nitrogen fixation. The interior of these cells is micro-oxic as a result of increased respiration, inactivation of O_2-producing photosystem (PS) II, and formation of a thickened envelope outside of the cell wall. Nitrogenase, sequestered within these cells, transforms dinitrogen into ammonium at the expense of ATP and reductant—both generated by carbohydrate metabolism, a process supplemented, in the light, by the activity of PS I. Carbohydrate, probably in the form of glurose, is

synthesized in vegetative cells and moves into heterocysts. In return, nitrogen fixed in heterocysts moves into the vegetative cells, at least in part in the form of amino acids.

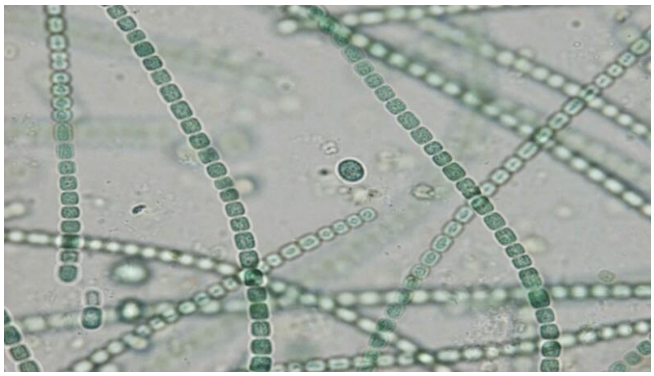

Fig. 4: Microscopic image of *Anabaena variabilis*

Chapter-3 Materials and Method

Algal cultures *Haematococcus pluvialis*, *Chlorella vulgaris* procured from ABCA Biosolutions and *Anabaena variabilis* (ARM 441) procured from IARI, were grown in BG-11 and BBM (+N and -N) medium at 28 ± 2ºC and 2500-3000 lux light intensity for 21 days with 16:8 light/dark cycle. Algal cultures were grown in the flasks for 21 days and pH was maintained at 7. After 3 day time interval for 21 days, growth was estimated in terms of biomass, cell count and astaxanthin content. Heterocyst was also checked microscopically in *Anabaena variabilis*.

Fig. 5: Algal growth in flasks

3.1 Composition of BG11 (Stanier *et al.*, 1971) : pH - 7

Components	**Amount (g/L)**
Sodium nitrate	1.5
K_2HPO_4	0.040
$MgSO_4.7H_2O$	0.075
$CaCl_2.H_2O$	0.036
Citric acid	0.006
Ferric ammonium citrate	0.006
EDTA	0.001
Sodium carbonate	0.20
Trace metal mix	1 ml

3.1.1 Trace metal mix

Components	Amount (g/L)
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.39
$CO(NO_3).6H_2O$	0.079
$CuSO_4.5H_2O$	0.0494
Distilled water	1000 ml

3.2 Composition of BBM (Kanz and Bold, 1969): pH - 7

Components	Amount (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

3.2.1Trace elements (for preparation of 1L stock solution)

Components	Amount (g/L)
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

3.3 Growth studies

Growth of *Haematococcus* sp., *Chlorella* sp. and *Anabaena* sp. was studied by estimation of astaxanthin, dry biomass and cell count (*Haematococcus pluvialis* and *Chlorella vulgaris*). Algal species were grown in the different medium (BG- 11 and BBM) and after every three day time interval for 21 days, 15 ml sample was taken for estimation of different parameters.

3.3.1 Dry biomass estimation: Biomass was estimated by using method given by Richmond and Gobbelaar, (1986).

Procedure:

1. The whatman filter was soaked in water to saturate and dried overnight at 60ºC. The weight of dried filter paper was noted as the initial reading. Took some beads and autoclave them.

2. The cultures were homogenized by vigorous shaking by adding dried beads to it and 5 ml of culture was taken and filtered through previously dried paper using vacuum filtration assembly. Wet weight was measured.

3. This was kept for drying and transferred to hot air oven maintained at about 60ºC, till constant weight was recorded at room temperature.

Calculations:

Dry biomass = Final reading of filter paper – Initial reading of filter paper

3.3.2 Astaxanthin Estimation

Astaxanthin was estimated by DMSO method given by Imamoglu *et al*., 2009

Procedure:

1. 5 ml algal culture was centrifuged at 2000×g for 5 minutes.
2. Pellet was saponified with 5% KOH in 30% (V/V) methanol at 70ºC for 5 min to degrade chlorophyll.

3. Supernatant was discarded and 3-5 drops of acetic acid were added to reduce pH .

4. Remaining pellet was extracted twice with 5 ml DMSO at 70ºC for 5 min to recover astaxanthin..

5. The absorbance of the combined extracts was measured at 490 nm.

Calculations:

Astaxanthin (%) = Extract volume (ml) × Abs 490 × 1.1/ Wt of biomass × 190.8

3.3.3 Microscopic determination of cell count

Cell count was determined using hemocytometer as per the method given by Provost and Wallert (1998).

Procedure:

1. With a cover-slip in place, used a pasteur pipette and transferred a small amount of the cell suspension to a chamber on the hemocytometer.
2. This was done by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action.
3. Took the average cell count from each of the sets of 16 corner squares.
4. Multiplied by 10^4.

3.3.4 Mass cultivation of *Haematococcus pluvialis* in BG11 and BBM

The mass cultivation of algal culture was carried out in plastic tubs in open conditions. The tubs were filled with 10lts of BG11 or BBM media. Water level, temperature and pH was checked after every 3 day time interval for 21 days during the mass multiplication of algal cultures. Starter cultures of *Haematococcus pluvialis* were inoculated in each tub followed by addition of few drops of Malathion to prevent insect breeding in the tubs. Biomass and astaxanthin content was checked after 21 days of incubation under outdoor conditions.

Fig. 6: Algal culture (*Haematococcus pluvialis*) grown in tubs

Two different unicellular algal species *Haematococcus pluvialis* and *Chlorella vulgaris* belonging to chlorophyceae were grown in BG11 (+N) medium and one heterocystous filamentous blue- green alga *Anabaena variabilis* (-N) was grown in BG11 (-N) media. Growth was monitored at interval of 3 days and astaxanthin content was estimated and compared.

4.1 Growth of *Haematococcus pluvialis*

4.1.1 Cell count

Haematococcus pluvialis was grown in BG11 (+N) media for 21 days at 25ºC. It was observed that the average cell count increased gradually from day 0 to day 21. The average cell count observed on day 0 was 0.3×10^4 cell/ml which increased upto 12.7×10^4 cell/ml on day 21 (Table 5, Fig. 7). There was a 35 fold increase in the average cell count from day 0 to day 21. Bocanegra *et al* (2004), reported the growth (green cells) of *Haematococcus pluvialis* in different media at 28º C with continuous illumination and manual agitation. Maximum growth obtained in BBM was 18×10^4 cell/ml and in BG-11 was 14×10^4 cell/ml. When cultures were aerated, an increase was observed in BBM 35×10^4 cell/ml and BG-11 27×10^4 cell/ml. When the same experiments were conducted using the 12 h light/12 h dark cycle, growth was lower in both the medias. But in our case, experiments were conducted using the 16 h light/ 8 h dark cycle and the growth increased gradually in both the cases.

Days	Cell count ($\times 10^4$ per ml)
0	0.3 ± 0.2
3	0.4 ± 0.08
6	0.7± 0.14
9	1.8 ± 0.5
12	4 ± 0
15	6 ± 0.58
18	10.9 ± 1.40
21	12.7 ± 1.44

Table 5: Cell count of *Haematococcus pluvialis* in BG11 (+N) media

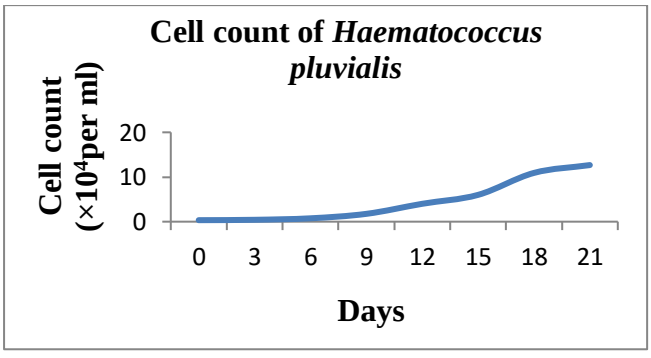

Fig. 7: Cell count of *Haematococcus pluvialis* in BG11 (+N) media

4.1.2 Dry Biomass

Biomass increased from 0.2 mg/ml to 0.8 mg/ml from day 0 to day 21. During 12-15th day, the biomass remained constant (0.53 mg/ml). It slightly increased on day 21 (0.87 mg/ml). Maximum increase in biomass was observed on day 21 (Table 6, Fig. 8).

Days	Dry Biomass (mg/ml)
0	0.20 ± 0
3	0.27 ± 0.07
6	0.33 ± 0.07
9	0.47 ± 0.07
12	0.53 ± 0.07
15	0.53 ± 0.07
18	0.60 ± 0.07
21	0.87 ± 0.07

Table 6: Dry Biomass of *Haematococcus pluvialis* in BG11 (+N) media

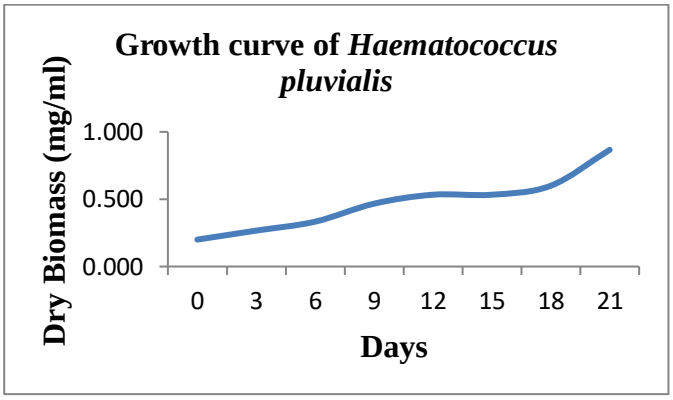

Fig. 8: Dry Biomass of *Haematococcus pluvialis* in BG11 (+N) media

4.1.3 Astaxanthin estimation

Astaxanthin is mainly produced by green algae *Haematococcus pluvialis.* Under unfavourable growth conditions, the alga changes from thin wall flagellated stage to red thick wall resting stage due to the accumulation of astaxanthin. BG11 is a stress media for *Haematococcus plu*vialis. The growth of *Haematococcus pluvialis* in this media is much higher as compared to other existing medias. No astaxanthin was extracted between day 0 to day 3 whereas 0.0077% astaxanthin was extracted on day 6. Maximum astaxanthin (0.0148%) was extracted on day 18 and 21 respectively (Table 7, Fig. 9). There was a 2 fold increase in the astaxanthin production from day 0 to day 21. Boussiba *et al* (1999) reported, that the kinetics of astaxanthin accumulation depends on the nature of the stress imposed on the green *Haematococcus* cells, in cultures starved either to phosphate or nitrogen. Nitrogen starvation may cause severe damage to the cell and results in increase in astaxanthin biosynthesis rate as compared to phosphate-starved cultures. Orasa *et al* (2001) found that if there is either nitrogen or phosphate starvation in

the media, *Haematococcus* green alga accumulates astaxanthin up to 4% of cell dry wt. Bocanegra *et al* (2004) reported, that the highest production of astaxanthin obtained with continuous illumination (345 µmol photon m^2/s) was 98 mg/g cells whereas 83 mg/g cells were obtained with the 12 h light/12 h dark cycle.

Days	Absorbance (490nm)	Astaxanthin (%)
0	0.003	0
3	0.010	0
6	0.051	0.007
9	0.070	0.010
12	0.108	0.012
15	0.114	0.012
18	0.155	0.015
21	0.223	0.015

Table 7: Astaxanthin estimation of *Haematococcus pluvialis* in BG11

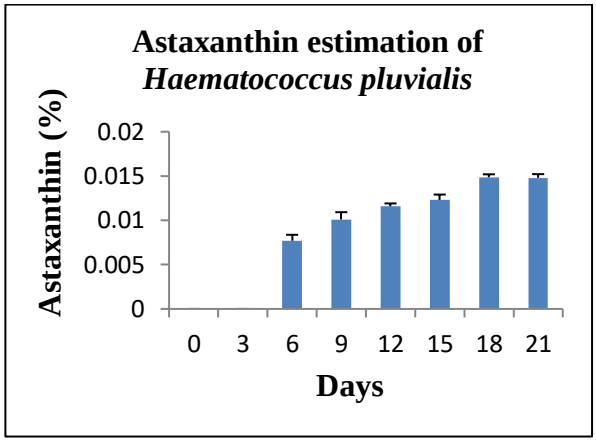

Fig. 9: Astaxanthin estimation of *Haematococcus pluvialis* in BG11 (+N) media

4.2 Growth of *Chlorella vulgaris*

4.2.1 Cell count

Chlorella was grown in BG11 (+N) media for 21 days at 25ºC and pH was maintained at 7. It was observed that there was a continuous increment in the average cell count from day 0 to day 21. 0.1×10^4 cell/ml were observed during day 0 whereas 2.9×10^4 cell/ml were observed during day 21 (Table 8, Fig. 10). There was 18 fold increase in the average cell count from day 0 to day 21. Zhang *et al* (2009) reported that the initial cell count of microalgae was 2.3×10^5 cells/ml. The cultures were cultivated for 10 days at 26 °C in the dark and the cell count was $3.2\times10^{6.}$ The number of cells continuously increased until the end of cultivation. However in our case, the cultures were cultivated for 21 days at 25º C in the light/dark cycle.

Days	Cell count ($\times 10^4$ per ml)
0	0.1 ± 0.08
3	0.4 ± 0.08
6	0.5 ± 0
9	0.6 ± 0.08
12	1 ± 0.25
15	1.5 ± 0.29
18	2 ± 0.52
21	2.9 ± 0.36

Table 8: Cell count of *Chlorella vulgaris* in BG11 (+N) media

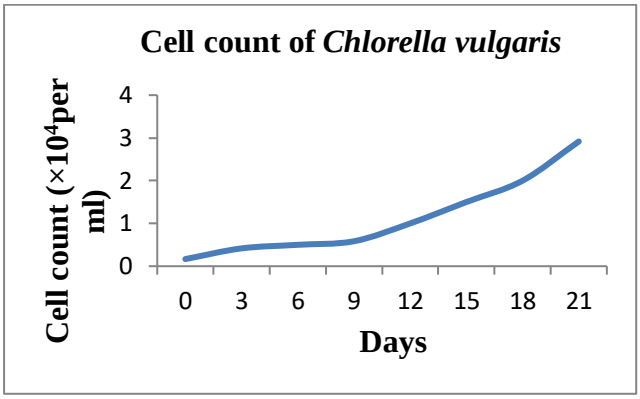

Fig. 10: Cell count of *Chlorella vulgaris* in BG11 (+N) media

4.2.2 Dry Biomass

0.06 mg/ml biomass was observed during day 0 whereas 0.9 mg/ml biomass was observed during day 21. The biomass remained constant when it switched from day 0 to day 3 (0.2 mg/ml), day 12 to day 15 (0.6 mg/ml) and day 18 to day 21 (0.9 mg/ml) respectively (Table 9, Fig. 11). Miao and Wu 2004; Xu *et al*., 2006; Li *et al*., 2007 reported, that the nitrogen has a prominent effect on the growth of microalgae. When no nitrate was added to the medium, growth of *Chlorella vulgaris* was decreased but not to a significant extent when compared with presence of nitrate. However in our case, *Chlorella vulgaris* was grown in (+N) media and the biomass increased eventually from day 0 to day 21.

Days	Dry Biomass (mg/ml)
0	0.07 ± 0.07
3	0.27 ± 0.07
6	0.27 ± 0.07
9	0.33 ± 0.13
12	0.6 ± 0
15	0.67 ± 0.18
18	0.93 ± 0.07
21	0.93 ± 0.07

Table 9: Dry Biomass of *Chlorella vulgaris* in BG11 (+N) media

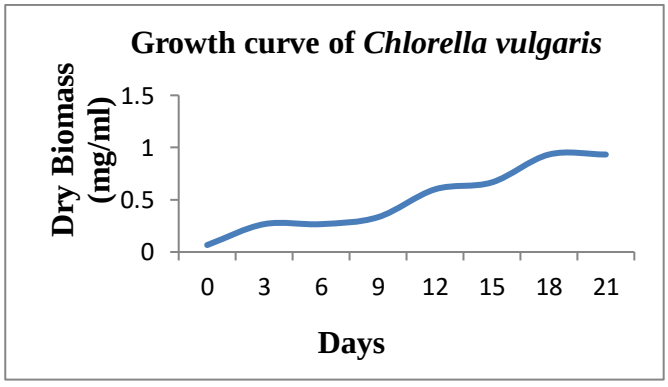

Fig. 11: Dry Biomass of *Chlorella vulgaris* in BG11 (+N) media

4.2.3 Astaxanthin estimation

It could be inferred that there was no astaxanthin accumulation from day 0 to day 9. Day 12 showed 0.0033% astaxanthin accumulation whereas 0.0099% astaxanthin was accumulated on day 21 (Table 10, Fig. 12).

Days	Absorbance (490nm)	Astaxanthin (%)
0	0.004	0
3	0.009	0
6	0.018	0
9	0.024	0
12	0.035	0.0033
15	0.047	0.0041
18	0.101	0.0062
21	0.160	0.0099

Table 10: Astaxanthin estimation of *Chlorella vulgaris* in BG11 (+N) media

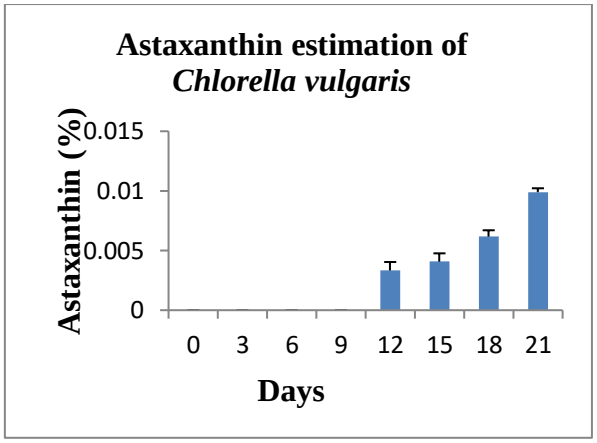

Fig. 12: Astaxanthin estimation of *Chlorella vulgaris* in BG11 (+N) media

4.3 Growth of *Anabaena variabilis*

4.3.1 Dry Biomass

0.06 mg/ml biomass was observed during day 0 whereas maximum biomass 1.06 mg/ml biomass was obtained on day 21. Constant biomass was observed during days 15 and 18 but increased slightly during day 21 (Table 11, Fig. 13).

Days	Dry Biomass (mg/ml)
0	0.07 ± 0.07
3	0.27 ± 0.07
6	0.27 ± 0.07
9	0.4 ± 0.12
12	0.73 ± 0.07
15	0.87 ± 0.07
18	0.87 ± 0.18
21	1.07 ± 0.07

Table 11: Dry Biomass of *Anabaena variabilis* in BG11 (-N) media

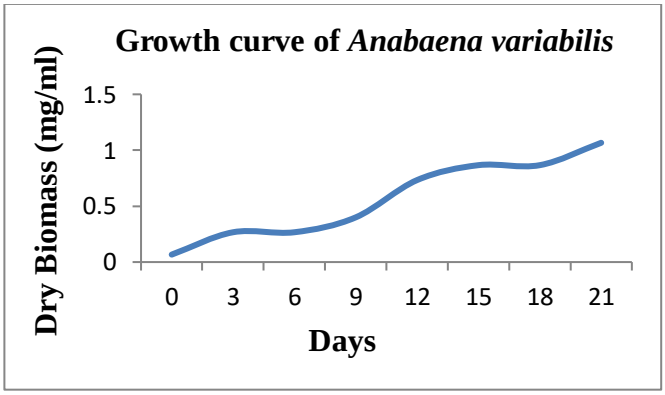

Fig. 13: Dry Biomass of *Anabaena variabilis* in BG11 (-N) media

4.3.2 Astaxanthin estimation

No astaxanthin was extracted during days 0 to 9. 0.0042% astaxanthin was extracted on day 12 whereas maximum astaxanthin (0.0088%) was extracted on day 21 (Table 12, Fig. 14).

Days	Absorbance (490nm)	Astaxanthin (%)
0	0.008	0
3	0.016	0
6	0.028	0
9	0.041	0
12	0.054	0.0042
15	0.078	0.0051
18	0.125	0.0083
21	0.164	0.0088

Table 12: Astaxanthin estimation of *Anabaena variabilis* in BG11 (-N) media

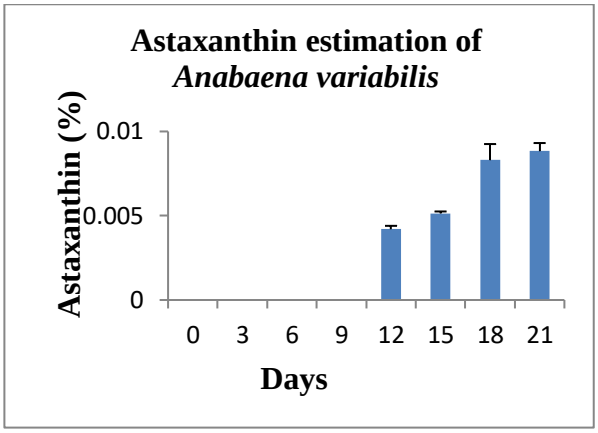

Fig. 14: Astaxanthin estimation of *Anabaena variabilis* in BG11 (-N) media

4.4 Comparative analysis of astaxanthin in *Haematococcus pluvialis, Chlorella vulgaris* and *Anabaena variabilis*

No astaxanthin was obtained from day 0 to day 3 in any of the cases. 0.0077 % astaxanthin was obtained on day 6 in case of *Haematococcus pluvialis* whereas it increased upto 0.148% on day 21. 0.0042% astaxanthin was obtained from *Anabaena variabilis* on day 12 and it increased upto 0.0088% on day 21 whereas 0.0033% astaxanthin was obtained from *Chlorella vulgaris* on day 12 and its value increased upto 0.0099% on day 21 (Table 13, Fig. 15). Astaxanthin content was highest in *Haematococcus pluvialis* followed by *Chlorella vulgaris* and *Anabaena variabilis*.

Days	*Anabaena variabilis*	*Chlorella vulgaris*	*Haematococcus pluvialis*
0	0.008	0.004	0
3	0.016	0.009	0
6	0.028	0.018	0.007
9	0.041	0.024	0.010
12	0.004	0.003	0.012
15	0.005	0.004	0.012
18	0.008	0.006	0.015
21	0.009	0.010	0.015

Table 13: Comparative analysis of astaxanthin content in different algae grown in BG11 media

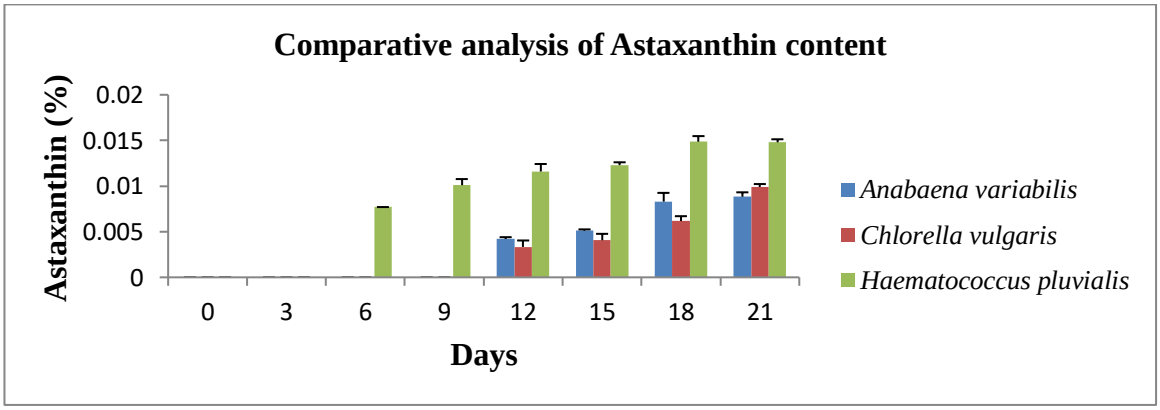

Fig. 15: Comparative analysis of astaxanthin content in different algae grown in BG11 media

4.5 Comparative analysis of biomass

Biomass is defined as the the total quantity or weight of organisms in a given area or volume. 0.2 mg/ml biomass was observed during day 0 in case of *Haematococcus pluvialis* and it increased upto 0.8 mg/ml on day 21. 0.06 mg/ml biomass was obtained on day 0 in case of *Chlorella vulgaris* and its value increased upto 0.9 mg/ml on day 21.

0.06 mg/ml biomass was obtained on day 0 in case of *Anabaena variabilis* and its value increased upto 1.06 mg/ml on day 21 (Table 14, Fig. 16). Maximum biomass was obtained from *Anabaena* variabilis between day 0 to day 21.

Days	*Anabaena variabilis*	*Chlorella vulgaris*	*Haematococcus pluvialis*
0	0.07 ± 0.07	0.07 ± 0.07	0.2 ± 0
3	0.27 ± 0.07	0.27 ± 0.07	0.27 ± 0.07
6	0.27 ± 0.07	0.27 ± 0.07	0.33 ± 0.07
9	0.40 ± 0.11	0.33 ± 0.13	0.47 ± 0.07
12	0.73 ± 0.07	0.60 ± 0	0.53 ± 0.07
15	0.87 ± 0.07	0.66 ± 0.18	0.53 ± 0.07
18	0.87 ± 0.17	0.93 ± 0.07	0.60 ± 0
21	1.06 ± 0.07	0.93 ± 0.07	0.87 ± 0.07

Table 14: Comparative analysis of biomass content in different algae grown in BG11 media

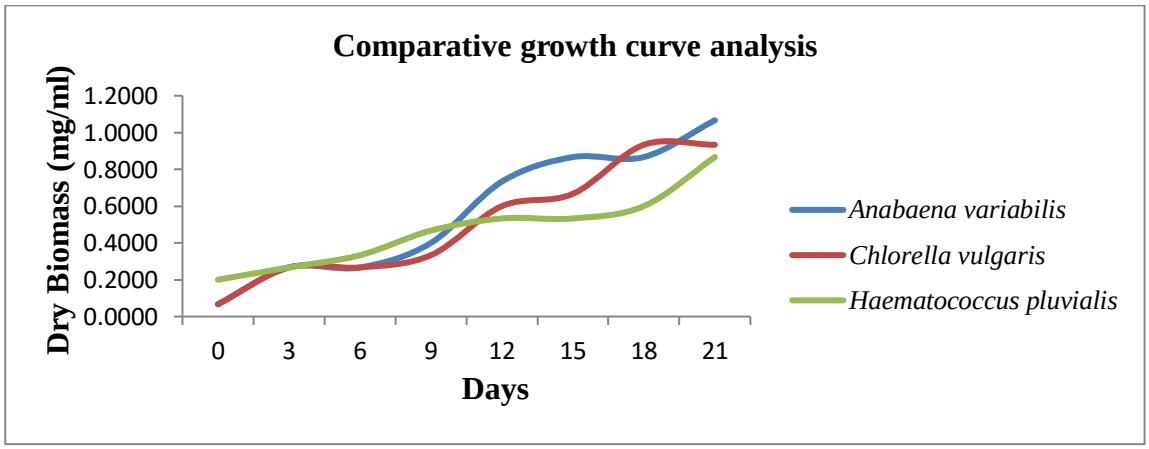

Fig. 16: Comparative analysis of biomass content in different algae grown in BG11 media

4.6 Comparative analysis of cell count

Haematococcus pluvialis and Chlorella vulgaris were grown in BG11 media for 21 days at 25ºC and the pH was maintained at 7. It was observed that the average cell count in case of *Haematococcus pluvialis* increased gradually from day 0 to day 21. The average cell count observed on day 0 in case of *Haematococcus pluvialis* was 0.3×10^4 cell/ml and it increased upto 12.7×10^4 cell/ml on day 21. 0.1×10^4 cell/ml were observed on day0 in case of *Chlorella vulgaris* and its value increased upto 2.9×10^4 cell/ml on day 21 (Table 15, Fig. 17). *Haematococcus pluvialis* is the fast growing algal species as compared to *Chlorella vulgaris*.

Days	*Haematococcus pluvialis*	*Chlorella vulgaris*
0	0.3 ± 0.2	0.1 ± 0.08
3	0.4 ± 0.08	0.4 ± 0.08
6	0.7 ± 0.14	0.5 ± 0
9	1.8 ± 0.5	0.6 ± 0.08
12	4 ± 0	1 ± 0.25
15	6 ± 0.58	1.5 ± 0.29
18	10.9 ± 1.40	2 ± 0.52
21	12.7 ± 1.44	2.9 ± 0.36

Table 15: Comparative analysis of cell count in different algae grown in BG11 (+N)

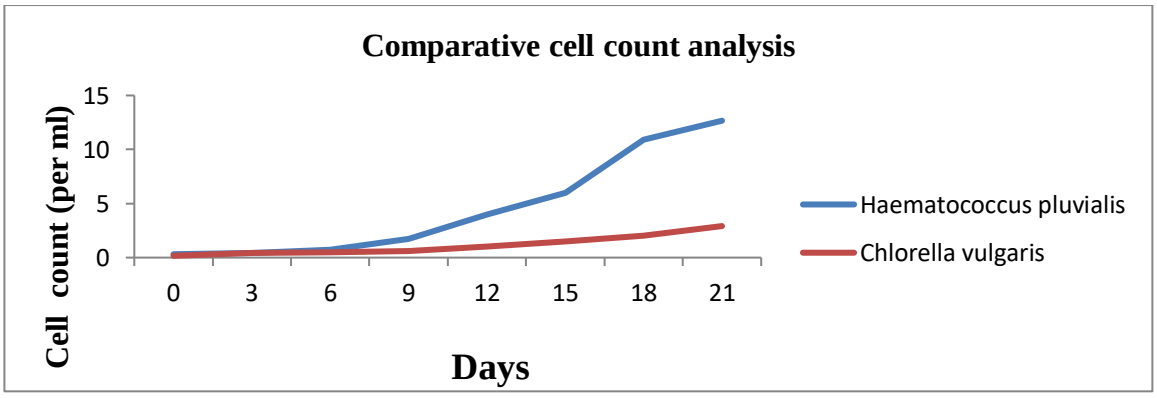

Fig. 17: Comparative analysis of cell count in different algae grown in BG11 (+N)

Anabaena variabilis (-N) wasn't able to produce higher amounts of astaxanthin. So, out of three algal species, only two of them (*Haematococcus pluvialis* and *Chlorella vulgaris*) were further taken for growth studies in two different media- BG11 and BBM.

BG11- BG11 broth is a universal medium for the cultivation and maintenance of blue green algae (cyanobacteria). But it works as a stress medium for *Haematococcus pluvialis*. Due to unfavourable conditions, the green algae (*Haematococcus pluvialis*) changes from thin wall flagellated stage to red thick wall resting stage. It is due to the accumulation of red coloured pigment astaxanthin (Stainer *et al*., 1971).

BBM- This medium is highly enriched and is used for many of the green algae (Kanz and Bold, 1969).

4.7 Growth of *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11 and BBM

4.7.1 Cell count

Haematococcus pluvialis was grown in BG11 (+N) and BBM (+N) media for 21 days at 25ºC and the pH was maintained at 7. It was observed that the average cell count increased gradually from day 0 to day 21 in both the media. The average cell count observed on day 0 was 0.3×10^4 cell/ml which increased upto 4.9×10^4 cell/ml on day 21 in BG11 whereas 0.08×10^4 cell/ml were observed on day 0 and increased upto 3.9×10^4 cell/ml on day 21 in BBM (Table 16, Fig. 18). There was a 17 fold increase in the average cell count from day 0 to day 21 in BG11 whereas 50 fold increase was observed in BBM. Bocanegra *et al* (2004), reported the growth (green cells) of *Haematococcus pluvialis* in different media at 28º C with continuous illumination and manual agitation. Maximum growth obtained in BBM was 18×10^4 cell/ml and in BG-11 was 14×10^4 cell/ml. When cultures were aerated, an increase was observed in BBM 35×10^4 cell/ml and BG-11 27×10^4 cell/ml. When the same experiments were conducted using the 12 h light/12 h dark cycle, growth was lower in both the medias. However in our case, experiments were conducted using the 16 h light/ 8 h dark cycle and the growth increased gradually in both the cases.

Onca *et al*(2000)., stated that the maximum cell number obtained with *Haematococcus pluvialis* was about 33×10^4 cell mL^{-1} on day 15 whereas in our case, 2.2×10^4 cell/ml were observed during day 15.

Tripathi *et al* (1999), said that the BBM was found to be the best for growth of cells. Maximum cell count (1.5×10^3 cell/ml) was obtained on the 10^{th} day whereas in our case, maximum cell count (3.9×10^4 cell/ml) was obtained on day 21.

Chlorella vulgaris was grown in BG11 (+N) and BBM (+N) media for 21 days at 25ºC and the pH was maintained at 7. Gradual increase in cell count was observed from day 0 to day 21 in both the media. The average cell count observed on day 0 was 0.2×10^4 cell/ml which increased upto 3.8×10^4 cell/ml on day 21 in BG11 whereas 0.08×10^4 cell/ml were observed on day 0 and increased upto 3.1×10^4 cell/ml on day 21 in BBM (Table 16, Fig. 18). There was a 19 fold increase in the average cell count from day 0 to day 21 in BG11 whereas 39 fold increase was observed in BBM. Zhang *et al* (2009) reported, that the initial cell count of microalgae in BG11 was 2.3×10^5 cell/ml and the cultures were cultivated for 10 days at 26 °C in the dark and the cell count was $3.2\times10^{6.}$ However in our case, the cultures were cultivated for 21 days at 25º C in the light/dark cycle.

Days	***Haematococcus pluvialis* (×10⁴)**		***Chlorella vulgaris* (×10⁴)**	
	BG11	**BBM**	**BG11**	**BBM**
0	0.3 ± 0.08	0.08 ± 0.08	0.2 ± 0	0.08 ± 0.08
3	0.6 ± 0.08	0.4 ± 0.08	0.4 ± 0.08	0.2 ± 0
6	1 ± 0	0.5 ± 0	0.5 ± 0.08	0.3 ± 0.08
9	1.8 ± 0.14	0.8 ± 0	1.1 ± 0.08	0.5 ± 0.08
12	2.2 ± 0.22	1.5 ± 0	1.8 ± 0.14	1.2 ± 0.08
15	3 ± 0.14	2.2 ± 0.08	2.6 ± 0.08	1.8 ± 0
18	3.9 ± 0.08	3 ± 0.08	3.2 ± 0.08	2.6 ± 0.08
21	4.9 ± 0.08	3.9 ± 0.08	3.8 ± 0.08	3.1 ± 0.08

Table 16: Comparative cell count analysis of *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11 and BBM media

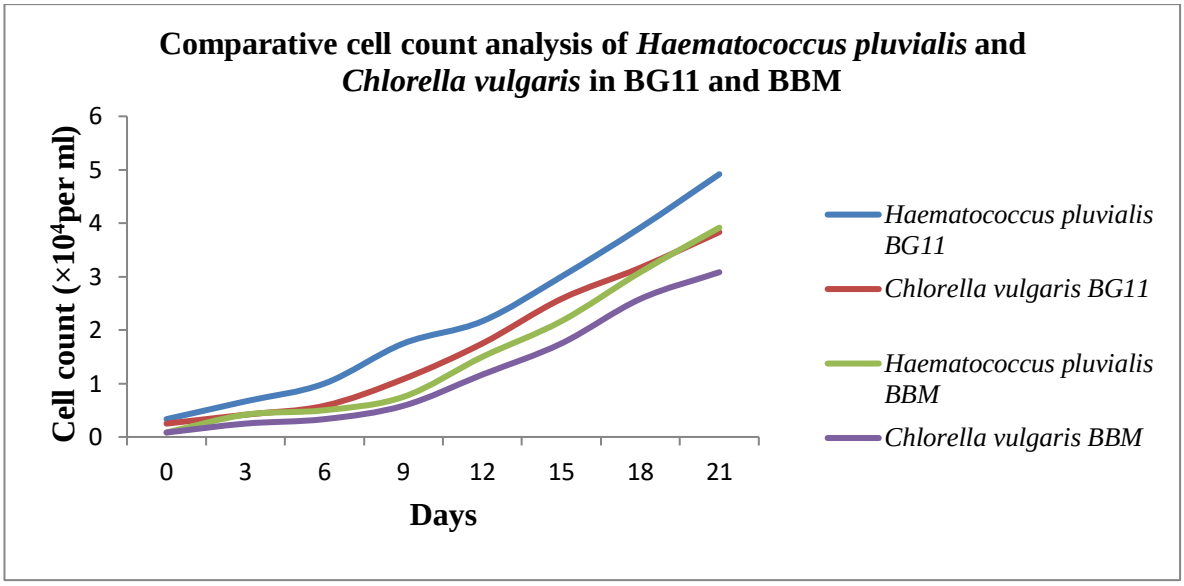

Fig. 18: Comparative cell count analysis of *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11 and BBM media

4.7.2 Biomass

Haematococcus pluvialis was grown in BG11 (+N) and BBM (+N) media for 21 days at 25ºC and the pH was maintained at 7. 0.07 mg/ml biomass was observed during day 0 and it increased upto 0.6 mg/ml on day 21 in BG11 whereas 0.07 mg/ml biomass was observed on day 0 which increased upto 0.47 mg/ml on day 21 in BBM (Table 17, Fig. 19). Linear growth was observed from day 0 to day 21 in BG11. Constant biomass growth (0.47 mg/ml) was observed during days 18 and 21 respectively in BBM. There was 9 fold increase in biomass from day 0 to day 21 in BG11 whereas 7 fold increase in biomass was observed in BBM. It was observed that *Haematococcus pluvialis* grew well on BBM media as compared to other media (Onca *et al*., 2000). The biomass production rate of *Haematococcus pluvialis* was 0.56 g L^{-1} on day 1. However in our case, biomass obtained on day 1 was 0.10 mg/ml.

Chlorella vulgaris was grown in BG11 (+N) and BBM (+N) media for 21 days at 25ºC and the pH was maintained at 7. There was a linear biomass growth from day 0 to day 21 in BG11. 0.07 mg/ml biomass was observed during day 0 and it increased upto 0.53

mg/ml biomass on day 21 in BG11 whereas no biomass was observed during day 0 which increased upto 0.4 mg/ml biomass on day 21 in BBM (Table 17, Fig. 19). Constant biomass growth (0.4 mg/ml) was observed during days 18 and 21 respectively in BBM. There was 8 fold increase in biomass from day 0 to day 21 in BG11 whereas 6 fold increase in biomass was observed in BBM. Nitrogen has a prominent effect on the growth of microalgae. When no nitrate was added to the medium, growth of *Chlorella vulgaris* was decreased but not to a significant extent when compared with presence of nitrate (Miao and Wu 2004; Xu *et al.*, 2006; Li *et al.*, 2007). However in our case, *Chlorella vulgaris* was grown in (+N) media and the biomass increased gradually from day 0 to day 21.

Days	**(*H.pluvialis*) Dry biomass (mg/ml)**		**(*C.vulgari*) Dry biomass (mg/ml)**	
	BG11	**BBM**	**BG11**	**BBM**
0	0.07 ± 0.07	0.07 ± 0.07	0.07 ± 0.07	0 ± 0
3	0.2 ± 0	0.13 ± 0.07	0.13 ± 0.07	0.07 ± 0.07
6	0.27 ± 0.07	0.2 ± 0	0.2 ± 0	0.13 ± 0.07
9	0.33 ± 0.07	0.27 ± 0.07	0.27 ± 0.07	0.2 ± 0
12	0.40 ± 0	0.33 ± 0.07	0.33 ± 0.07	0.27 ± 0.07
15	0.47 ± 0.07	0.4 ± 0	0.4 ± 0	0.33 ± 0.07
18	0.6 ± 0	0.47 ± 0.07	0.47 ± 0.07	0.4 ± 0
21	0.6 ± 0	0.47 ± 0.07	0.53 ± 0.07	0.4 ± 0

Table 17: Comparative biomass analysis of *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11 and BBM media

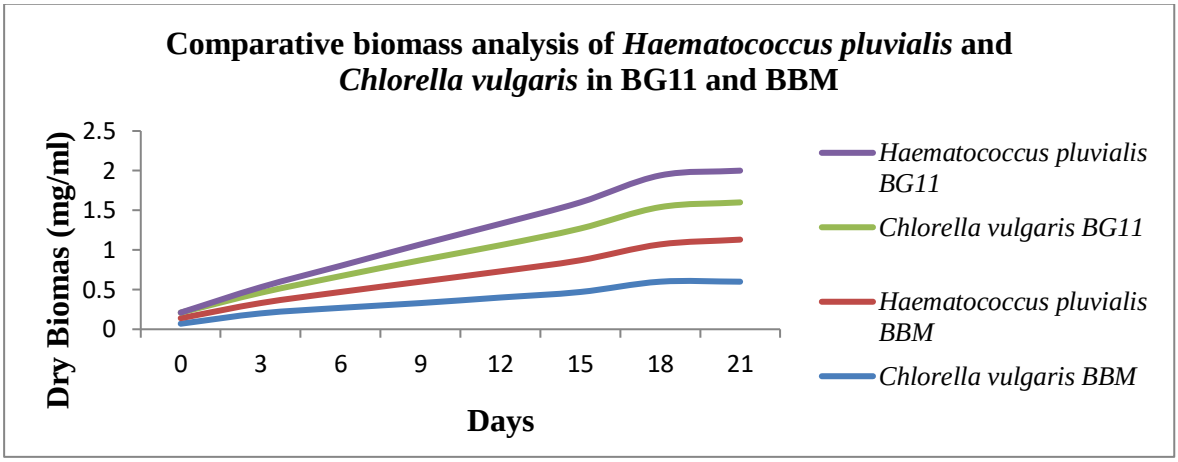

Fig. 19: Comparative biomass analysis of *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11 and BBM media

4.7.3 Astaxanthin estimation

Haematococcus pluvialis was grown in BG11 (+N) and BBM (+N) media for 21 days at 25ºC and the pH was maintained at 7. No astaxanthin accumulation was observed from day 0 to day 9 in both the media. 0.0011% astaxanthin was accumulated on day 12 which increased upto 0.0059% astaxanthin on day 21 in BG11 whereas 0.0010 % astaxanthin was extracted on day 12 which increased upto 0.0049% astaxanthin on day 21 in BBM (Table 18, Fig. 20). There was 5 fold increase in the astaxanthin production from day 12 to day 21 in both the media. Boussiba *et al* (1999) reported, that the kinetics of astaxanthin accumulation depends on the nature of the stress imposed on the green *Haematococcus* cells, in cultures starved either to phosphate or nitrogen. Nitrogen starvation may cause severe damage to the cell and results in an increase of the astaxanthin biosynthesis rate compared to the phosphate-starved cultures. Orasa *et al* (2001), said that the if there is either nitrogen or phosphate starvation in a media, *Haematococcus* green alga accumulates astaxanthin up to 4% cell dry wt. Bocanegra *et al* (2004) reported, that the highest production of astaxanthin obtained with continuous illumination (345 µmol photon m^2/s) was 98 mg/g cells whereas 83 mg/g cells were obtained with the 12 h light/12 h dark cycle. However in our case, highest production of

astaxanthin (0.0059% and 0.0050%) was obtained on day 21 in BG11 and BBM respectively with 16 h light/ 8 h dark cycle. Tripathi *et al* (1999), stated that the astaxanthin content was higher at 35ºC in both autotrophic and heterotrophic media. In a heterotrophic medium at low C/N ratio, *Haematococcus pluvialis* growth was higher with prolonged vegetative phase, while high C/N ratio favoured early encystment and higher astaxanthin formation. *Chlorella vulgaris* was grown in BG11 (+N) and BBM (+N) media for 21 days at 25ºC and the pH was maintained at 7. No astaxanthin was extracted from day 0 to day 9 in both the media. 0.0011% astaxanthin was extracted during day 12 which increased upto 0.0041% astaxanthin on day 21 in BG11 whereas 0.0011% astaxanthin was extracted during day 12 which increased upto 0.0038% astaxanthin during day 21 in BBM (Table 18, Fig. 20). There was only 4 fold increase in astaxanthin production from day 12 to day 21 in BG11 whereas 3 fold increase in biomass was observed in from day 12 to day 21 in BBM.

Days	***Haematococcus pluvialis***				***Chlorella vulgaris***			
	BG11		**BBM**		**BG11**		**BBM**	
	Absorbance (490 nm)	**Astaxanthin (%)**	**Absorbance (490 nm)**	**Astaxanthin (%)**	**Absorbance (490 nm)**	**Astaxanthin (%)**	**Absorbance (490 nm)**	**Astaxanthin (%)**
0	0.001	0	0.001	0	0.001	0	0.001	0
3	0.003	0	0.002	0	0.001	0	0.002	0
6	0.004	0	0.004	0	0.002	0	0.003	0
9	0.006	0	0.004	0	0.004	0	0.003	0
12	0.008	0.0012	0.006	0.0010	0.006	0.0010	0.005	0.0011
15	0.014	0.0018	0.008	0.0012	0.008	0.0012	0.006	0.0012
18	0.042	0.0041	0.0028	0.0035	0.0024	0.0030	0.020	0.0029
21	0.061	0.0059	0.0040	0.0050	0.0038	0.0041	0.026	0.0038

Table 18: Comparative astaxanthin analysis of *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11 and BBM media

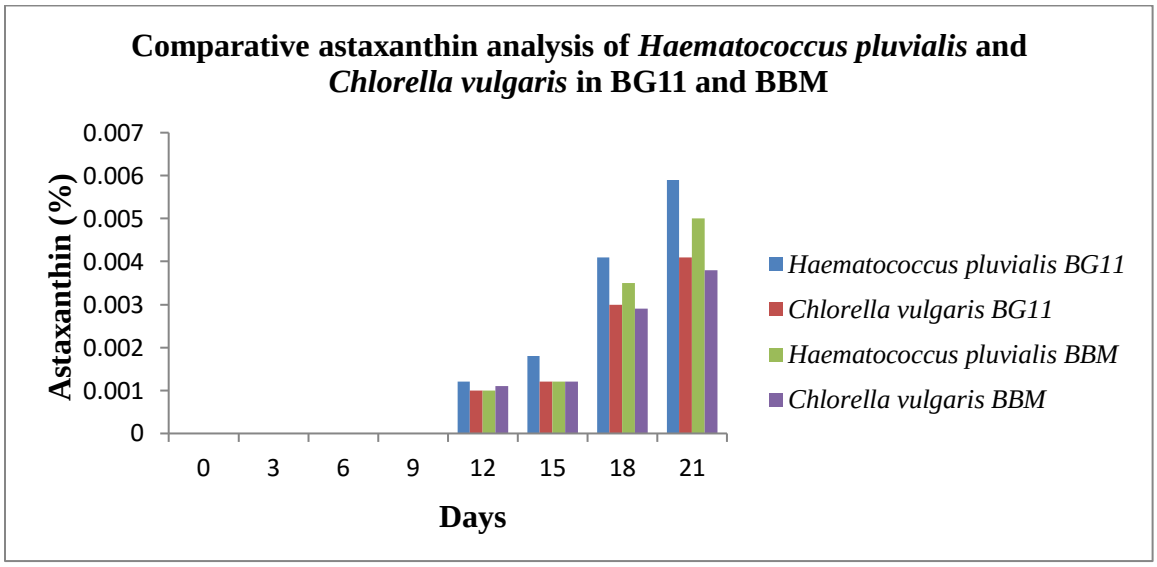

Fig. 20: Comparative cell count analysis of *Haematococcus pluvialis* and *Chlorella vulgaris* in BG11 and BBM media

Since *Haematococcus pluvialis* produced maximum astaxanthin as compared to *Chlorella vulgaris*. Hence, *Haematococcus pluvialis* was further taken for mass production in open tubs in BG11 and BBM and their results were then compared.

4.8 Mass production of astaxanthin from *Haematococcus pluvialis* in BG11 and BBM

Haematococcus pluvialis culture was inoculated in 10 lts BG11 and BBM media in triplicates. Depth and diameter of the tub were 18 cm and 35 cm respectively. Tubs were placed in polyhouse for 21 days and were covered with perforated polythene sheets so as to avoid entry of insects. Temperature, pH were observed after every three day time interval for 21 days. Biomass and astaxanthin content were estimated at 3 day interval for 21 days. pH increased from 7.04 to 8.96 in BG11, whereas pH increased from 7.01 to 8.39 in BBM. Temperature showed increase from day 0 to day 21, since the work was performed during summer between March - April.

Days	BG11			BBM		
	Temperature Morning (10 a.m.)	**Temperature Evening (5 p.m.)**	**pH**	**Temperature Morning (10 a.m.)**	**Temperature Evening (5 p.m.)**	**pH**
0	26.8 ± 0.18	28.3 ± 0.41	7.04	27.4 ± 0.15	29.2 ± 0.21	7.01
3	27 ± 0.10	28.3 ± 0.32	7.21	27.5 ± 0.32	28.8 ± 0.09	7.19
6	28 ± 0.15	29.3 ± 0.15	7.63	28.8 ± 0.09	29.4 ± 0.12	7.32
9	28.5 ± 0.12	29.8 ± 0.09	7.96	29.1 ± 0.06	29.6 ± 0.12	7.43
12	29.5 ± 0.21	30.3 ± 0.03	8.29	29.3 ± 0.03	29.6 ± 0.03	7.72
15	30.2 ± 0.06	30.8 ± 0.09	8.63	30.1 ± 0.09	30.6 ± 0.09	7.95
18	30.6 ± 0.20	31.8 ± 0.09	8.82	30.8 ± 0.06	31.3 ± 0.15	8.10
21	33.4 ± 0.20	35.2 ± 0.23	8.96	32.7 ± 0.22	34.3 ± 0.18	8.39

Table 19: Comparative analysis of temperature and pH during morning and evening in BG11 and BBM media

BG11 produced 0.4276 g/lt biomass and 0.096% astaxanthin whereas BBM produced 0.2463 g/lt biomass and 0.028% astaxanthin. This amount of astaxanthin was produced from 10lts of respective medias. Zhang *et al* (2009), stated that the the astaxanthin content of *Haematococus pluvialis* cultured in 100 m^2 open ponds (BG11) was similar to that produced in the laboratory, but required an average duration of 15 days, longer than obtained in the laboratory system, mainly because of the shorter daily illumination time and the influence of raining and cloudy days. The result demonstrated that production of astaxanthin from *Haematococcus* in open system by two-stage growth one-step process was feasible and practical. Olaizola (2000), said that after an optimization period-BBM (pond management, biomass processing and drying) results indicate a slight improvement from 2.8% to 3.0% of the dry weight. It has been reported that laboratory scale cultures of *Haematococcus* accumulate as much as 4–6 % of the dry weight as astaxanthin (Bubrick, 1991; Young *et al*., 1996; Margalith,1999).

Media	*Haematococcus pluvialis*
BG11	0.096 ± 0.002
BBM	0.028 ± 0.002

Table 20: Mass production of astaxanthin from *Haematococcus pluvialis* in BG11 and BBM

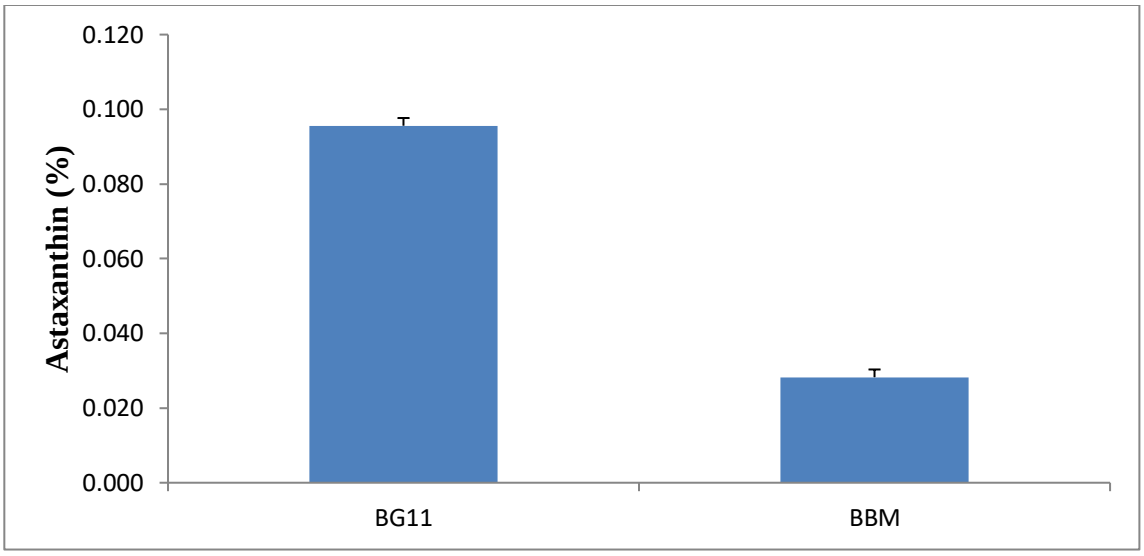

Fig. 21: Mass production of astaxanthin from *Haematococcus pluvialis* in BG11 and BBM media

Chapter-5 Conclusion

1. The present work focused on production of astaxanthin from three different algal species: *Haematococcus pluvialis, Chlorella vulgaris* and *Anabaena variabilis*. Growth was monitored in terms of biomass and cell count.
2. *Anabaena variabilis* (-N) wasn't able to produce higher amounts of astaxanthin. So, out of three algal species, only two of them (*Haematococcus pluvialis* and *Chlorella vulgaris*) were further taken for growth studies in two different media- BG11 and BBM.
3. *Haematococcus pluvialis* produced maximum astaxanthin as compared to *Chlorella vulgaris*. *Haematococcus pluvialis* was further mass produced in open tubs in BG11 and BBM and their results were then compared. *Haematococcus pluvialis* produced biomass of 4.276 mg/ml with 0.096% astaxanthin in BG11 whereas 2.463 mg/ml biomass and 0.028% astaxanthin was produced in BBM.
4. *Haematococcus pluvialis* was the best organism for the production of astaxanthin as compared to *Chlorella vulgaris* and *Anabaena variabilis*.

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