Impact of CO_2 sparging on growth of *Haematococcus pluvialis* and astaxanthin production

Dissertation

Submitted in partial fulfillment of the requirement of award of degree of

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Submitted
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Declaration

I, hereby declare that the work presented in the dissertation entitled"**Impact of CO_2 sparging on growth of *Haematococcus pluvialis* and astaxanthin production**" in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET), Patiala, is an authentic record of my own work during the period of six months from January 2018 to June 2018, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, TIET. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Dated: 21.8.2018

Place: Patiala

Shipra

Shipra Thakur

Certificate

This is certify that the thesis entitled, submitted by Ms. Shipra Thakur, "**Impact of CO_2 sparging on growth of *Haematococcus pluvialis* and astaxanthin production**" in partial fulfillment of the requirements for the award of degree of Master in Science in Biotechnology at Thapar Institute of Engineering and Technology (TIET), Patiala, is a record of student's own work carried out by her under my supervisor. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

Dr. Dinesh Goyal
Professor & Supervisor

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Abstract

Haematococcus pluvialis is known to accumulate secondary ketocarotenoid astaxanthin, a potent antioxidant. Production of astaxanthin from *H. pluvialis* can be achieved in a two stage process, where first step involves the production of high quantity of biomass followed by its induction to accumulate astaxanthin. High quantity of biomass was achieved by inclusion of carbon dioxide sparging in the growth medium. Growth measurements showed increase in cell number and chlorophyll content with CO_2 sparging in the medium. Owing to increase in photosynthetic activity led to increase in biomass production. Indoor conditions produce of 0.387 mg/L biomass with carbon dioxide sparging.

Accumulation of astaxanthin was induced by addition of salicylic acid and ferrous sulphate. Biochemical studies of the culture upon induction indicated presence of unfavourable conditions. Treatment with 200 µM of Salicyclic acid and 450 µM of ferrous sulphate gave highest astaxanthin accumulation of 0.868 mg/L, which was 54% higher than the astaxanthin obtained in the untreated cells of *Haematococcus*. Microscopic observation of treated *Haematococcus* cells also showed the increased accumulation of astaxanthin. It was observed that accumulation of astaxanthin is associated with environmental and nutritional stresses, which may cause interference in the normal cell division of *Haematococcus.*

Introduction

Microalgae are recognized as one of the oldest living organisms. Microalgae are found in almost every type of environment of the earth, they are found in desserts to snow and even in harsh habitat such as, the hot springs. Algae are not only important from environmental perspective; they are also the source of important molecules. Most of the algae derived molecules are recognized under the category of "Nutraceuticals". Algae have an advantage of higher production in comparison to plants. Microalgae have 15-300 times higher production than the traditional crops on an area basis. Microalgae possess very short harvesting cycle, thus allows multiple harvesting. Algae are efficient fixators of carbon dioxide. They utilize the carbon dioxide present in the air as source of inorganic carbon and convert it into biomass. They can be utilized as tool for biological mitigation and biomass obtained can be utilized as the feedstock forhigh value products.

One of such nutraceutical obtained from algae is astaxanthin, which is most potent anti-oxidant. Most important source of astaxanthin is *Haematococcus pluvialis*, green unicellular algae, that accumulates astaxanthin in high quantities. Besides this, most of the astaxanthin available in the market is synthetically derived. Only less than 1% of the astaxanthin available in market is obtained from *Haematococcus pluvialis*. There are several attempts to increase the production of astaxanthin from *H. pluvialis*.

Astaxanthin, is a secondary keto-carotenoid pigment, that accumulates only during stress conditions. Various chemical and physical methods are used to induce cells to produce astaxanthin. Production of astaxanthin from *H. pluvialis* is usually carried out in a two step cultivation process. The first step is dedicated to the production of large quantity of biomass and second step includes the induction of biomass to produce astaxanthin.

Another challenge is in the extraction of astaxanthin from *H. pluvialis*. The thick sporopollelin wall, generated during its transition from a green to aplanospore cell, causes barrier for efficient removal. There are various methods available, that are designed carefully, keeping in mind the strength of sporopollelin wall. This aspect is important, as this step of downstream processsing

adds to the price of product. Present work was an attempt to achieve high production of biomass of *Haematococcus*, with simple inclusion of carbon dioxide sparging and manipulating conditions for higher production of astaxanthin by the cells.

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Review of literature

Algae are one of the oldest living organism lacking true roots, stem and leaves, and are called as thallophytes (Brennan and Owende, 2010). They fix atmospheric CO_2, by the process of photosynthesis, similar to the process occurring in higher plants but is more efficient, simply because of their structure (Dragone *et al.,* 2010) and presence of chlorophyll an as a primary pigment required for photosynthesis (Brennan and Owende, 2010).

I) Classification and structure of microalgae

Classification of algae can be simply based on cell type that broadly classifies them into two large groups of, prokaryotes and eukaryotes. Prokaryotic algae, consists of those algal cells, that lack membrane enclosed, specialized organelles such as plastids, mitochondria, nuclei, golgi bodies, and flagella, example of prokaryotic algae is cyanobacteria. The remaining, algae forms the other class of eukaryotic algae and have membrane-bound specialized cell organelles.

A eukaryotic cell is protected by a tough cell wall that is composed of polysaccharides, which are partially produced and modified by the golgi bodies. The plasma membrane, found inner to the cell wall. It is a living structure responsible for controlling the influx and outflow of substances in the cell. Some algae may also possess the locomotory organs include the flagella, that helps in forward movement in the medium upon beating.

The nucleus contains the genetic material of the cell. It is well enclosed in the double layered membrane that is regularly interrupted with the pores. Nucleus itself encloses chromosomes, nucleolus suspended in matrix called as karyolymph. The chloroplasts have membrane sacs called thylakoids that carry out the light reactions of photosynthesis. Algae have double membrane mitochondria that within which lies 70S ribosomes and DNA in the mitochondrian matrix and contain the respiratory apparatus (Lee *et al.*, 2008).Microalgae were been classified according to their colour (pigment), (Alam *et al*., 2012) (Table 1).

Table 1: Classification of algae based on Colour (pigment) (Alam *et al.,* 2012)

Rhodophyceace	**Red algae**	**Pigments**
Phaeophyceace	Brown algae	Chlorophyll a, β-Carotene, xanthophyll, Phycoerythrin
Chrysophyceace	Golden algae	Chlorophyll a and c, fucoxanthin
Chlorophyceace	Green algae	Chlorophyll a and b, β- carotene, xanthophyll
Xanthophyceace	Yellow-green algae	Chlorophyll a and c, β-carotenoid, carotenoid diadinoxanthin
Cyanophyceace	Blue-green algae	Chlorophyll a, Phycocyanin, phycoerythrin, allophycocyanin

II) Mode of nutrition in microalgae

Microalgae nutrition have either autotrophic or heterotrophic mode of nutrition. Autotrophic algae, they utilize inorganic compounds as a source of carbon. Whereas, heterotrophic, microalgae use organic compounds for growth. Autotrophic algae include green and red algae. Autotrophs can be further divided into photoautotrophs and chemoautotrophs. Photoautotrophs are those that entraps light energy from the sun, and chemoautotrophic, that utilizes oxidizing inorganic compounds for energy. Similar to autotrophs, heterotrophscan also divided into either photoheterotroph that uses light energy as a source of energy, or chemoheterotrophs, oxidizing organic compounds for energy. However, some photosynthetic microalgae have mixotrophic mode of nutrition, and combines heterotrophy and autotrophy by photosynthesis (Lee *et al.,* 2008).

Autotrophic algae produce energy currency, ATP by converting solar radiation and fixing atmospheric by the process of photosynthesis. This energy produced is then utilized in the growth, reproduction and maintenance of algal cells (Brennan and Owende, 2010). The process of photosynthesis occurring in algal cells is similar to higher plants. However, algal cells have more efficient photosynthetic capability. The possible reasons for this are its aquatic habitat and simpler cellular structure in comparison to the higher plant (Chisti *et al*., 2007). Figure 1, illustrates the different mode of nutrition found in microalgae. Table 2, illustrates the findings on different modes of nutrition found in microalgae.

Figure 1: Different modes of nutrition found in microalgae

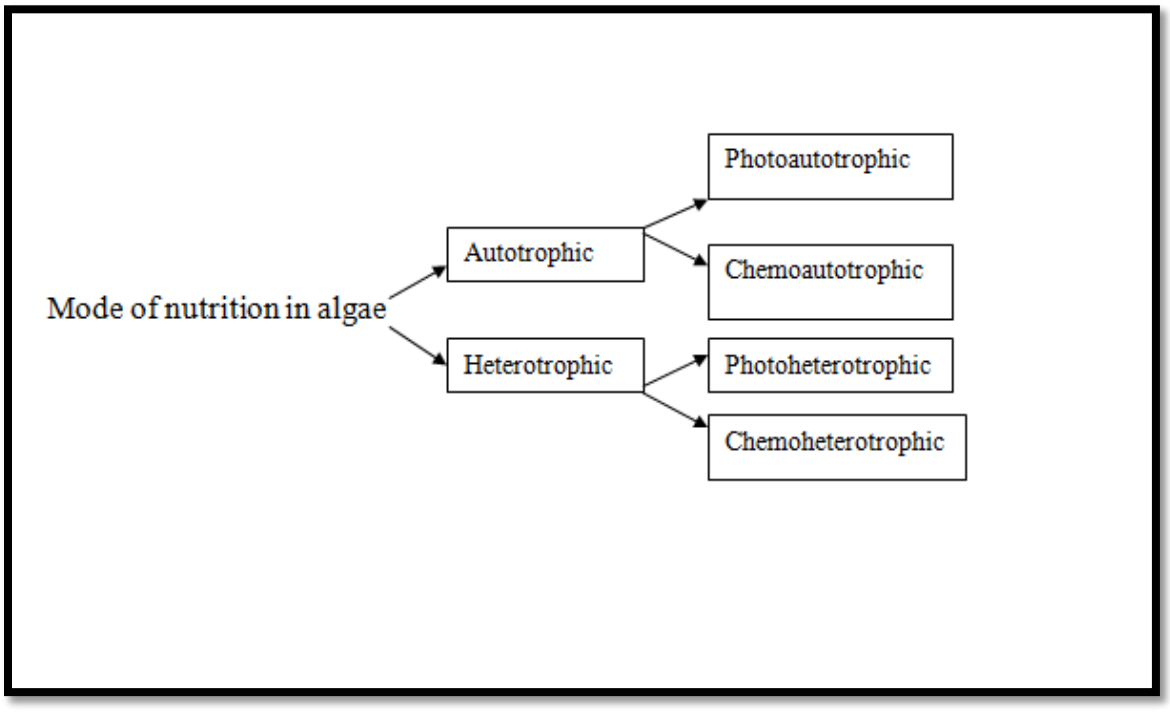


Table 2: Different nutritional modes in microalgae

Nutritional mode	Energy source	Carbon source	Advantage	Disadavantage
Photoautotrophic	Light	Inorganic carbon	Industrial CO_2 uptake; less contamination; cost efficient;	Lower cell density; light penetration limitation
Chemoautotrophic	Oxidizing inorganic compound	Inorganic carbon	Industrial CO_2 uptake; less contamination; high quality metabolites	Lower cell density; high operational costs; high substrate cost
Photoheterotrophic	Light	Organic matter	High biomass productivity; wastewater treatment	High operational costs; high substrate cost; contamination
Chemoheterotrophs	Oxidixing inorganic compound	Organic matter	High biomass productivity; wastewater treatment	High operational costs; high substrate cost; contamination
Mixotrophic	Light and oxidizing inorganic compound	Inorganic carbon	Independent growth parameters	Contamination; High operational costs

Cultivation of algae in laboratory conditions is carried out using an appropriate growth medium. A growth medium must be able to provide all the inorganic elements that are required to forms all the components the algal cell. Essential elements of a growth medium for microalgae include nitrogen (N) and phosphorus (P). Algal cell, minimal nutritional requirements was represented using molecular formula, $CO_{0.48}\ H_{1.83}\ N_{0.11}\ P_{0.01}$ (Chisti *et al*., 2007).

Nitrogen is an important component for algal nutrition and is mostly supplied in the form of nitrate, however, ammonia and urea are also brought into use. Out of all nitrogen sources, Urea is the most favorable, nitrogen source because, with equivalent nitrogen concentration, it produces higher yields and has advantage of low pH changes (Chisti *et al*., 2007).

Phosphorus is supplied in the form of complex with metal ion and thus it supplied in greater amount, since only some part of the nutrient added, is bioavailable to the algal cell for nutrition (Chisti *et al*., 2007). Other macronutrient includes magnesium ion, chlorideion, sulphate ion and these have important role in growth of algae. Important micronutrients include iron, manganese, zinc, cobalt, copper and molybdenum, they have pivotal role to play in algal growth and reproduction (Chisti *et al*., 2007).

III) Application of microalgae

Algae have been used by human for a long time. They are not only important source of several important biomolecules that have important cosmetic application and nutritional roles to play. There were several studies that included algae as photosynthetic gas exchangers for space travel (Borowitzka *et al*., 1999). Algae was also studied as source of many biologically active substances such as antibiotics (Pulz *et al*., 1998).

i) Human nutrition

There are several examples of microalgae species that are been produced commercially for human nutrition. They are sold in form on tablets, liquid and capsules. They are also used as one of the ingredient for ready to eat food items such as pastas, candies (Yamaguchi *et al*., 1997, Liang *et al*., 2004). They not only have nutritional benefit but also act as food colorants. This all would not have been possible if algae did not have diverse chemical properties (Borowitzka *et al.,* 1999). Microalgae that have extensively commercialized for human consumption includes four major strains: *Arthrospira, Chlorella, Dunaliella salina* and *Aphanizomenon flos-aquae* (Spoloare *et al*., 2006).

ii) Animal nutrition

Algae are not limited to human nutrition only. About one third of algal production is dedicated to the animal feeds. The algal biomass produced is utilized in aquaculture and is also included in the feed of farm animals and house pets (Becker *et al*., 2004). Algae form the natural food source for the animals. In aquaculture, algae are utilized as feed for the fishes such as salmons and molluscs that gives there meat a characteristic colour and adds a consumer appeal to the product. Algae are also utilized for larval nutrition. Feeding algae to the animal can be done directly in the form of food or indirectly as prey fed to small fishes. Examples of algal species utilized for animal nutrition are *Nannochloropsis, Isochrysis, Chlorella, Skeletonema* (Yamaguchi *et al.,* 1997; Apt *et al*., 1999; Mueller-Feuga *et al*., 2000).

iii) High value molecules

Algae have an advantage of possessing high value biomolecules in very high concentration, which can be, extracted in pure forms. Algae are the source of several important biomolecules such as pigments, fatty acids and source of stable isotope biochemicals that have important role in research and development (Spolaera *et al*., 2006).

a) Fatty acids

Animals and plants, lack suitable enzymes required to produce the poly-unsaturated fatty acids (PUFAs) of more than 18 carbon. Thus they have to obtain it from the diet (Certik *et al.,* 1999; Gill *et al.,* 1997). Possible sources of these fatty acids are fish and fish oil. But, many concerns associated with its safety such as, poor oxidative stability and presence of unpleasant fishy smell makes it an unfavorable choice (Certik *et al*., 1999; Luiten *et al.,* 2003). Fishes consumes microalgae as their part of diet and that acts

the source of PUFA, therefore it was found advantageous to consider microalgae as sources of PUFA (Spolaera *et al*., 2006).

b) Pigments

There are about 400 carotenoid obtained from algae. These include beta-carotene, astaxanthin and other carotenoids such as lutein, bixin, lycopene and zeaxanthin, which are of little importance (Vitchez *et al.*, 1997; Del *et al*., 2000). They are used as colorant in fruit juices and in other food and as an additive in animal feed (Del *et al*., 2000).

Astaxanthin has important application in salmon feed industry (Garcia-Gonzalez *et al*., 2005). Astaxanthin has ability to accumulate in tissue of the fish, giving it a characteristic pink color (Hejazi *et al*., 2004). All carotenoids has important intrinsic property to quench free oxygen species, due to which it acts as anti-oxidant, anti-inflammatory and has cancer preventive effect (Del *et al.*, 2000; Garcia-Gonzalez *et al.*, 2005).

Phycobilliproteins includes phycoerythrins and phycocyanins are important pigments, they mainly obtained from *Arthrospira* and *Porphyridium* (Viskeri *et al.*, 2003). Earlier they were used as natural dyes, it was later, and that several investigations confirmed its health promoting effects. It has range of pharmaceutical applications due to its health promoting effects (Becker *et al*., 2004).

c) Stable isotope biochemicals

Algae when provided with low cost inorganic molecules such as $^{13}CO_2$, $^{15}NO_3$, $^{2}H_2O$, can produce high value compounds by incorporating these stable isotopes such as ^{13}C, ^{15}N and ^{2}H, by the process of photosynthesis. High value compound includes biomolecules such as amino acids, carbohydrates, lipids, nucleic acids. These compounds have important roles to play in determination of structure of these biomolecules at atomic level and also been helpful in metabolic studies (Apt *et al*., 2004).

iv) Biofertilizer

Algae are used worldwide, as a biofertiliser, because of their ability to hold water and manage the mineral composition of the soil. They are used in the form of liquid fertilizer, in abandoned mining areas, where they act to protect soil from erosion and helpful in initiating the floral succession (Critchley and Ohno, 1998). Other benefits include release of polymers around plant roots that help in particle to adhere and increase water storage in soils. They also carry out nitrogen–fixation. Along with that they also release bioactive compounds that are helpful in growth of higher plants (Critchley and Ohno, 1998).

Important strains that are used as biofertiliser are *Anabaena* and *Nostoc*. They are used in sub tropical areas, where they fix atmospheric nitrogen, and are beneficial to the crops such as rice. Several studies suggest, the production of plant growth regulators from algae, that are helpful in seed germination, and growth of leaves, stem and induce flowering in the plant (Pulz *et al.*, 2004).

v) Antioxidants

Microalgae, have adapted themselves to the extreme habitats on earth. The adaptation is the result of evolution that took place over billions of years. Algae prominent mode of nutrition is autotrophy through photosynthesis. Photosynthesis results in the production of high oxygen and other radical stresses. To cope, this microalgae has developed numerous mechanisms that is able to prevent the free radical reaction in the algae (Pulz *et al.,* 2004).

vi) Algae in environmental protection

Algae are traditionally utilized in treatment of wastewater. This is due to its ability to obtain nitrogen and phosphorus from the wastewater. This characteristic of algae is important in the treatment of organic waste in wastewater. Not only this, it has other advantage that the wastewater can be used to replace the cultivation medium entirely. The biomass produced can be utilized as the feedstock, for extraction of compound of interest. This can also be utilized in treatment of organic waste from agro-food industries (Cantrell *et al*., 2008).

Other advantage is that natural sunlight can be utilized for the commercial production. However, it has limitation of the availability of sunlight, due to diurnal cycles of algae and seasonal variation (Brennan and Owende, 2010). It is known that algae can fix carbon dioxide from three different sources i.e., carbon dioxide in the atmosphere, in the gases released from the industries and carbon dioxide provided in the form of soluble salts (Wang *et al., 20*08). Atmospheric carbon dioxide concentration is estimated to be around 360 ppm. Microalgae, can typically tolerate up to 150,000 ppm of carbon dioxide (Bilanovic *et al*., 2009; Chius *et al*., 2009). Therefore, it is advantageous to treat the gases released by the industries such as power plants to be directly fed into the algae cultivation medium. And carbon dioxide can be directly fed into the medium in the form carbonates such as sodium carbonate and sodium bicarbonate (Emma *et al*., 2000).

IV) *Haematococcus pluvialis*

Microalgae are known to be a very good source of several carotenoids, chlorophyll, phenolics, phycocyanins, phycobiliproteins, phycoerythrin and many other health beneficiary compounds (Shah *et al*., 2016; Ranga Rao *et al*., 2010). One of such commercially important substances is astaxanthin. *H. pluvialis* is the richest source of astaxanthin (Shah *et al*., 2016).

Astaxanthin (3,3'-dihydroxy-β-carotene-4,4'-dione), a secondary keto-carotenoid also produced by chemical synthesis (Witting reaction), has safety concern relating to its presence of synthetic intermediates (Krause *et al*.,1997). Thus, natural astaxanthin is preferred over synthetic for high end markets (Li *et al*., 2011). *Haematococcus pluvialis*, a green alga which typically accumulates astaxanthin up to 2-3% of its dry weight (Guerin *et al*., 2003). *H. pluvialis* is considered to be the best source for the commercial production of astaxanthin (Ranga Rao *et al*., 2010).

Astaxanthin obtained from *H. pluvialis*, is approved as one of the ingredients in several health supplements and as a color additive in salmon feeds in U.S.A, Japan and many countries of Europe (Yuan *et al*., 2011). There is ever increasing demand for the naturally produced astaxanthin, thus it requires to highly efficient production (Shah *et al*., 2016). It is important to understand the biology of *Haematococcus pluvialis* lifecycle.

i) Biology of *Haematococcus pluvialis*

Haematococcus pluvialis is a unicellular freshwater microalgae, distributed throughout the world (Ranga Rao *et al.,* 2010). It is biflagellate green microalgae, belongs to class Chlorophyceae, order Volvacoles and family Haematococcaceae (Bold and Wynne, 1985). They are also named *H. lacustris* and *Sphaerella lacustris*. *H. pluvialis* was discovered in 1842 by J. Von Flotow and later in 1844, Tracy Elliot Hazen gave insights into the biology and lifecycle of *H. pluvialis* (Shah *et al.,* 2016).

Its habitat includes temporary stagnant water bodies such as rain pools, natural or man-made pools and bird bath (Czygan *et al.,* 1970). They have been found in the diverse environment and climatic conditions (Chikanov *et al*., 2004). Its ability to encyst, when it encounters unfavorable conditions have helped the microalgae to survive in extreme habitat such as extreme light, temperature and high salt concentrations, where other organisms fail to thrive (Shah *et al.,* 2016).

ii) Life cycle

H. pluvialis life cycle consists of four easily identifiable stages: microzooid, macrozooid, palmella and last stage called aplanospore or haematocyst stage (Hazen *et al*., 1899). Microzooid, macrozooid and palmella stage is referred as a green vegetative stage, whereas aplanospore stage is defined as the phase of encystment, along with the accumulation of astaxanthin (Shah *et al., 20*16). *H. pluvialis* reproduction rarely occurs by the sexual reproduction,it is dominated by the vegetative reproduction (Triki *et al*., 1997).

The microzooid are small of size around 10 µm, compared to zoospores that is of size in range of 20 to 50 µm. Along with that macrozooid, exhibit high-speed motility after the release from gametophyte (Shah *et al.,* 2016). Macrozooid stage of the lifecycle can be identified with its shape that can be either spherical, ellipsoidal or pear-shaped, with two flagella, of equal length attached, anteriorally to the cell. Microscopy shows, cup-shaped chloroplast, with numerously scattered pyrenoids. Macrozooid cells can give rise to 2-32 daughter cells and show fastidious growth (Wayama *et al.,* 2013).

Macrozooid stage is predominant only during favourable conditions. As unfavourable conditions prevails, cells looses flagella and develop multilayered amorphous structure inner to the cellular matrix, this stage is called as palmella stage and is characterized as non motile resting but vegetative cells (Hagen *et al.,* 2002). If, favourable conditions such as nutrient deprivation, high salinity and high light intensity stress continous, cells reversibly enters the aplanospore stage. Aplanospore is characterised by the development of trilaminar structure and secondary cell wall. This results in the highly resistant aplanospore cell (Hagen *et al.,* 2002).

Another feature of aplanospore, is its distinctive bright red appearance due to high accumulation of astaxanthin, a secondary keto carotenoid. Astaxanthin occurs in the cytoplasm of the cell, in form lipid droplets, esterified with the fatty acids (Hagen *et al.,* 2002). These mature aplanospore when provided optimal conditions of growth, these cells are capable of reattaining the vegetative form of reproduction to produce new daughter cells (Shah *et al.,* 2016).

iii) Cultivation of *Haematococcus pluvialis*

Both chemical and physical parameters such light, temperature, nutrient medium are important factors that control the growth rate of *H. pluvialis* (Fabregas *et al*., 2000; Fan *et al*., 1994). Thus for developing an optimal procedure for carrying out, growth of microalga and harvesting the products two major aspects are always considered. These include the factors such as temperature, light intensity, pH, aeration and agitation. The second aspect is a selection of appropriate medium (Sarra *et al.,* 1993; Gong *et al*., 1997).

It is important to note the importance of appropriate media selection as, cultivation medium not only affects the growth and productivity but also affects the cell composition and yield of the product itself (Gong *et al.,* 1997; Imamglou *et al.,* 2007). The green algae, *H. pluvialis* can grow autotrophically as well as, heterotrophically, in the presence of acetate as carbon source, in the dark conditions (Lee *et al*.,1991). Today *H. pluvialis* is considered to richest source of astaxanthin but the main problem that is faced is the relatively slow growth rate of *H. pluvialis*. Due to this reason, *H. pluvialis* is susceptible to the contamination by the fast-growing blue-green algae (Orasa *et al.,* 2000).

a) Light

H. pluvialis being phototrophic algae, is very much affected by the light and its intensity. However, its important to know that light conditions provided should be within the optimal range. As excess light can cause photo-inhibition and low light intensity can cause decrease in light penetrance within culture. Light penetrance is a factor that depends upon the cell concentration (Boussiba *et al.,* 2000).

Light intensity requirement of culture is dependent upon the nutritional status (Droop *et al*., 1954). As the cell concentration increases the light requirements of the culture also increases. The range of light intensity is adjusted between 30-150 µmol Photon $m^2s^{-1,}$ depending upon the volume of culture for *H.pluvialis* (Imamglou *et al.,* 2007).

b) Culture medium

As already discussed appropriate selection of the medium is important and it depends upon the growth requirement of the algae of interest. Constituents of the Culture medium also affects the quality of the final product and direct effect on the market cost of the (Borowitzka *et al.,* 2005). Nitrogen source play very important role and various studies have been done, regarding different nitrogen source and their strength. Goksan *et al* (2011) tested different nitrogen sources (NS), it included, $NaNO_3$, KNO_3, NH_4NO_3, and urea. The BG11 medium was used as the basis for all the groups, and $NaNO_3$ was replaced with the other NS. With ammonium, it was required to modify the pH as it results in the

release of hydrogen ion and causes the pH of the medium to become acidic. Whereas, growth on, the nitrate medium, causes the pH to increase (Mengel *et al.,* 1983).

Borowitzka *et al* (1991) reported, using KNO_3 within the range of 0.5-1.0 g/L, to achieve maximum cell density. Similarly, Gong and Chen (1997) and Fabregas *et al* (2000) found, 0.37 and 0.41g/L as optimal range of KNO_3. For urea, it was found that lowest concentration of 0.1 g/L produced best result as, higher concentrations inhibited the growth. It was also concluded by Goksan *et al* (2000), that urea is suitable for *H. pluvialis* nutrition. There are many media optimized for algae the contains vitamin as a growth stimulator. Examples of such media is Basal culture medium, OHM (optimal Haematococcal media). Basal and OHM contain vitamin such as thiamine, cyanocobalamin, biotin. Presence of vitamins results in higher cost of this media (Imamglou *et al., 200*7). Among vitamins, thiamine as a growth factor for microalgae was found to be more effective than cyanocobalamine, whose effect was there but was not essential (Imamglou *et al.,* 2007). Goksan *et al* (2011), found that cell counts of cultures supplemented with vitamins were higher. The inclusion of vitamins induces the cell to divide and also causes the mean cell diameter to increase. Imamglou *et al* (1991) showed that vitamins are efficient in lower concentration and it is not essential to use all vitamin altogether. Thiamine when used alone was sufficient to achieve an increase in the growth of about 15%.

c) Aeration

It was seen that aeration caused the amount of chlorophyll and other carotenoids to increase with increase in the flow rate. It was however observed that aeration, also lead to the formation the turbulence that causes the cell damage. But there was an increase in the amount of chlorophyll and other carotenoids with a high flow rate. Another important effect of aeration was that, aeration above optimal range cause the cessation of growth and also increased the cell diameter. Thus aeration can be utilised as one of factor to induce astaxanthin synthesis in the cell (Chen *et al.,* 1997; Garcia-Malea *et al*., 2006).

iv) The composition of *H.pluvialis* cell

It is known that *Hematococcus pluvialis* has a unique lifecycle, consists of a motile green stage and non-motile red stage (Shah *et al.,* 2016; Boussiba *et al.,* 2000).

a) Protein - Green stage, i.e, in the favorable and earlier stage of the lifecycle of *H. pluvialis* is found to be rich in protein content (Shah *et al.,* 2016). It was noted that 21% of the cell content was protein, during red stage (Kim *et al.,* 2015). Lorenz *et al* (1999), observed slightly higher percentage, that is, 23% of its cellular content during red stage. Amino acids such as glutamic acid, aspartic acid, leucine and alanine forms major part of upto 46% of total amino acid content (Shah *et al.,* 2016).

b) Carbohydrates - During, the green, vegetative stage, carbohydrates form 15-17% of its cellular content, that increases to nearly 50% during red stage (Shah *et al.,* 2016). During unfavorable conditions, the cell tends to accumulate starch and thus carbohydrate content of the cell increases, that consumed during prolonged stress conditions (Shah *et al.,* 2016).

c) Lipid - The green stage has lipid content that fairly varies between 20-25% of cell content. 10% of which are predominantly short-chain (C16, C18) poly-unsaturated fatty acids that are deposited in the chloroplast (Shah *et al.,* 2016). Under prevalent stress conditions, biochemistry of the cell is directed towards the synthesis of triacylglycerols (TAGs) and other neutral lipids, that can accumulate upto 40% of its total cellular weight and exist as cytoplasmic lipid droplets, that holds significant amounts of carotenoid content (Boussiba *et al*.,1992; Saha *et al.,* 2013). It was interesting to note that when cell transits from green to red stage, it tends to accumulate glycolipids, such that it nearly doubles. Whereas, phospholipid content during green and red stage remains constant. Linolenic acid (18:3), linoleic acid (18:2) and palmitic acid (16:0) are most abundant. The higher lipid content of *H. pluvialis*, produced during nutrient straved conditions, it as an attractive option for bio-diesel production (Damiani *et al*., 2013).

V) Astaxanthin

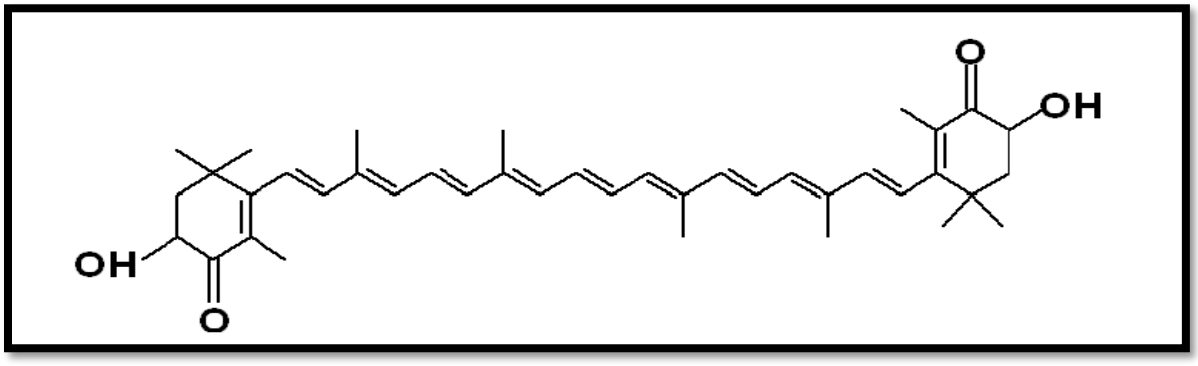
3, 3'-dihydroxy-β, β'-carotene-4, 4-dione, commonly called as astaxanthin, is a keto-carotenoid oxidized from *β*-carotene. It is found in many fishes such as salmons, crustaceans and in plants, where it is synthesized from lycopene. It has very important advantage in aquaculture due to its pigmentation effect that adds the consumer appeal to the product (Higuera-Ciapara *et al*., 2006).

Not only its attractive pink colour, it also has biological activity that it acts as a precursor for vitamin A. Due to its attractive pink colour, biological activity, such as it acts as a vitamin A precursor, and have antioxidant activity, which is higher than that found in β-carotene and vitamin E. Thus, astaxanthin has application as a food colorant in food industry and in medicine (Johnson and An, 1991).

Astaxanthin exists as major carotenoid in the algae *Hematococcus pluvialis* and exists in the form of esters of fatty acids (Johnson and An, 1991). Figure 2, shows the planar structure of astaxanthin. Astaxanthin has two chiral centre or two asymmetric carbons at C-3 and C-6, in its structure. Due to which it has three different stereo-isomer: (3*S*,3'*S*)-astaxanthin, (3*R*,3'*R*)-astaxanthin, and (3*S*,3'*R*)-astaxanthin, all with trans configuration (Vecchi and Muller, 1979).

Molecular formula of astaxanthin is $C_{40}H_{52}O_4$ (Johnson and An, 1991). *H. pluvialis* possesses 3*S*, 3′*S* stereoisomer mostly. This isomer is most valuable amongst all other isomer (Yang *et al*., 2013; Al-Bulishi *et al*., 2015). If we compare astaxanthin with all other anti-oxidant available, astaxanthin is 54 times stronger than β-carotene, 10 times stronger than canthaxanthin, zeaxanthin and lutein, 100 times stronger than α-tocopherol and 65 times more potent than vitamin C (Miki *et al*., 1991; Borowitzka *et al.,* 2013; Koller *et al*., 2014; Pérez-López *et al*., 2014). Current status of market shows that synthetically derived astaxanthin dominates naturally derived astaxanthin. Over 95 % of astaxanthin available in market is synthetically derived and only less than 1% of astaxanthin available is derived from *H. pluvialis* (Koller *et al*., 2014).

Figure 2: Planar structure of astaxanthin



Synthetic astaxanthin is derived from Witting reaction using asta-C_{15}–triaryl phosphonium salt and the C_{10}-dialdehyde as precursors (Krause *et al.,* 1997). However it was observed that synthetically derived astaxanthin has lower activity, when compared to its natural counterpart and till now has not been approved for human use. There are several safety issues associated with the usage of synthetic astaxanthin (Lorenz and Cysewski, 2000; Koller *et al*., 2014). Synthetically derived astaxanthin, has risk of unwanted presence of reaction intermediates and they have different stereochemistry. It is because of these reasons that natural astaxanthin is preferred over the synthetic one (Li *et al*., 2011).

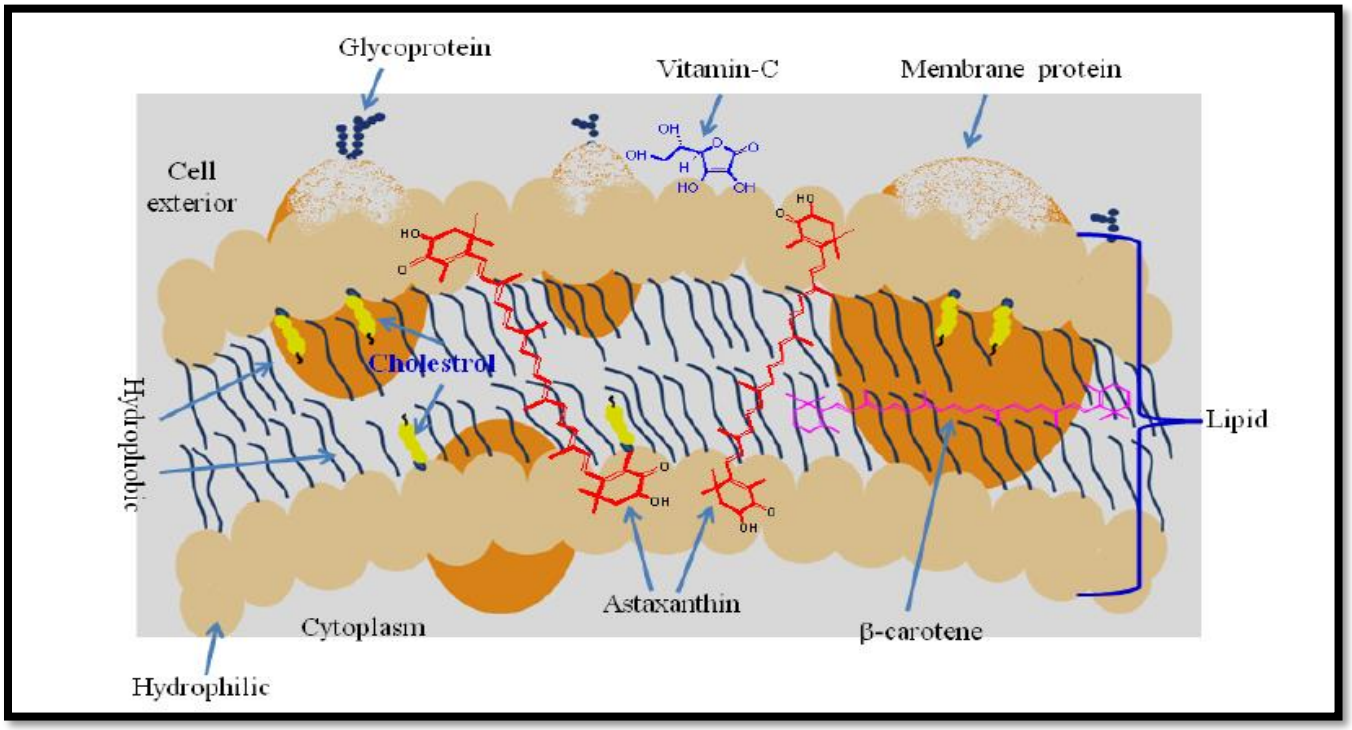
a) Biochemistry of astaxanthin

Apart from the presence of two chiral centres, astaxanthin also contains hydroxyl, keto groups and conjugated double bonds. Due to the presence of these both groups, this carotenoid has both the lipophilicity and hydrophilicity (Higuera-Ciapara *et al.,* 2006). The distinctive red color of this pigment is due to the presence of several conjugated double bond. Double bond is not only responsible for red color; it is also responsible for the anti- oxidant activity of the molecule. This double bond breaks and releases the electrons that neutralize the nearby free radicals. Thus, it terminates the free radical chain reaction (Guerin *et al.,* 2003). Number of studies claims the superior activity of astaxanthin than other antioxidants, mostly because it could easily bind with cell membrane from inside and outside (Rao *et al.,* 2014). Figure 3, shows the superior position of astaxanthin the cell membrane.

b) Extraction of astaxanthin

Countries such as India, USA and China are largest producer of biomass of *Haematococcus.* Current value of astaxanthin, is estimated to be approximately to be $ 7150 per kg. 300 tons of *Haematococcus* biomass is produced annually (Brennan and Owende, 2010). *Haematococcus* sp. has high amounts of astaxanthin, which is reported to have number of pharmaceutical application (Wayama *et al*., 2013). Extraction of astaxanthin is high energy consuming and cost-intensive downstream processing (Gunerken *et al*., 2015; Lee *et al*., 2015). A number of methods utilizing solvents, edible oils and microwave assisted methods are utilized to obtain astaxanthin from biomass.

Figure 3: Position of Astaxanthin in cell membrane (Ranga Rao *et al.*, 2014)



Steps such as cell wall disruption, for products that are intercellular in nature are major technical and economical challenges for the industry. Strong multilayered structure of *H.pluvialis* acts as barrier, between the solvent and astaxanthin, situated in the extra-plastidial bodies (Ranga Rao *et al.*, 2014).

Thus, sporopollelin wall, present during the red stage acts as strong barrier and also reduces its bio-availability in fishes such as salmons. Thus there is a need to remove cell wall, prior to the consumption as feed (Good & Chapman, 1979; Sommer *et al.*, 1991). There are various techniques carried out that causes cell-wall disruption. Table 3, shows various methods used to extract astaxanthin from *H. pluvialis.* These include cell homogenization, cells subjected ultra-sonication and microwave, treatment with solvent and with acid or base, Fenton chemicals, use of hydrolyzing enzyme and supercritical CO_2 treatment (Kim *et al*., 2014; Martinez-Guerra *et al*., 2014; Park *et al*., 2015, 2014). Selection of appropriate method for cell disruption, followed by extraction is dependent on the strain, as well as its stage of lifecycle (Praveenkumar *et al*., 2015). The efficacy of each method can be affected by physical parameters such as temperature, pressure, condition of biomass and as well as quantity of biomass (Rawat *et al*., 2011).

Table 3: Methods used for extraction of astaxanthin from *H. pluvialis*

Method	**Result**	**Reference**
Chemical treatment using 5% KOH in 30% methanol, followed by treatment with DMSO	Highest yield of astaxanthin obtained was 58.7 pg/cell, after treatment of cells under phosphate starved conditions	Boussiba and Vonshak, 1992
Chemical treatment using methanol/acetone with heat followed by treatment with enzyme actinase E, cellulose, kitalase	Heat treated acetone produced improved the extractability of the cell upto 60 %. Enzyme treatment with Kitalase produced positive results.	Kobayashi *et al.*, 1997
Biomass autoclaved, treated with 0.1 M HCl, 0.1 M NaOH and enzymatic treatment of proteinase K and Mechanical disruption using cell homogenizer and spray drying. Followed by re-suspension of cells in Diethyl ether: ethanol (1:1) and treatment with n-hexane.	Highest Percentage of Astaxanthin was achieved using Spray drying method. It produced 30 mg/g of biomass	Mendes-Pinto *et al*., 2001
Cells treated with dodecance. Dodecane extract treated with methanol and NaOH.	95% yield of astaxanthin was obtained.	Kang *et al*., 2007
Cells treated with commercial vegetable oil (soya bean oil, olive oil) and stirred.	A recovery yield of 87.5% astaxanthin was, obtained over the period of 48 hours.	Kang *et al*., 2008
Cells treated with pressure produced by the liquid CO_2, Followed by treatment with ethanol.	83.8% of recovery yield was obtained from combination of different conditions	Pan *et al.*, 2012
Ultra-sonic treatment to cells ,followed by the treatment with ethanol and ethyl acetate used in 50%-70% concentration	27.58 mg/g of recovery yield was obtained with48 % ethanol in ethyl acetate	Zou *et al.*, 2013
Four different methods : Cells were treated with HCl and Acetone, binary system of hexane and isopropanol, methanol and acetone and soyabean oil extraction	Highest astaxanthin yield of 19.8 mg/g was obtained using HCl and acetone treatment ,	Dong *et al.*, 2014

c) Biological Activities of astaxanthin

i) Antioxidant effects

Aerobic organism produces several free radicals that are result of aerobic respiration. While, the free radicals generated has important role to play. Sometime excess of these reactive free radicals causes damage to the systemic biological macromolecules such DNA, lipids and protein. This could have devastating effects. This whole process of radical chain reaction occurs constantly in body and is known as oxidative stress (Di Mascio *et al., 19*91).

Prolong oxidative stress can lead to retinopathy, arteriosclerosis, Alzheimer disease and various other ailments. While most organisms possess a repertoire of internal systems, to cope such free radicals, these include catalase, superoxide dismutase and peroxide enzyme system (Maher *et al*., 2000). Carotenoids, such as astaxanthin quench oxygen species and cease the free radical chain reaction. It was observed by Ranga Rao *et al* (2010), that feeding of rats with *Hematococcus* biomass, caused increased activity of catalase, superoxide dismutase, peroxidase and thio-barbituric acid reactive substances.

ii) Anti-lipid peroxidation activity

Due to unique molecular structure of astaxanthin, it is enable to bind to inner and outer portions of the cell wall. Because of the better positioning of astaxanthin within lipid bilayer, it safeguards, by inhibiting the lipid peroxidation (Ranga Rao *et al.,* 2013). Kamath *et al* (2008) showed than rats induced with gastric ulcers and skin cancer using ethanol, showed 80% decrease in lipid peroxidation. Kang *et al* (2001) also reported that astaxanthin activity against lipid peroxidation induced by Tetra-chloromethane by inhibition of lipid peroxidation and help stimulate the cell's own anti-oxidant systems.

iii) Anti-inflammation activity

Astaxanthin is found to be effective against inflammation. Several studies have supported the anti-inflammatory activity of astaxanthin. Extracts of *Haematococcus* and *chlorococcum*, was found to be highly effective in reducing the bacterial load and reducing the gastric inflammation in mice infected with *Helicobacter pylori* (Ranga rao*et al.,* 2013). Haines *et al* (2011) reported, lowering of the broncho-alveolar inflammation affected cells and increased the cAMP and cGMP levels in lungs, on feeding the subjects astaxanthin along with *Ginko biloba* extract and ascorbic acid.

iv) Anti-diabetic activity

Patients of Diabetes mellitus are observed to have high oxidative stress. High oxidative stress can result in condition called as hyperglycemia, which could be result of dysfunctional pancreatic cells and tissue damage. Astaxanthin has the ability to reduce the oxidative stress in such patients and can directly have healthier impact in treatment of diabetes mellitus. It can also be helpful in balancing the blood sugar and insulin levels (Uchiyama *et al*., 2002).

v) Cardiovascular disease prevention

Astaxanthin has most potent antioxidant activity along with anti-inflammatory activity and it has been studied in animal model as well as in the human subjects. Oxidative stress and inflammation are patho-physiological features of atherosclerotic cardiovascular disease, which is a combination of both oxidative stress and inflammation. Astaxanthin is found to be an effective therapeutic agent for the treatment of atherosclerotic cardiovascular disease (Fassett *et al.,* 2011).

Khan *et al* (2010), during his study found astaxanthin in the blood plasma and platelets, heart, liver, and also had effect in increasing basal arterial blood flow when mice was fed with astaxanthin derivative. Nakao *et al* (2010) found mice had increased heart mitochondrial membrane potential and greater contractility index compared to the control group, when fed with 0.08 % astaxanthin

vi) Anti-cancer activities

It is already discussed that several reactive species are generated as a result of aerobic respiration. These reactive species include hydrogen peroxide, superoxide and hydroxyl radical. Photochemical pathway of metabolism also generates the singlet oxygen. It is seen that these reactive species contribute to oxidation of the lipids and other biomolecules that, may contribute to aging, several degenerative disease like cancer (Ryu *et al.,* 2012).

These anti-oxidant compounds reduce the carcinogenesis by decreasing mutagenesis events. They inhibit the oxidation based damage of cells and tissues. It is observed that cancerous cells have low cell-cell interactions through gap junctions and its replenishment is an important step to reduce the proliferation of malignant cells (Ranga Rao *et al.,* 2014). Protein product of Connexin 43 gene was responsible for the gap junction based interaction between the cells. It was seen that consumption of astaxanthin and canthaxanthin enhanced the expression of connexin 43 gene in the cells (Wolf *et al*., 1996).

vii) Other beneficial effects

Ultraviolet radiation possesses significant threat to the health. Ultraviolet radiations trigger the chain reaction that results in the generation of the free radicals. These free radicals are responsible for the oxidation of the biomolecules such as lipids, DNA and protein. This may result in ailments such as skin cancer in future.

Astaxanthin, along with being a powerful anti- oxidant, is also an important immune system modulator. It prevents oxidation and delays sunburn. O'Connor and O'Brien, 1998 and Lyons and O'Brien, 2002 showed the astaxanthin activity against DNA damage by UV radiation in cultured rat kidney fibroblast and human skin cell respectively.

There are various astaxanthin supplements available as inject-able solution, topical creams and capsules, which have activity against the sunburn (Lorenz *et al*., 2002). Other health effects is in treatment of

rheumatoid arthritis (Lignell and Bottiger, 2001), anti-cataract activity (Guyen *et al.*, 1998), and in carpal tunnel syndrome (Lignell and Bottiger, 2001)

VI) Carbon dioxide fixation

There is ever-increasing anxiety about the issue of global warming, which is associated with the increased carbon dioxide concentration in the atmosphere (Roman-Leshkov *et al*., 2007). Microalgae are fast growing carbon dioxide fixators. They can fix carbon dioxide directly from air or from exhaust gases. Coupling of carbon dioxide fixation, along with the algal growth has an advantage of production of high value compounds, is a favourable strategy for carbon dioxide mitigation.

i) Strategies for carbon dioxide fixation

Diverse strategies have been explored, which can be broadly classified into two groups (Wang *et al*., 2008):

a) Chemical based techniques
b) Biological carbon dioxide mitigation

A commonly used chemical based technique includes cyclic carbonation and decarbonation of gaseous carbon dioxide. Other methods include partitioning carbon dioxide from the rest of the gaseous mixture and treating with the aqueous amine solution (Remnik *et al*., 2004). A typical chemical based process involves three main steps of separation, transportation and sequestration.

Separation along with compression using pressure of 110 bars cost 30-50 US dollars per ton carbon dioxide. Transportation cost is around, 1-3 US dollars for every 100 kilometers per ton of carbon dioxide. Sequestration cost ranges between 1-3 US dollars per tons of carbon dioxide. Since the chemical based Carbon dioxide capturing is cost intensive and energy consuming, the carbon dioxide mitigation benefits from chemical process is insignificant.

Biological mitigation is been studied promptly, as a substitute strategy. As it has an advantage of biomass output formation, by the means of photosynthesis (Ragauskas *et al*., 2006). Biological carbon dioxide mitigation can be carried out by both the terrestrial plants and many forms of photosynthetic microorganisms. But, the capacity of the terrestrial plants is low and contributes only 3-6 % of carbon dioxide fixation, released as result of fossil fuel combustion. The possible reason for such low contribution is due to low growth rate of terrestrial plants (Skjanes *et al*., 2007).

Whereas, a group of microalgae are not only fast growing but are 10-50 times more efficient in capturing carbon dioxide (Li *et al*., 2008). Other very important advantage of microalgal based mitigation is that it completely recycles the carbon dioxide. The fixed carbon dioxide is utilized as chemical energy in the process of photosynthesis. That can be used as source of biofuels and other valuable compounds, by the use of current technologies. In case of chemical based technologies, they

have a demerit associated with the disposal, as both the adsorbent and as well as the captured carbon dioxide need to be disposed off safely (Yeh *et al*., 2001).

ii) Carbon dioxide sources for microalgae

Microalgae can be used to capture carbon dioxide from three varying sources, which can be put into three groups

a) Atmospheric carbon dioxide

Concentration of carbon dioxide in the atmosphere exists between 0.03-0.06%, such low concentration of carbon dioxide, severely affects the mass transfer rates, by slowing down the exchange of carbon dioxide from air to the algae in the pond (Chelf *et al*., 1993).

b) Carbon dioxide generated from the industries as exhaust gases

Industrial exhaust gas contains up to 15% of carbon dioxide. Exhaust gases such as flue gases can be utilized as rich source of carbon dioxide for the micro algal growth (Wang *et al*., 2008).

c) In the form of carbonate salts

The third route of bio-fixation of carbon dioxide is via use of soluble carbonate salts such as Sodium carbonate and Sodium bicarbonate. This route involves first step of fixing carbon dioxide in the form salt, later followed by a second step is exploitation of these salts as source of carbon for the growth and development by the algae.

Various micro algal species are capable of exploiting the carbonates as source of carbon (Merett *et al*., 1996). Some microalgae show extracellular carboanhydrase activity on high rate, which is important for the generation of the free carbon dioxide, which is later assimilated. Many microalgae possess active transport system for bicarbonate uptake (Wang *et al*., 2008). Matsumoto *et al* (1997), in his published report, reviewed upon the high levels of carbon dioxide tolerance in micro algal species. Along with this, they also have tolerance to the moderate levels of the oxides of nitrogen and sulfur present in the exhaust gases. Other studies done in microalgae species for bio-mitigation of carbon dioxide is given in table 4.

VII) Astaxanthin accumulation in *H. pluvialis*

The freshwater microalgae, *H. pluvialis* is recognized as a top microbial source of the pigment astaxanthin, which is collected in high quantities in these cells, during its passage from green vegetative to the red aplanospore cell (Cordero *et al*., 1996). The requirement for production of astaxanthin from *H. pluvialis* is appreciably different from those of conditions for the growth (Harker *et al*., 1995).

Table 4: Studies on microalgal species for bio-mitigation of carbon dioxide

Micro-algae	**Carbon dioxide (%)**	**Temperature (°C)**	**Biomass productivity (g/L/Day)**	**Reference**
Dunaliella	3	27	0.17	Kishimoto *et al*., 1994
Chlorella sp.	40	42	N/A	Sakai *et al*., 1995
Botryococcus braunii	-	25-30	1.1	Murakami & Ikanuchi, 1997
Chlorococcum littorale	40	30	N/A	Iwasaki *et al*.,1998
Chlorella vulgaris	air	25	0.040	Scargg *et al*., 2002
Scenedesmus obliquus	air	Not specified/Not controlled	0.009	Gomez-Villa *et al*., 2005
Scenedesmus obliquus	air	Not specified/Not controlled	0.016	Gomez-Villa *et al*., 2005
Scenedesmus obliquus	18	30	0.14	De Morais & Costa,2007
Spirulina	12	30	0.22	De Morais & Costa, 2007
Hematococcus pluvialis	16-34	20	0.076	Huntley & Redalje, 2007

To acquire high productivity of pigment from high quantities of biomass, a two-stage system is believed to be more effective (Choi *et al*., 2002). The commercial production system utilizes two-step process. Where, the first step is dedicated to the production of large quantities of green cells and the second step, committed to induce the cells for the higher accumulation of keto-carotenoid, astaxanthin (Lorenz *et al*., 2002). It is noted that the transition of the green cell to red cell of *H. pluvialis* is encouraged under the growth-limiting situation of culture. The biochemical and morphological changes, in the green cells, converts them to red aplanospore cell (Droop *et al*., 1955). In order, to improve accumulation, of pigments in algae, several chemicals, as well as metabolic set-offs or enhancers are used, that directly regulate the cellular metabolism of the cell (Yu *et al*., 2015).

Contrary, to the genetic modification, this strategy is dependent upon phenotypic screening and do not require knowledge about the specific molecular target in metabolic pathways (Yu *et al*., 2015). It is proposed that nitrogen deficiency, high light intensity cause enormous accumulation of astaxanthin.

Other assumption, associate astaxanthin production, with the parameters that hinder the cell division without affecting the capability of the microalgae cells to assimilate carbon (Yu *et al*., 2002).

Crucial advancement in the productivity of several microbial fermentation methods generally involves the development of high yielding strain through the process of genetic manipulation (Franz *et al*., 2013). But there are several other factors that modify the growth and product yield considerably. These include the factors such as nutrient availability and physical environment, to which, the microalgae of interest is exposed (Yu *et al*., 2002). Table 5, shows various chemicals and conditions used for astaxanthin accumulation in *H. pluvialis*.

It is acknowledged that photochemical and non-photochemical stress produces reactive oxygen species (ROS). Examples of ROS, produced are superoxide ion radicals, hydrogen peroxide, hydroxide radicals and singlet oxygen species (Mittler *et al*., 2002). The generation of ROS can cause damage to cellular machinery. This process is called as oxidative stress (Apostol *et al*., 1989). To overcome, the oxidative stress produced, cells have, various mechanism that involves enzyme such as catalase, Superoxide dismutase, glutathione and peroxidase and several antioxidant such as tocopherol, ascorbic acid, and carotenoids that neutralise, ROS (Moulin *et al*., 2010).

H. pluvialis accumulating astaxanthin is a strategy to survive these conditions (Boussiba and Vonshank, 2002). Kobayashi *et al* (1997), observed higher tolerance to ROS in aplanospore than in the vegetative stage cell. Along with the its role as protectant against ROS, it also has a preventative effect against strong irradiance. Some authors also suggest, that astaxanthin has no preventative role against strong irradiance but rather, astaxanthin is the product of protecting agents against high irradiance. These agents are unknown (Lemoine *et al*., 2010).

A class of chemicals called phytohormones is found to effective, in manipulating the growth and development in microalgae. Table 6 shows the effects of phytohormones in higher plants and algae.

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Table 5: Chemicals and conditions used for enhancing astaxanthin accumulation in *H. pluvialis*

Chemical/Condition used	**Concentration**	**Reference**
Ferrous Sulphate	450µM	Kobayashi *et al*.,1991
Light intensity	170 µmol/m^2 /sec	Boussiba & Vonshak, 1992
Low nitrogen with high light intensity	Nitrogen concentration of 0.15g/Litre with 170 µmol/m^2 /sec	Boussiba & Vonshak, 1992
Phosphate starvation	Cells were suspended in phosphate deprived medium	Boussiba & Vonshak, 1992
Low phosphate ,high nitrogen and high light intensity	0 g/Litre \ -- \170 µmol/m^2 /sec	Boussiba & Vonshak, 1992
Salt stress	0.8% Sodium chloride	Boussiba & Vonshak, 1992
Sulfate starvation	Cells suspended in medium containing $MgCl_2$ instead of $MgSO_4$ (0.06 g/ Liter)	Boussiba & Vonshak, 1992
Cell division inhibitor - Vinblastine	--	Boussiba & Vonshak, 1992
Sodium acetate	--	Kobayashi *et al*., 1992
Ferrous Sulphate	22.5mM (pH 1.4)	Kobayashi *et al*.,1993
Sodium acetate	2.25M (pH 7.0)	Kobayashi *et al*., 1993
Salt stress	10-90 mM	Harker *et al*., 1995
Ferrous sulfate	18-72 µM	Harker *et al*., 1996
Sodium acetate and Salt stress	0.025 – 0.1 g/L and 0.1 – 0.4 % Sodium Chloride	Cordero *et al*., 1996
Abscisic acid and its analog	10^{-3} M	Kobayashi *et al*., 1998
Sodium acetate and Salt stress	2.2 mM and 0.25 -2 % w/v Sodium chloride	Sarada *et al*., 2002
Methyl Jasmonate	2 and 20 mg/L	Lu *et al*., 2012
Gibberellic acid	2 and 20 mg/L	Lu *et al*., 2012
Salicylic acid with the low and high intensity of light	10-500µM with light intensity 30 µmol/m^2 /sec and 60 µmol/m^2 /sec	Raman *et al*., 2011
Methyl Jasmonate with the low and high intensity of light	10-500µM with light intensity 30 µmol/m^2 /sec and 60 µmol/m^2 /sec	Raman *et al*., 2011
Salicylic acid	50 and 25 mg/L	Gao *et al*.,2012
2,4- Epibrassinolide	20 and 50 mg/L	Gao *et al*., 2013
Gibberellic acid	20 and 40 mg/L	Gao *et al* 2013

Table 6: Effect of phytohormone in higher plants and *H. pluvialis*

Phytohormone	**Role in Higher plant**	**Finding in *H. pluvialis***	**Significance in microalgae**	**Reference**
Abscisic acid (ABA)	Tolerance to stress	Exogenous application of ABA, induced stress tolerance to dehydration	Improvement in stress tolerance	Kobayashi *et al*., 1997
Cytokinin (CK)	Growth and development	Exogenous application of CK, had positively affected cell cycle progression	Improved growth rate, fatty acid accumulation, biomass productivity and stress tolerance	Maor *et al*., 2010
Auxin (AX)	Growth and development	Exogenous application of AX , improved growth rate	Improved growth rate,fatty acid accumulation, biomass productivity and stress tolerance	Maor *et al*., 2010
Gibberllic acid (GA)	Growth and development	Exogenous application stimulated astaxanthin production	Secondary carotenoid production	Lu *et al*., 2010
Salicylic acid (SA)	Secondary metabolite production	Exogenous application stimulated astaxanthin production	Secondary carotenoid production	Raman *et al* ., 2011

Since, algae share similar carotenoid pathway,with higher plant (Cunmingham and Gantt, 1998). It is indicated that phytohormones system in higher plants, have evolved from pre-existing microalgal system. Phytohormones such as auxin, cytokinin, gibberellic acid, salicylic acid are crucial element of complicated signalling mechanism, occurring during the defense reponse (Walia *et al*., 2007). Mostly, all phytohormones of higher plants are found in algae but the knowledge about it is in adequate (Tarakhovskaya *et al*., 2007).

Material and Methods

I) Effect of carbon dioxide sparging on biomass production

Culture of *H. pluvialis* was obtained from ABCA biosolution, Mohali, Punjab and was grown in BG-11(+N) medium at 25 $\pm$ 2 ˚C, with 2500-3000 lux light intensity and pH maintained at 7.0. *H. pluvialis* was grown in 500 mL volume, Erlenmeyer flask for 21 days. *H. pluvialis* culture was sparged with carbon dioxide gas to maintain the pH at 7.0. Cultivation of *H. pluvialis* was carried out under indoor and outdoor conditions in culture flask. Effect of carbon dioxide sparging was studied, by studying the growth and astaxanthin accumulation and by estimation of phosphate and nitrate content of the medium. All growth measurements were performed in triplicates.

i) Composition of BG-11(+N) medium

For Preparation of 1000x stock of BG-11(1 Litres) (Stanier *et al.*, 1971), pH – 7.0

S.No	Component	Quantity(g/L)
1.	$NaNO_3$	--
2.	K_2HPO_4	40
3.	$MgSO_4.7H_2O$	75
4.	$CaCl_2.2H_2O$	36
5.	Citric acid(Di-hydrate)	6
6.	Ammonium Ferric Citrate	6
7.	$Na_2EDTA.2H_2O$	1
8.	Na_2CO_3	20
9.	Trace metal	Recipe given below

Trace Metal Composition

S.No	Component	Quantity(g/L)
1.	H_3BO_3	2.86
2.	$MnCl_2.4H_2O$	1.81
3.	$ZnSO_4.7H_2O$	0.22
4.	$Na_2MoO_4.2H_2O$	0.39
5.	$CuSO_4.5H_2O$	0.08
6.	$Co(NO_3)_2.6H_2O$	0.05

For preparation of 1x working stock of BG-11 medium (1 Litre)

S.No	Component	Volume in mL
1.	$NaNO_3$	1.5 g
2.	K_2HPO_4	1 mL
3.	$MgSO_4.7H_2O$	1mL
4.	$CaCl_2.2H_2O$	1mL
5.	Citric acid(Di-hydrate)	1mL
6.	Ammonium Ferric Citrate	1mL
7.	$Na_2EDTA.2H_2O$	1mL
8.	Na_2CO_3	1mL
9.	Trace metal	1mL

ii) Growth measurement

To study the effect of carbon dioxide sparging in *H. pluvialis*, the growth studies were carried out using spectro-photometric analysis, cell count, dry biomass estimation and estimation of chlorophyll. For this, 18 mL of culture was withdrawn regularly for 21days at an interval of 3 days.

a) Spectro-photometric analysis

Requirement

1. Cuvette
2. Spectrophotometer at 680 nm

Procedure

1. 3 mL sample was withdrawn from the culture flask after shaking to obtain homogenous culture.
2. Sample was transferred to cuvette.
3. Sample was analyzed at wavelength of 680 nm and absorbance obtained was noted.

b) Dry biomass estimation

Requirement

1. Cleaned and dried 10 mL volume of Centrifuge tube
2. Hot air oven set at 50◦C
3. Centrifuge

Procedure

1. Weighed the empty centrifuge tube (W_1).
2. Completely homogenized the culture, by shaking. Transferred, 10 mL of culture from culturing flask to the centrifuge.
3. Centrifuged the culture at 6000 rpm for 10 minutes. Discarded the supernatant.
4. Dried the pellet in hot air oven at 50°C, until a constant weight was obtained.
5. Weighed the centrifuge tube again, with dried pellet (W_2).

Calculation

Weight of dry biomass = $W_2 - W_1$

c) Cell count

Cell count was done using hemocytometer.

Requirement

1. Hemocytometer
2. Distilled water
3. Clean and dried vials
4. Micropipette

Procedure

1. 100 µL of culture was taken from culture flask and was transferred to the vial
2. To this, 400 µL of distilled water was added, to obtain dilution by the factor of 5.
3. Hemocytometer was wiped clean and dried. The gridded area of Hemocytometer chamber was covered by placing square cover slip.
4. The Hemocytometer was charged with sample, by careful pipetting diluted sample to one of the corner of the coverslip.
5. Sample is when distributed homogenously is observed under the microscope, at magnification of 40X.
6. Average cell count in four corners was determined. Multiplied by 10^4 and dilution factor of 5, to obtain the cell count.

Calculation

Number of cell / mL = Average number of cells in four corners x 10^4 x 5 (Dilution factor)

iii) Estimation of chlorophyll

Estimation of chlorophyll *a* and *b* was determined by the method given by Wellburn & Lichtenthaler, 1983

Requirements

1. 96% methanol
2. Centrifuge
3. Water bath at 70° C
4. Centrifuge tube of 10 mL volume
5. Spectrophotometer (666,653 and 470 nm)

Procedure

1. A volume of 3 mL of culture was withdrawn from the culture flask, after shaking and was centrifuged at 3500 rpm to obtain pellet of *Haematococcus* cells.
2. The supernatant was discarded and pellet is re-suspended in 96% methanol and was incubated at 70 ° C for 30 minutes in water bath.
3. After cooling, the sample was centrifuged at 6000 rpm for 10 minutes to obtain the supernatant.
4. Repeated, the process, until white Pellet is obtained. Collected the supernatant.
5. Supernatant obtained was analyzed by measuring absorbance at wavelength 666, 653 and 470 nm.

Calculation involved

Chlorophyll a (mg/L) = $(15.65 * A_{666} - 7.34 * A_{653}) * (U/V)$

Chlorophyll b (mg/L) = $(27.65 * A_{653} - 11.21 * A_{666}) * (U/V)$

A_{666} -Absorbance at 666 nm

A_{653} - Absorbance at 653 nm

U - Volume of methanol

V- Volume of Culture

iv) Estimation of astaxanthin

Estimation of astaxanthin was carried out according to the method given by Boussiba & Vonshank, 1992

Requirements

1. 10 mL centrifuge tube
2. Benchtop Centrifuge
3. Micropipette
4. DMSO (Dimethyl Sulfoxide)
5. 5% KOH prepared in 30% methanol
6. Distilled water

Procedure

1. 10 mL volume of *H. pluvialis* culture sample was withdrawn from homogenously shaken culture in 10 mL centrifuge tube.
2. Centrifuged the sample at 6000 rpm for 10 minutes. The Supernatant obtained was discarded.
3. Saponified the Pellet with 5% KOH prepared in 30%methanol and incubated for 10 min at 70 ˚C, in water bath.
4. Allowed it to cool and centrifuged it at 6000 rpm for 10 min. The supernatant obtained was discarded.
5. The remaining pellet is extracted with 10 mL DMSO and incubated it in a water bath for 5 min at 60 ˚C.
6. Repeated the process until white pellet is obtained.
7. The absorbance of the combined extract is obtained at 490 nm.

Calculation

$$\text{Concentration of astaxanthin (mg/L)} = \frac{[(4.5 * A_{490}) * V_a]}{V_b}$$

A_{490} – Absorbance of the extract at 490 nm

V_a – Volume of DMSO

V_b --- Volume of microalgal culture

v) Estimation of Phosphate

Phosphate estimation was carried out by the method given by Strickland & Parsons, 1968.

Requirements

1. Sulphuric acid
2. Ammonium molybdate
3. Potassium antimonyl tartrate
4. Ascorbic acid
5. Distilled water
6. BG-11 medium without dipotassium hydrogen phosphate
7. Spectrophotometer

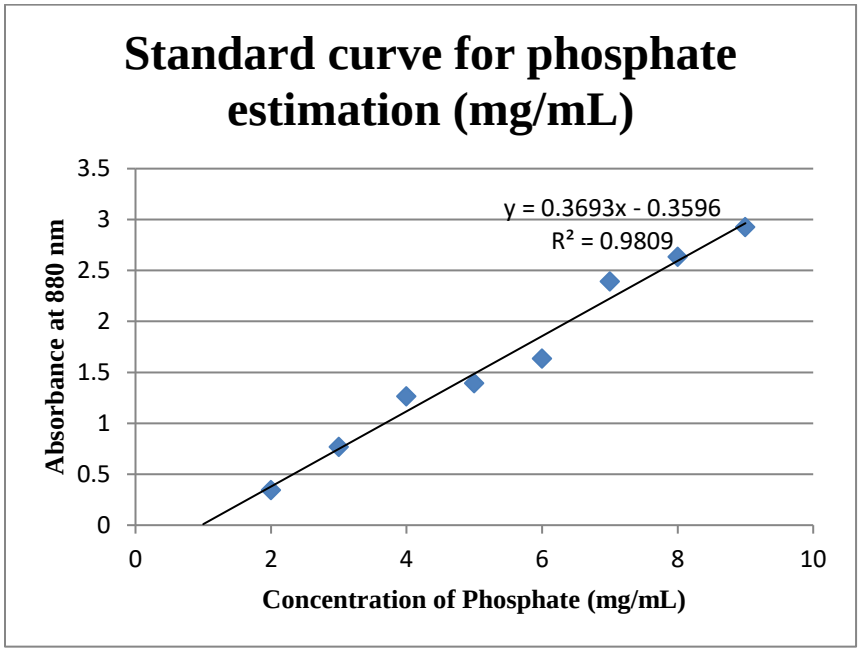
Reagent preparation

1. **Reagent A** - Dissolve 14 mL concentrated sulphuric acid and dilute it to 100 mL with distilled water.
2. **Reagent B**– Dissolve 0.055 gram of potassium antimonyl tartrate in 20 mL distilled water.
3. **Reagent C** – Dissolve 0.8 gram of ammonium molybdate in 20 mL distilled water.
4. **Reagent D** – Dissolve 0.88 gram of ascorbic acid in 50 mL distilled water (**Note**: stability of the solution is 1 week and should be stored at $4^{\circ}C$.
5. **Combined Reagent** – Mix the above reagent in the following ratio Reagent A: Reagent B: Reagent C: Reagent D, 10:1:3:6. (**Note**: Stability of this solution is for about 1 hour, thus should be prepared fresh)

Procedure

1. 5 mL of *Haematococcus* culture was transferred to the centrifuge tube. Culture was centrifuged at 6000 rpm for 10 min, to separate cells and media.
2. The supernatant was obtained and transferred to the test tube. Prepared blank by using 5 mL BG-11 medium without dipotassium hydrogen phosphate.
3. Added 800 µL of freshly made combined reagent in all test sample and blank.
4. Absorbances of the samples were measured at 880 nm (**Note**: the stability of the solution is not more than half an hour).

Figure 4: Standard curve for phosphate estimation (mg/mL)



Concentration of Phosphate (mg/mL)	Absorbance at 880 nm
0.005	0.347
0.01	0.771
0.015	1.265
0.02	1.394
0.025	1.637
0.03	2.394
0.035.	2.637
0.04	2.927

vi) Estimation of Nitrate

Nitrate estimation was carried out using method given by Strickland & Parsons, 1968

Requirements

1. Sodium nitrate
2. White phenol
3. Sulphuric acid
4. Potassium hydroxide
5. Distilled water
6. BG-11 media without sodium nitrate
7. Hot air oven set at 100 ˚C
8. Spectrophotometer

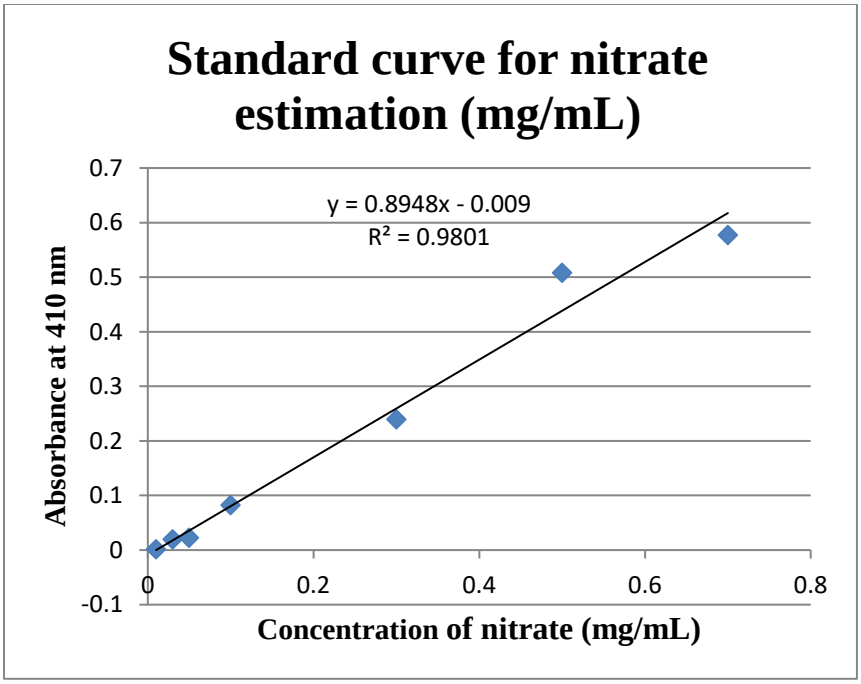
Reagent preparation

1. **Reagent A** – Dissolve 25 grams of white phenol in 100 mL of concentrated Sulphuric acid. Heat it for 2 hours by incubating it in water bath at 50˚C. The solution prepared should be kept in dark bottle.
2. **Reagent B** – Dissolve 10.09 grams of Potassium hydroxide in 15 mL of distilled water. (**Note**: it is required that Potassium hydroxide to be prepared fresh)

Procedure

1. 5 mL of *Haematococcus* culture was taken in centrifuge tube and centrifuged at 6000 rpm for 10 minutes, to obtain supernatant.
2. Supernatant obtained is transferred to the flask and dried completely in hot air oven. Blank was also prepared using BG-11 medium without sodium nitrate.
3. The flask was allowed to cool. On cooling 0.5 mL of Reagent A was added.
4. Followed by the addition 5 mL of distilled water and 5 mL of potassium hydroxide.
5. Measure the absorbance of each sample and blank at 410 nm.

Figure 5: Standard curve for nitrate estimation (mg/mL)



Concentration of nitrate (mg/ml)	Absorbance at 410 nm
0.01	0.001
0.03	0.0196
0.05	0.0224
0.1	0.082
0.3	0.239
0.5	0.508
0.7	0.577

II) Induction of *Haematococcu*s biomass to accumulate astaxanthin

The cells were induced to accumulate astaxanthin by inclusion of ferrous sulphate and salicylic acid. The culture of *Haematococcus* was grown in BG-11 medium. When culture attained, logarithmic phase, the cells were treated with the external astaxanthin inducing molecules. The culture was given treatment of ferrous sulphate at concentration of 450 µM and Salicylic acid treatment at various concentrations of 0, 50, 100, 200, 400, 500 µM. The experiment was carried out in triplicates.

The sample volume of 15 mL was withdrawn from the culture. The growth studies, of the culture were carried out for 12 days, along with the estimation of protein and total carbohydrate. These studies were carried out at the interval of 3 days.

i) Estimation of Protein

Protein estimation was carried out using method given by Lowry *et al*., 1951.

Requirements

1. Bovine serum albumin (BSA)
2. Sodium carbonate
3. Sodium hydroxide
4. Copper sulphate
5. Sodium potassium tartrate
6. Folin - Ciocalteau Reagent
7. Distilled water
8. Tri-chloro acetic acid

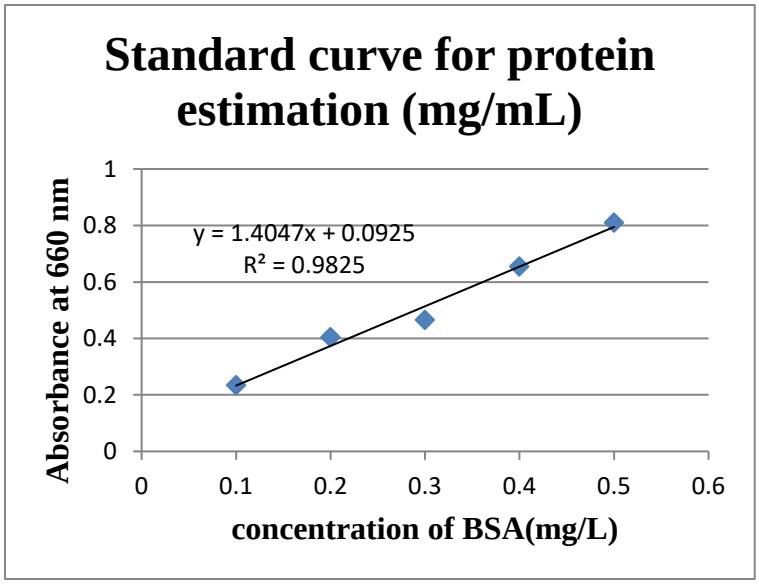
Reagents preparation

1. **BSA stock solution** – 0.1 gram of BSA in 100 mL of distilled water.
2. **Reagent A** – 50 mL of 2% sodium carbonate added to 50 mL 0.1 N sodium hydroxide.
3. **Reagent B** – 5 mL of 1.56% of copper sulphate mixed with 5 mL of 2.3 %Sodium potassium tartrate solution.
4. **Analytical reagent** – add 2 mL of reagent B to 100 mL of reagent A.
5. **Folin–Ciocalteau reagent (1N)** – Dilute the commercial reagent (2N) with an equal volume of water.
6. **24 % tri-chloro acetic acid** – 960 µL of trichloro acetic acid, added to 3.04 µL of distilled water.

Procedure

1. 1 mL sample of *Haematococcus* culture was transferred to microcentrifuge tube. The Culture was centrifuged at 6000 rpm for 10 minutes. The Supernatant obtained was discarded.
2. The pellet obtained was treated 200 µL of 24% tri-chloro acetic acid. The sample was incubated in water bath for 30 minutes at 90˚C.
3. Centrifuged the sample at 6000 rpm for 10 minutes and removed the supernatant obtained. The pellet obtained was re-suspended in 200 µL of distilled water.
4. Transferred, the sample to the, test tube. Added 2 mL of analytical reagent and incubated for 10 minutes at room temperature. Blank was also prepared by adding 200 µL of distilled water.
5. Added 200 µL of Folin-Ciocalteau reagent and incubated at room temperature for half an hour.
6. Measure the absorbance at 660 nm.

Figure 6: Standard curve for protein estimation (mg/mL)



Concentration of BSA (mg/mL)	Absorbance At 660 nm
0.1	0.234
0.2	0.404
0.3	0.465
0.4	0.655
0.5	0.810

ii) Estimation of total carbohydrate

Total carbohydrate estimation was carried out using method given by Dubois *et al*., 1951.

Requirements

1. Sulphuric acid
2. Phenol
3. Distilled water
4. Water bath

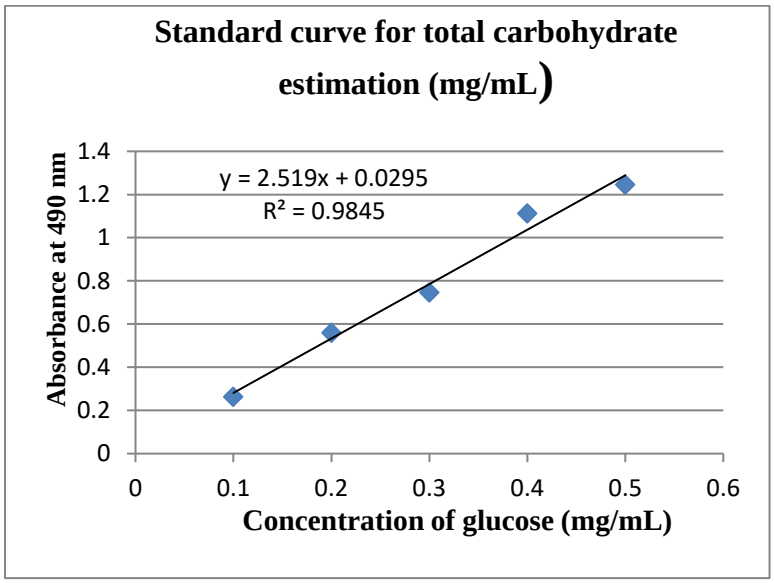
Reagent preparation

1. **72% sulphuric acid –** prepared by adding 72 mL concentrated sulphuric acid and total volume made to 100 mL by adding distilled water.
2. **5% phenol –** 5 mL phenol added to 95 mL of distilled water.

Procedure

1. 1 mL of culture was transferred to the test tube. Blank was prepared using 1 mL distilled water.
2. 3 mL of 72% sulphuric acid was added, followed by the addition of 5% phenol.
3. The tube were shaken well and incubated in water bath at 90˚C for 30 minutes.
4. Analyzed the absorbance at 490 nm.

Figure 7: Standard curve for total carbohydrate estimation (mg/mL)



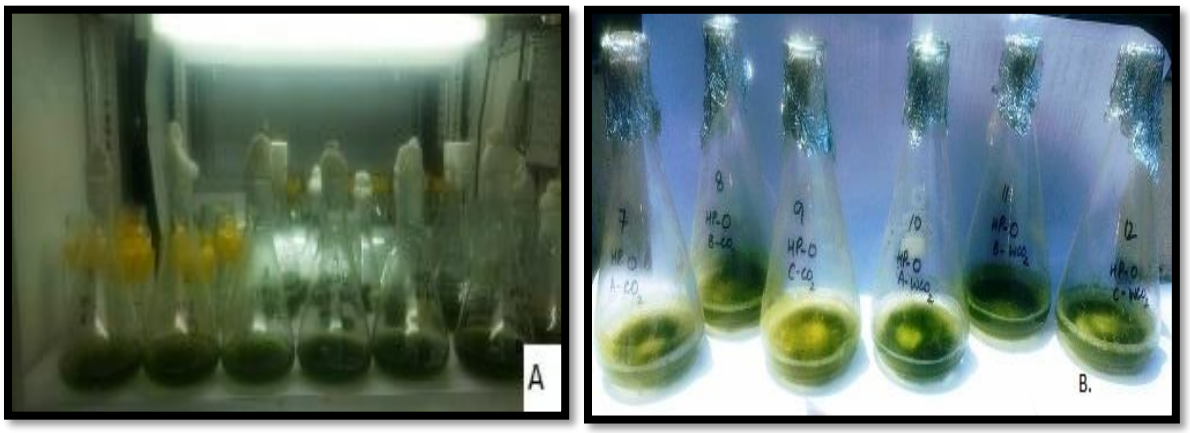
Concentration of glucose (mg/mL)	Absorbance at 490 nm
0.1	0.263
0.2	0.559
0.3	0.746
0.4	1.112
0.5	1.246

Results and Discussion

I) Effect of Carbon dioxide sparging on biomass production

Haematococcus pluvialis was grown in BG-11 medium at 25 ˚C in four different set of conditions, this includes indoor conditions with and without sparging of carbon dioxide (Figure 8 A) and outdoor conditions with and without sparging of carbon dioxide (Figure 8 B). The growth measurements and various other tests were carried out at an interval of 3 days up to 24 days.

Figure 8: A) Indoor cultivation of *Haematococcus pluvialis;* **B)** Outdoor cultivation of *Haematococcus pluvialis*



i) Biomass estimation

Biomass was estimated for all *Haematococcus* culture (Table 7; Figure 9). The biomass obtained in indoor culture with carbon dioxide sparging increased from 0.01 mg/mL to 0.39 mg/mL. Exponential increase in biomass was observed between 8th to 16th day. Maximum biomass of 0.39 mg/mL was observed on 20th day. The biomass of indoor culture without sparging of carbon dioxide increased from 0.02 mg/mL to 0.27 mg/mL. The exponential phase was observed between 8th to 16th day. Maximum biomass was observed on 24th day of 0.27 mg/mL.

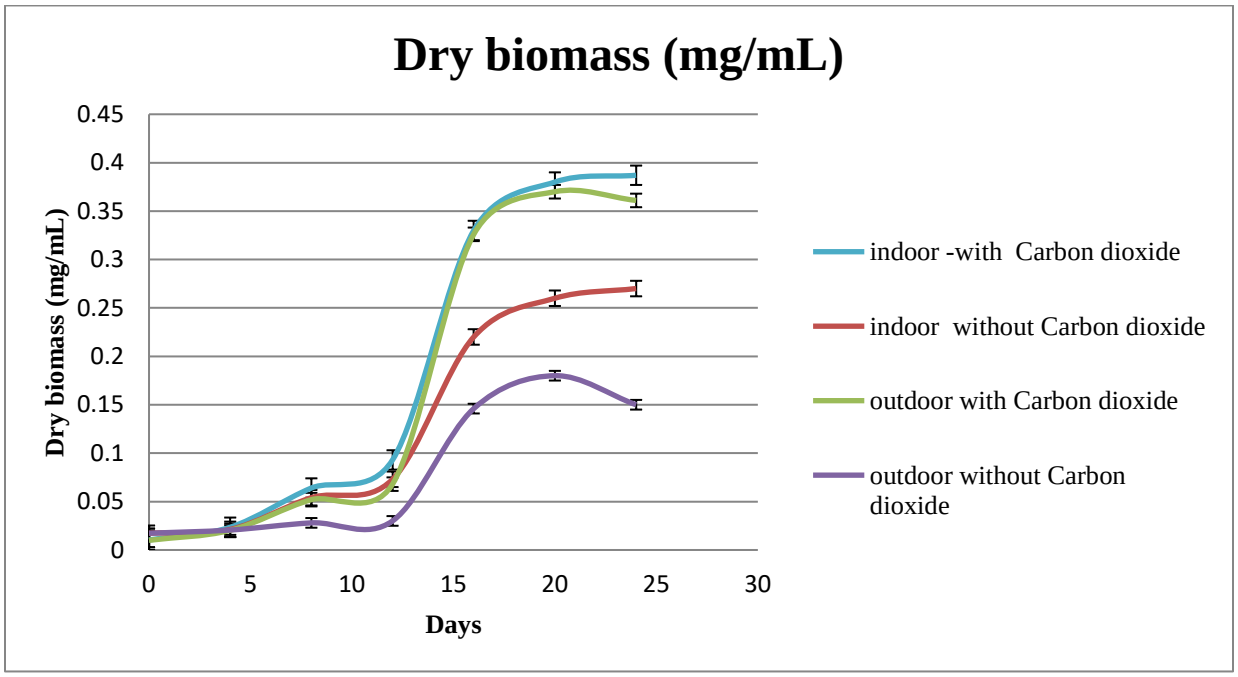
The biomass of outdoor culture with carbon dioxide sparging increased from 0.01 mg/mL to 0.36 mg/mL. Exponential phase of the growth was observed between 8th to 20th day. Maximum biomass of 0.37 mg/mL was obtained on 20th day. The biomass of outdoor culture without sparging of carbon dioxide increased from 0.02 mg/mL to 0.15 mg/mL. The exponential phase was observed between 8th to 16th days. Maximum biomass of 0.18 mg/mL was obtained on 20th day.

Table 7: Biomass (mg/mL) of *Haematococcus*

Days	Indoor with carbon dioxide (mg/mL)	Indoor without carbon dioxide (mg/mL)	Outdoor with carbon dioxide (mg/mL)	Outdoor without carbon dioxide (mg/mL)
0	0.01 ±0.008	0.02± 0.008	0.01±0.008	0.02±0.009
4	0.03± 0.008	0.02±0.008	0.02±0.008	0.02±0.008
8	0.06±0.009	0.05±0.008	0.05±0.007	0.03±0.007
12	0.09±0.008	0.07±0.007	0.07±0.008	0.03±0.006
16	0.33±0.008	0.22±0.008	0.33±0.008	0.14±0.008
20	0.38±0.008	0.26±0.008	0.37±0.007	0.18±0.008
24	0.39±0.008	0.27±0.008	0.36±0.007	0.15±0.008

*values given are average of three replicates

Figure 9: Biomass (mg/mL) of *Haematococcus*



Indoor culture with carbon dioxide sparging performed well in biomass production, in comparison to other cultures. The indoor culture with carbon dioxide sparging, adapted to the fresh medium earlier than culture without carbon dioxide, leading to the shorter lag phase. 1.5 fold increases in biomass is

seen in indoor culture with carbon dioxide sparging. Indoor culture without carbon dioxide sparging showed significant increase in biomass on 8th day of culture. Outdoor culture with carbon dioxide also has better rate of biomass production in comparison to indoor culture without carbon dioxide sparging and outdoor culture without carbon dioxide sparging. A drop in biomass production in outdoor culture was observed due to high temperature. Temperature during month of April was significantly higher than the optimum growth temperature of *Haematococcus*. This was also applicable for lower biomass production observed in outdoor culture without carbon dioxide sparging. On 20th day, culture was observed to enter the stationary phase, due to nutrient exhaustion of the medium.

ii) Cell count (per mL)

The cell count is another measurement for growth, since *Haematoccus* is unicellular. Similar to the dry biomass estimation, cell count also shows the better growth occurring in indoor culture with carbon dioxide sparging. Outdoor culture with carbon dioxide sparging also performs better in terms of growth in comparison to without carbon dioxide sparging. Cell count was obtained for *Haematococcus* culture (Table 8; Figure10).

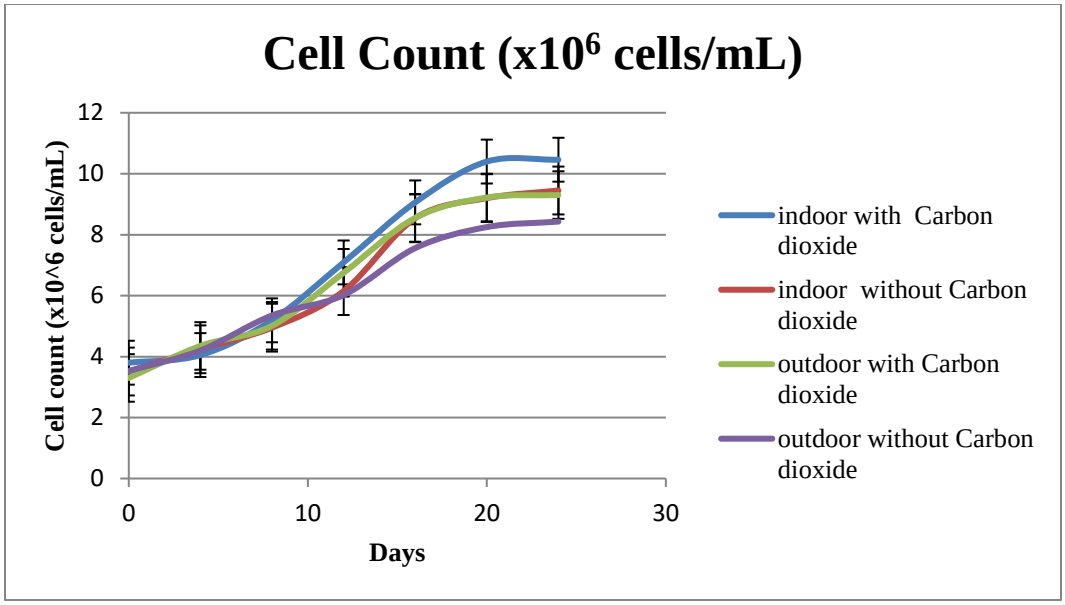
Cell count for indoor culture with carbon dioxide sparging initially had 3.8 x 10^6 cells/mL that increased to 10.5 x 10^6 cells/mL on 24th day. For indoor culture without carbon dioxide sparging 0th day had 3.5 x 10^6 cells/mL, that increased to 9.45x 10^6 cells/mL on 24th day.Cell count on 0th day for outdoor culture with carbon dioxide sparged was 3.3 x 10^6 cells/mL, that increased to 9.3 x 10^6 cells/mL on 24th day.. For outdoor culture, without carbon dioxide sparging, cell count on 0th day was 3.5 x 10^6 cells/mL that increased to 8.4 x 10^6 cells/mL on 24th day.

Table 8: Cell count (x 10^6cells/mL) of *Haematococcus*

Days	Indoor with carbon dioxide (per mL)	Indoor without carbon dioxide(per mL)	Outdoor with carbon dioxide (per mL)	Outdoor without carbon dioxide (per mL)
0	3.8±0.725	3.5±0.884	3.3±0.359	3.5 ± 0.221
4	4.05 ±0.580	4.2 ±0.651	4.3±0.484	4.2 ±0.654
8	5.2±0.489	4.9±0.489	5±0.653	5.3 ± 0.956
12	7.1±0.256	6.1±0.568	6.7±0.855	6 ±0.866
16	9.1±0.389	8.5±0.562	8.5±0.987	7.6 ± 0.698
20	10.4 ±0.247	9.2 ± 0.689	9.2±1.125	8.2±0.358
24	10.5 ±0.658	9.45 ±0.478	9.3±0.746	8.4± 0.415

*values given are average of three replicates

Figure 10: Cell count (x10^6 per mL) of *Haematococcus*



iii) Absorbance at 680 nm

Absorbance of *Haematococcus* culture was taken at 680 nm (Table 9; Figure 11). Absorbance of the Indoor culture with carbon dioxide at 680 nm was 0.32 on 0th day, which became 1.18 on 24th day. Maximum absorbance was obtained as 1.46 on 20th day that decreased to 1.18 on 24th day. Absorbance of Indoor culture without carbon dioxide sparging, the intial 0th day absorbance was 0.31 that increased to 1.26 on 24th day. Maximum average absorbance was obtained on 20th day was 1.26.

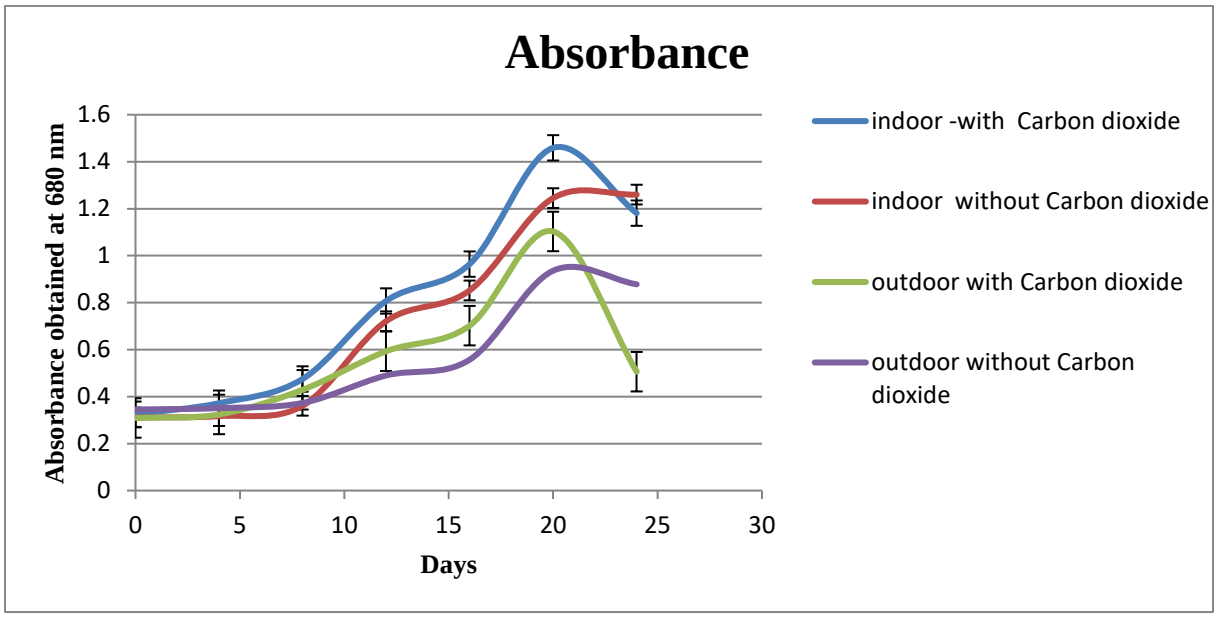
Table 9: Absorbance obtained at 680 nm for *Haematococcus*

Days	Indoor with carbon dioxide	Indoor without carbon dioxide	Outdoor with carbon dioxide	Outdoor without carbon dioxide
0	0.32±0.056	0.31±0.057	0.31±0.045	0.34±0.086
4	0.37±0.032	0.32±0.085	0.32±0.045	0.35±0.086
8	0.47±0.085	0.36±0.085	0.43±0.032	0.37±0.056
12	0.81±0.085	0.72±0.86	0.59±0.046	0.49±0.056
16	0.96±0.085	0.85±0.035	0.70±0.045	0.56±0.056
20	1.46±0.056	1.24+0.035	1.10±0.079	0.94±0.84
24	1.18±0.056	1.26±0.035	0.91±0.085	0.88±0.86

*values given are average of three replicates

Absorbance for outdoor culture with carbon dioxide sparging was initially on 0th day was 0.31, which became 0.91 on 24th day. Maximum absorbance was obtained on 20th day, which was of 1.11. Absorbance for outdoor culture without carbon dioxide sparging was 0.34 on 0th day, that became0.88 on 24th day. Maximum absorbance was obtained on 20th day of 0.94.

Figure 11: Absorbance at 680 nm of *Haematococcus*



All the growth measurement studies show that the maximum biomass is obtained in the indoor cultivation coupled with the carbon dioxide sparging. Indoor cultivation, when compared to the outdoor cultivation, is able to provide optimum temperature for growth. Outdoor cultivation has a serious limitation of uncontrolled conditions. In this case, it was high light intensity of natural sunlight and high temperature that slowed down the growth of *Haematococcus* sp. in outdoor culture.

Aerated carbon dioxide acts as carbon source for the production of biomass. This carbon dioxide is utilized by the algae by the process of photosynthesis. Fixed carbon dioxide is reflected as the increased biomass. Not only this, carbon dioxide also helps in the maintenance of the pH of culture medium that rises up to 2-3 units /day.

iv) Chlorophyll a & b content

Increase in chlorophyll *a* & *b* content of cell is a sign of growth. Chlorophyll *a* acts as primary pigment for photosynthesis and thus have significant role in growth. As cell become metabolically inactive, there is a decrease in the chlorophyll content. Chlorophyll content of the cell varies at different stages of life cycle. (Table 10; Figure 12 A & B) & (Table 11; Figure 12 C & D).

Table 10: Chlorophyll *a* (mg/L) of *Haematococcus*

Days	Indoor with carbon dioxide (mg/L)	Indoor without carbon dioxide (mg/L)	Outdoor with carbon dioxide (mg/L)	Outdoor without carbon dioxide (mg/L)
0	1.10±0.235	1.36±0.082	1.35±0.233	1.12±0.064
4	1.86±0.235	1.55±0.006	1.38±0.544	1.29±0.325
8	2.57±0.235	2.35±0.356	1.86±0.126	1.66±0.487
12	5.50±0.154	4.83±0.356	3.79±0.126	2.63±0.127
16	8.44±0.235	5.97±0.325	6.18±0.123	5.09+0.245
20	3.05±0.234	1.99±0.356	2.82±0.238	2.3±0.312
24	3.46±0.234	2.51±0.254	2.82±0.325	2.07±0.254

*values given are average of three replicates

Table 11: Chlorophyll *b* (mg/L) of *Haematococcus*

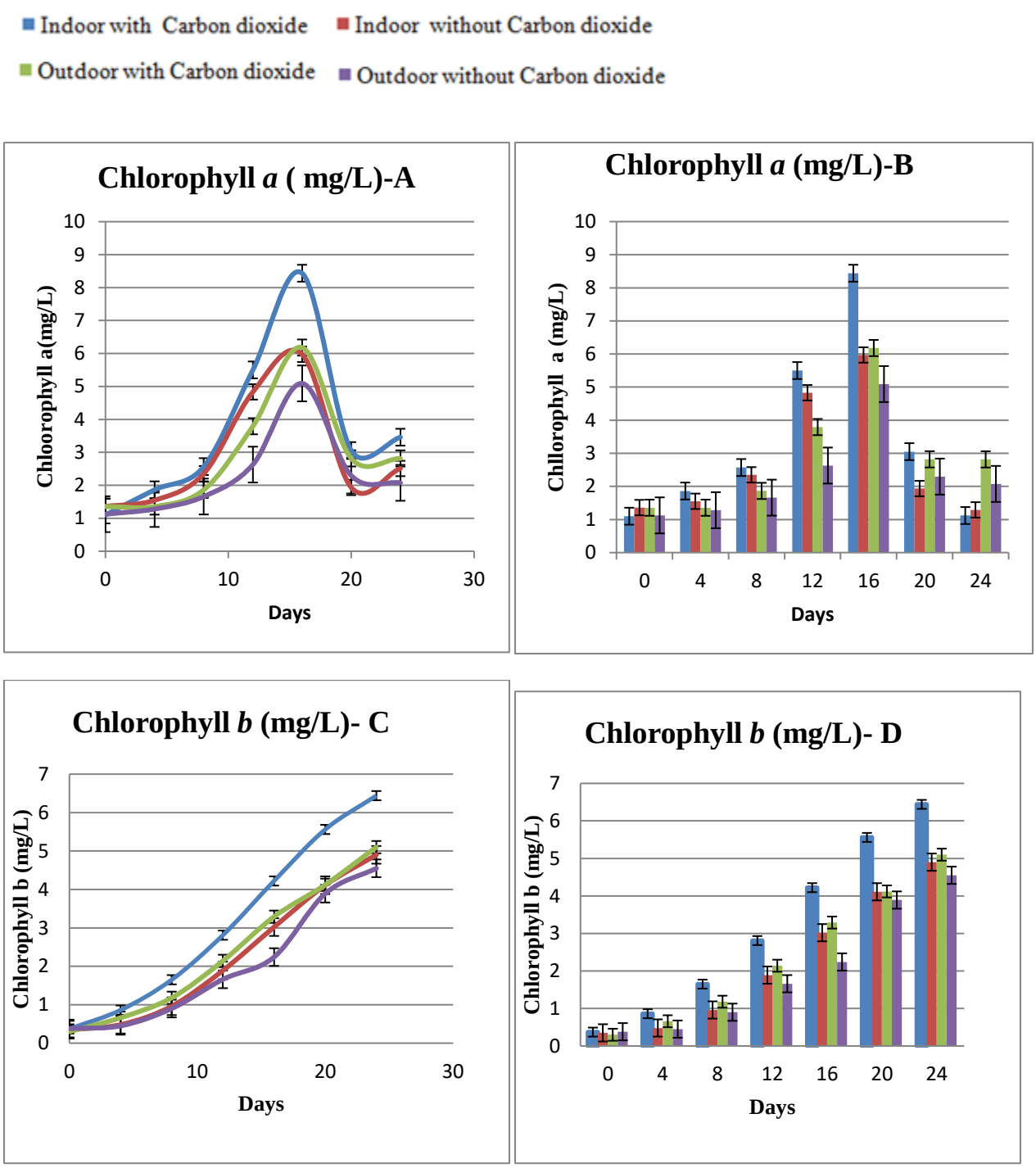
Days	Indoor with carbon dioxide (mg/L)	Indoor without carbon dioxide (mg/L)	Outdoor with carbon dioxide (mg/L)	Outdoor without carbon dioxide (mg/L)
0	0.37±0.05	0.35±0.08	0.3±0.07	0.38±0.06
4	0.86±0.05	0.48±0.16	0.66±0.07	0.45±0.05
8	1.65±0.12	0.96±0.15	1.18±0.15	0.90±0.06
12	2.81±0.06	1.89±0.25	2.14±0.07	1.66±0.06
16	4.22±0.07	3.02±0.15	3.29±0.23	2.24±0.12
20	5.56±0.05	4.11±0.24	4.12±0.24	3.89±0.12
24	6.44±0.05	4.9±0.24	5.1 ±0.28	4.55±0.33

*values given are average of three replicates

Aerating culture medium with carbon dioxide causes increase in chlorophyll and other carotenoids. However, aeration above the optimal range can cause cessation of growth (Garcia-Malea *et al*., 2006).

Figure 12: A) & B) shows Chlorophyll *a* (mg/L) of *Haematococcus*

C) & D) shows Chlorophyll *b* (mg/L) of *Haematococcus*



Chlorophyll *a* and *b* of indoor culture with carbon dioxide sparging was initially 1.1 mg/L and 0.37 mg/L. Chlorophyll *a* and *b* increased to maximum, 3.46 and 6.44 mg/L respectively, on 24th day.

Chlorophyll *a* and *b* content for indoor culture without carbon dioxide sparging was 1.36 mg/L and 0.35 mg/L respectively, on 0^{th} day, which increased to 2.5 mg/L and 4.89 mg/L on 24^{th} day.

Chlorophyll a and *b* content of outdoor culture with carbon dioxide sparging was 1.36 mg/L and 0.3 mg/L on 0^{th} day. Chlorophyll *a* and *b* content increased to 2.8 and 5.1 mg/L on 24^{th} day. Maximum chlorophyll *a* and *b* content was found on 20^{th} and 24^{th} day respectively. Chlorophyll *a* and *b* content of outdoor culture without carbon dioxide sparging was 1.12 mg/mL and 0.38 mg/L on 0^{th} day. Chlorophyll *a* and *b* content increased to 2.07 and 4.55 mg/L on 24^{th} day. Maximum chlorophyll *a* and *b* content was found on 24^{th} day.

Highest chlorophyll content is obtained in indoor cultivation coupled with carbon dioxide. Higher chlorophyll content of the *Haematococcus* cell indicates the active metabolism and increased photosynthetic activity of the cell. Which can be further linked with the increase in the biomass production, as a result of carbon dioxide fixation by photosynthesis**.**

v) Astaxanthin

Astaxanthin is a secondary ketocarotenoid, which is synthesized during unfavourable conditions. Initially, the astaxanthin present in cells on 0^{th} day, decreased slightly as cells encountered favourable conditions of fresh media. Table 12 and Figure 13 shows the astaxanthin estimated (mg/L) for *Haematococcus*. On day 16^{th}, all cultures subjected to different conditions, showed increase in astaxanthin content and further increased till 24^{th} day. This may be attributed to the changing nutrient status of the media.

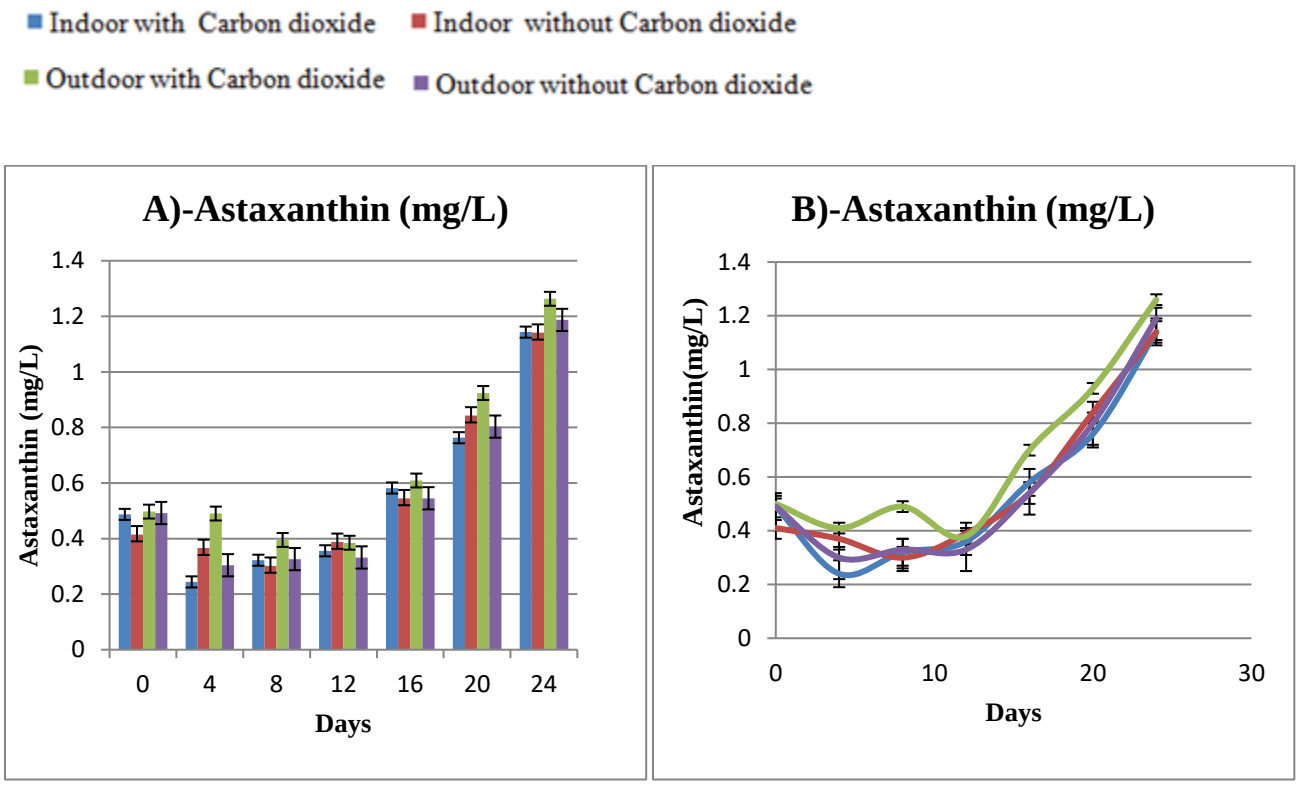
Table 12: Astaxanthin (mg/L) of *Haematococcus*

Days	Indoor with carbon dioxide (mg/L)	Indoor without carbon dioxide (mg/L)	Outdoor with carbon dioxide (mg/L)	Outdoor without carbon dioxide (mg/L)
0	0.49±0.003	0.41±0.002	0.50±0.001	0.49±0.005
4	0.24±0.003	0.37±0.003	0.41±0.002	0.30±0.005
8	0.32±0.003	0.30±0.003	0.49±0.002	0.33±0.003
12	0.36±0.001	0.39±0.003	0.38±0.002	0.33±0.002
16	0.58±0.003	0.54±0.003	0.7±0.002	0.54±0.003
20	0.76±0.002	0.84±0.002	0.93±0.005	0.80±0.005
24	1.14±0.003	1.14±0.003	1.26±0.002	1.19±0.002

*values given are average three replicates

Aeration of medium causes an increase in the amounts of pigments. The aeration is one of the factors that can be utilized to induce the synthesis of astaxanthin in the cell (Chen *et al*., 1997; Garcia-Malea *et al*., 2006) (Table 12; Figure 9). Astaxanthin content of indoor culture with carbon dioxide sparging was found to be 0.49 mg/L on 0thday, which became maximum, 1.14 mg/L on 24th day. Indoor culture without carbon dioxide sparging on 0th day had 0.41 mg/L of astaxanthin, which increased to 1.14 mg/L of astaxanthin on 24th day. Similar trends are shown in Outdoor culture with and without carbon dioxide sparging. Outdoor culture with carbon dioxide sparging, initially had 0.50 mg/L and outdoor culture without carbon dioxide sparging had 0.49 mg/L on 0th day, which became 1.26and 1.19 mg/L respectively on 24th day.

Figure 13: Astaxanthin (mg/L) in *Haematococcus*



vi) Nitrate estimation

Nitrate source in BG-11 medium is provided in form of potassium nitrate salt. Nitrate is consumed by the cells, its concentration in the medium decreases. Nitrate consumption is an indication of growth. High consumption of the nitrate indicates the higher production of biomass. Nitrate concentration of *Haematococcus* culture filtrate was estimated (Table 13; Figure 14).

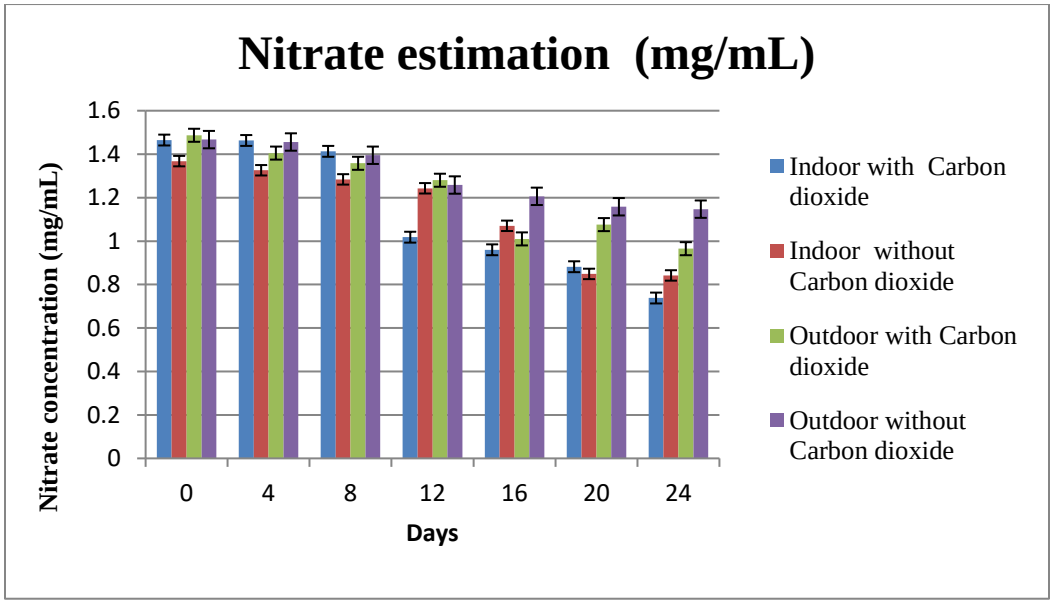
Indoor culture with carbon dioxide sparging initially had 1.46 mg/mL of nitrate concentration, which decreased to 0.74 mg/mL on 24th day. Indoor culture without carbon dioxide sparging, on 0th day had 1.37 mg/mL of nitrate concentration, which decreased to 0.84 mg/mL on 24th day. Similar trends were seen in outdoor culture with carbon dioxide sparging that initially had nitrate concentration of 1.49 mg/mL, which decreased to 0.96 mg/mL. In outdoor culture without carbon dioxide sparging, nitrate concentration was initially 1.47 mg/mL, which decreased to 1.15 mg/mL on 24th day.

Table 13: Nitrate concentration (mg/mL) in culture filtrate of *Haematococcus*

Days	Indoor with carbon dioxide (mg/mL)	Indoor without carbon dioxide (mg/mL)	Outdoor with carbon dioxide (mg/mL)	Outdoor without carbon dioxide (mg/mL)
0	1.46±0.007	1.36±0.011	1.49±0.007	1.47+0.007
4	1.46±0.007	1.33±0.007	1.40±0.007	1.46+0.007
8	1.41±0.007	1.28±0.007	1.36±0.004	1.39±0.007
12	1.02±0.009	1.24±0.006	1.28±0.007	1.26±0.007
16	0.96±0.008	1.07±0.006	1.01±0.007	1.21+0.004
20	0.88±0.007	0.85±0.006	1.08±0.004	1.16±0.004
24	0.74±0.007	0.84±0.006	0.96+0.007	1.15±0.004

*values are average of three replicates

Figure 14: Nitrate concentration (mg/mL) in culture filtrate of *Haematococcus*



Boussiba and Vonshak (1992) studied the effect of low nitrogen in the medium. They found that nitrogen concentration as low as 0.15 g/litre, along with light intensity of 170 $\mu M/m^2$/sec, caused the accumulation of the astaxanthin in the *Haematococcus* cells. However, in this case, nitrate concentration is sufficient to support further growth. Thus, this spent medium can be recycled, along with the addition of other exhausted components of the medium

vii) Phosphate estimation

Phosphate in BG-11 medium was provided in the form dipotassium dihydrogen phosphate. Phosphate is required by the cell for growth and cell maintenance. A growing cell consumes phosphate and multilplies to form new cells. Decrease in concentration of phosphate in culture medium is good indication of growth. The phosphate was estimated for the indoor and outdoor culture filtrate (Table 14; Figure 15).

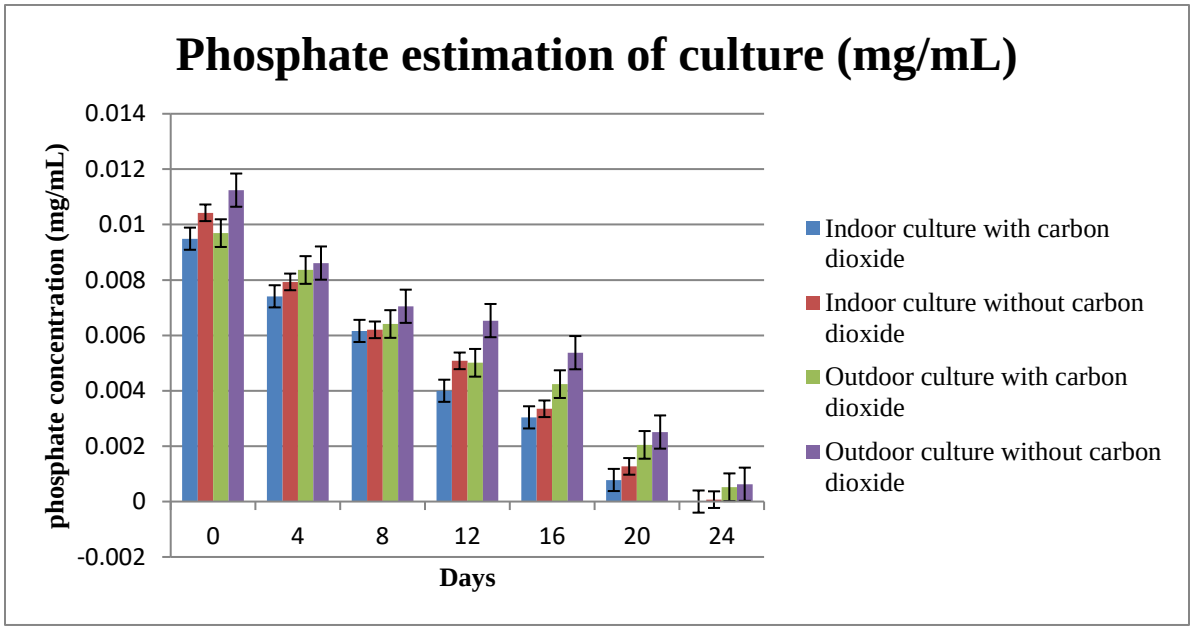
Phosphate initially in indoor culture with carbon dioxide sparging had 0.01 mg/mL. As growth occurs, the phosphate concentration decreased. On 24^{th} day the phosphate has completely exhausted. In case of indoor culture, without carbon dioxide sparging, phosphate concentration which initially on 0^{th} day was 0.01 mg/mL, decreased to 0.0001 mg/mL on 24^{th} day. In case of outdoor culture, with carbon dioxide sparging, the concentration of phosphate was 0.01 mg/mL on 0^{th} day, which decreased to 0.0005 mg/mL on 24^{th} day. For outdoor culture, without carbon dioxide sparging, the 0^{th} day concentration of phosphate was 0.011 mg/mL , that later on 24^{th} day decreased to 0.0006 mg/mL.

Table 14: Phosphate concentration (mg/mL) in culture filtrate of *Haematococcus*

Days	Indoor culture with carbon dioxide (mg/mL)	Indoor culture without carbon dioxide (mg/mL)	Outdoor culture with carbon dioxide (mg/mL)	Outdoor culture without carbon dioxide (mg/mL)
0	0.01±0.007	0.01±0.005	0.01±0.007	0.011±0.006
4	0.007±0.007	0.008±0.005	0.008±0.007	0.009±0.006
8	0.006±0.006	0.006±0.005	0.006±0.007	0.007±0.006
12	0.004±0.006	0.005±0.007	0.005±0.006	0.006±0.007
16	0.003±0.006	0.003±0.007	0.004±0.006	0.005±0.007
20	0.001±0.006	0.001±0.007	0.002±0.006	0.002±0.007
24	0±0	0.0001±0	0.0005±0.001	0.0006±0

*values are average of three replicates

Figure 15: Phosphate concentration (mg/mL) in culture of *Haematococcus*



II) Induction of *Haematococcus* biomass to accumulate astaxanthin

Haematococcus pluvialis culture in mid-logarithmic phase was distributed into 250 mL culture flask. The cultures are treated with different concentrations of salicylic acid and 450 µM concentration of ferrous sulphate. Treatment of cultures is carried out for 12 days. During which 15 mL culture sample is regularly withdrawn for growth studies and various other tests.

i) Absorbance

Spectrometric analysis of treated cultures (Table 15; Figure 16), showed the decrease in absorbance. This was due to of cell lysis occurring as a result of excessive unfavourable conditions produced by the application of salicylic acid and ferrous sulphate.

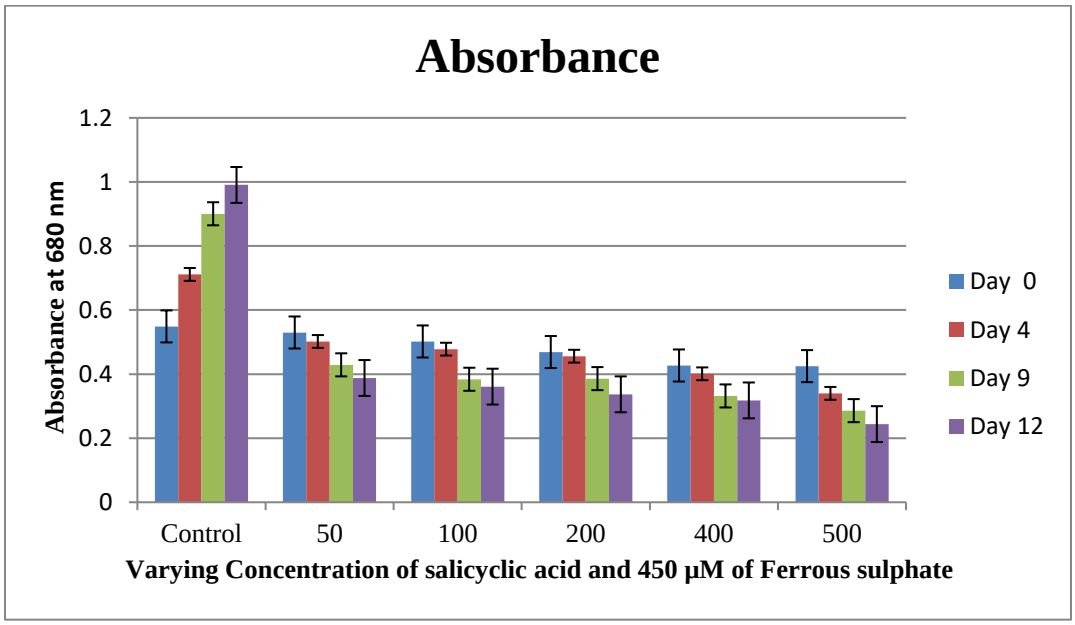
Culture D and E, treated with high concentration of salicylic acid, showed immediate decrease in absorbance on 8^{th} and 4^{th} respectively. Whereas, Cultures A, B and C, showed no increase in absorbance, indicating the growth arrest, but do not have conditions for causing cell lysis. Culture A, B and C on 12th day showed significant decrease in the absorbance, indicates the cell lytic conditions present in these cultures.

Table 15: Absorbance at 680 nm of treated culture of *Haematococcus*

Concentration of salicylic acid (µM)	0^{th} day	4^{th} day	8^{th} day	12^{th} day
Control (0 µM)	0.55±0.05	0.71±0.046	0.90±0.025	0.99±0.004
A (50 µM)	0.53±0.018	0.50±0.012	0.43±0.04	0.40±0.07
B (100 µM)	0.50±0.023	0.48±0.015	0.38±0.05	0.36±0.05
C (200 µM)	0.47±0.05	0.45±0.014	0.40±0.05	0.30±0.05
D (400 µM)	0.43±0.05	0.40±0.07	0.33±0.045	0.32±0.015
E (500 µM)	0.42±0.05	0.34±0.06	0.30±0.036	0.24±0.05

*values given are average of three replicates

Figure 16: Absorbance at 680 nm of treated culture of *Haematococcus*



ii) Chlorophyll *a* & *b*

Chlorophyll a &*b* content of culture is an indicator of growth. Decrease in chlorophyll content of the cultures, is an evidence of growth inhibiting conditions in the cultures. Chlorophyll *a* (Table 16; Figure 17) and *b* (Table 17; Figure 18) in mg/L was determined.

Culture A, B and C showed no considerable change in chlorophyll *a* & *b* content. However culture C showed change in the chlorophyll content on 8th day. Culture D and E contained high concentration of salicylic acid, showed a drop in chlorophyll content on 4th day. This is the result of cell lytic conditions, prevalent in the medium.

Table 16: Chlorophyll *a* (mg/L) of treated culture of *Haematococcus*

Concentration of salicylic acid (µM)	0th day (mg/L)	4th day (mg/L)	8th day (mg/L)	12th day (mg/L)
Control (0)	3.30±0.04	3.41±0.04	4.0±0.04	5.00±0.07
A (50)	3.30±0.04	3.17±0.05	1.58±0.06	1.60±0.07
B (100)	3.32±0.04	3.24±0.05	2.04±0.05	1.87±0.07
C (200)	2.60±0.04	2.34±0.07	2.06±0.05	1.77±0.05
D (400)	2.83±0.05	2.05±0.06	1.75±0.05	1.63±0.08
E (500)	2.85+0.05	2.02±0.05	1.63±0.06	1.48±0.08

*values given are average of three replicates

Table 17: Chlorophyll *b* (mg/L) of treated culture of *Haematococcus*

Concentration of salicylic acid (µM)	0th day (mg/L)	4th day (mg/L)	8th day (mg/L)	12th day (mg/L)
Control (0)	1.80±0.85	2.81±0.45	2.99±0.47	3.72±0.56
A (50)	1.53±0.25	1.50±0.34	1.26±0.59	0.79±0.26
B (100)	1.36±0.64	1.26±0.48	1.16±0.58	0.74±0.47
C (200)	1.46±.35	1.16±0.64	0.96±0.41	0.68±0.49
D (400)	1.54±0.47	0.84±0.86	0.64±0.24	0.55±0.35
E (500)	1.45±0.48	0.78±41	0.55±0.40	0.45±0.24

*values given are average of three replicates

Growth studies including absorbance at 680 nm and chlorophyll content of the culture is substantial to indicate that high concentration of more than 200 µM of salicylic acid can cause reduction in growth and

cell lysis. For cultures treated with concentration, lesser than 200 µM showed decrease in absorbance and chlorophyll content on 8th day. This data is essential in deciding the duration of treatment.

Chlorophyll a and b is a sign of active metabolism taking place in the cell. Decreasing chlorophyll indicates the diminishing metabolism of the cell. The treated cell, prepares itself to enter into the aplanospore stage.

Figure 17: Chlorophyll *a* (mg/L) of treated *Haematococcus*

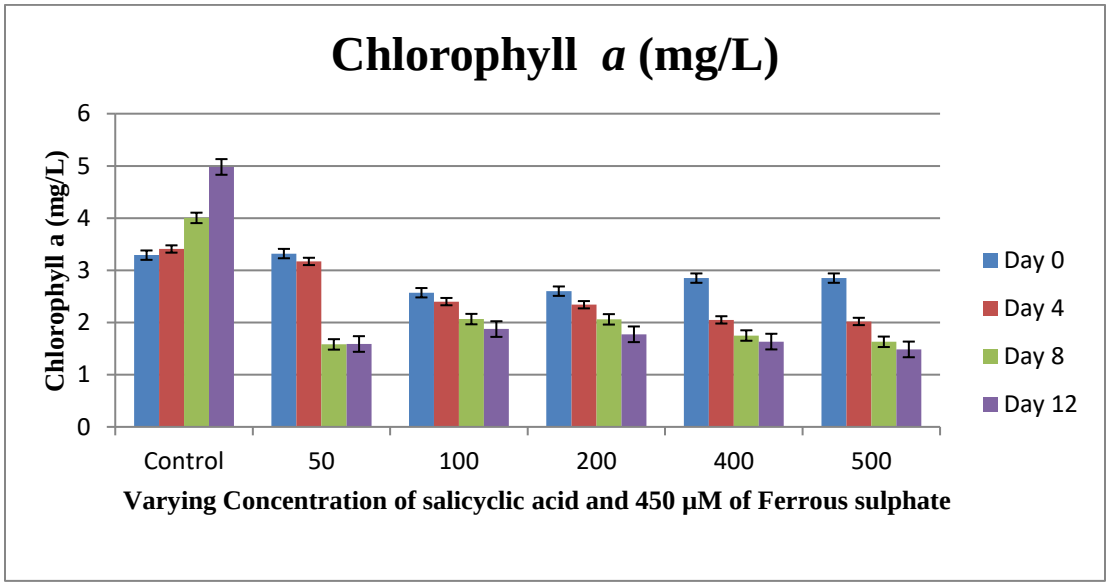
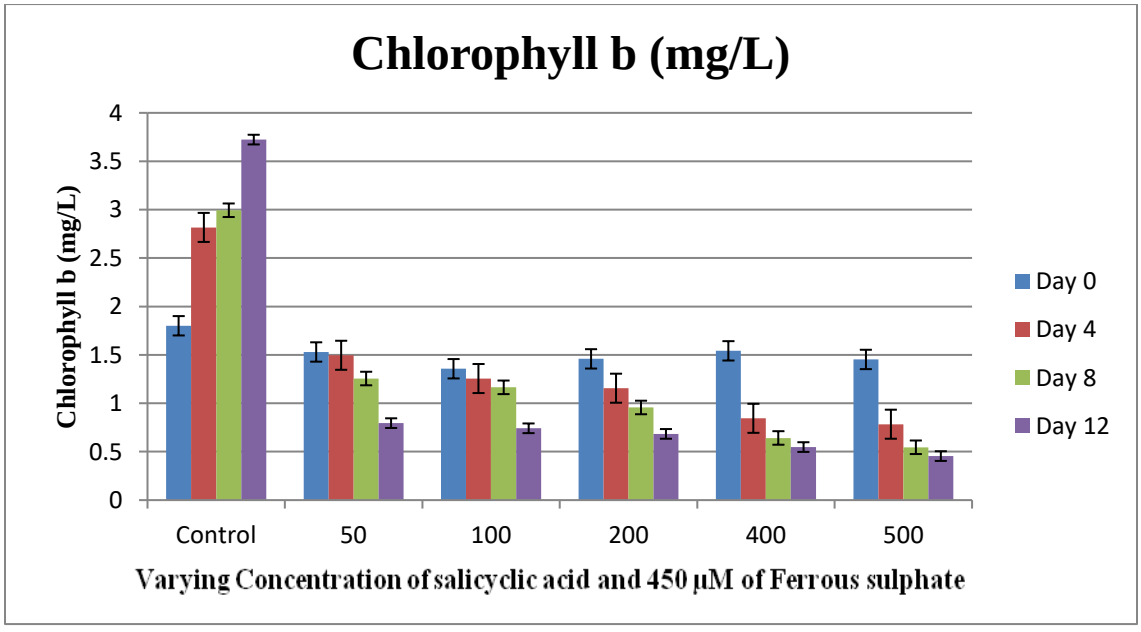


Figure 18: Chlorophyll *b* (mg/L) of treated culture of *Haematococcus*



iii) Total carbohydrate estimation

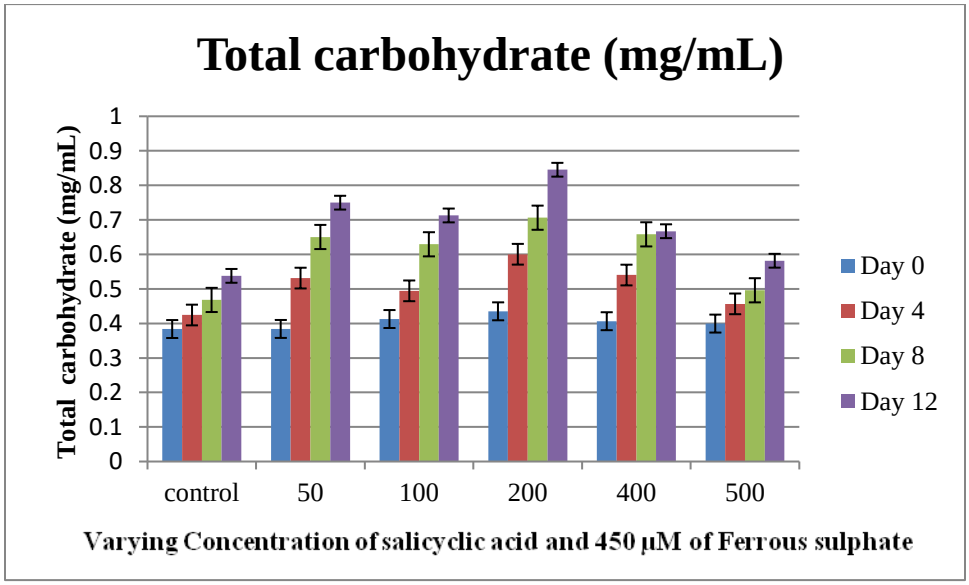
H. pluvialis cell during unfavourable condition tend to accumulate carbohydrate. Thus, increase in the carbohydrate content of the cell, is indicative of cell undergoing stress. Total carbohydrate content of treated culture was estimated (Table 18; Figure 19).

Table 18: Total carbohydrate (mg/mL) of treated culture of *Haematococcus*.

Concentration of salicylic acid (µM)	0^{th} day (mg/mL)	4^{th} day (mg/mL)	8^{th} day (mg/mL)	12^{th} day (mg/mL)
Control (0)	0.38±0.056	0.42±0.049	0.47±0.054	0.54±0.051
A (50)	0.38±0.045	0.53±0.036	0.65±0.024	0.75±0.048
B (100)	0.41±0.047	0.50±0.057	0.63±0.058	0.71±0.037
C (200)	0.43±0.058	0.60±0.048	0.71±0.047	0.84±0.035
D (400)	0.41±0.047	0.54±0.048	0.66±0.048	0.67±0.027
E (500)	0.40±0.048	0.41±0.045	0.50±0.048	0.58±0.054

*values given are average of three replicates

Figure 19: Total carbohydrate (mg/L) of treated culture of *Haematococcus*



Total carbohydrate content of the culture A, B and C increased with the duration of treatment. Highest increase in carbohydrate content was shown by culture C. Culture D and E , showed increase in

carbohydrate content, but increase was not comparable to the increase observed in the culture A, B and C. As number of live cell in culture D and E, have decreased substantially. As the numbers of live cells have decreased, there is lesser number of cells available to accumulate carbohydrate. All cultures, showed highest total carbohydrate content on 12th day of treatment. Culture C, with highest content of total carbohydrate. Green stage has carbohydrate content of up to 15% to 17% of its cellular content which increases to nearly 50% during red stage (Shah *et al*., 2016). During unfavourable conditions, the cell tends to accumulate starch and thus carbohydrate content of the cell increases, that is consumed during prolonged stress conditions (Shah *et al*., 2016)

iv) Protein Estimation

Protein is an essential component of cell. It has important structural and functional role. Cell undergoes from green vegetative stage to red aplanospore stage. It becomes more metabolically inactive, thus protein has lesser role to play. For cell, in stress, there is decrease in protein concentration of the cell. Protein content of treated culture was determined (Table 16; Figure 20).

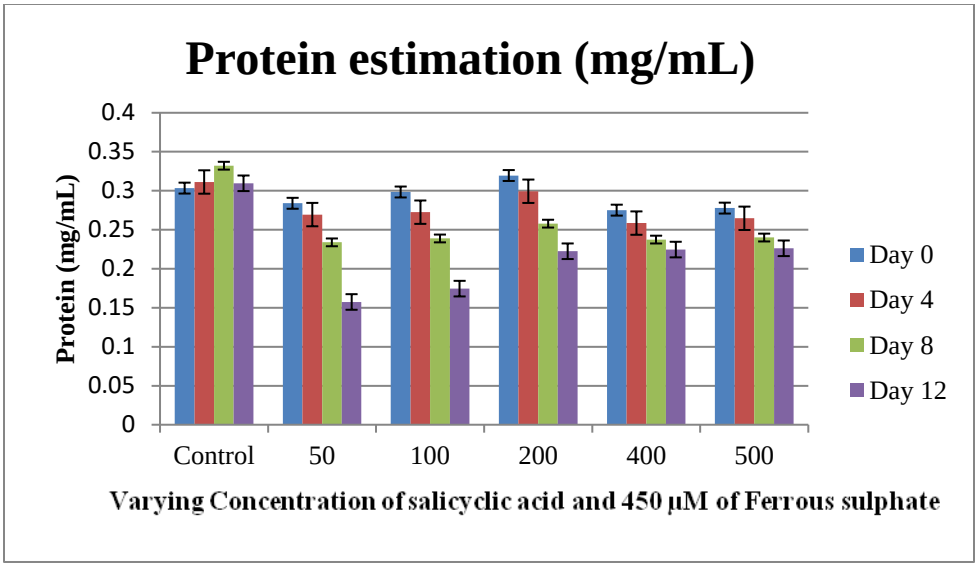
Table 19: Protein content (mg/mL) of treated culture of *Haematococcus*

Concentration of salicylic acid (µM)	0th day (mg/mL)	4th day (mg/mL)	8th day (mg/mL)	12th day (mg/mL)
Control (0)	0.43±0.010	0.44±0.011	0.47±0.045	0.43±0.054
A (50)	0.40±0.015	0.38±0.011	0.33±0.026	0.22±0.028
B (100)	0.42±0.015	0.38±0.025	0.33±0.048	0.24±0.048
C (200)	0.45±0.015	0.42±0.13	0.36±0.011	0.31±0.014
D (400)	0.40±0.015	0.36±0.024	0.33±0.25	0.31±0.024
E (500)	0.40±0.015	0.37±0.055	0.34±0.025	0.32±0.056

*values given are average of three replicates

Cultures A, B and C showed significant decrease in protein content, over the course of 12 days. Culture A, B and C showed 41-46% decrease in the protein content. Culture D and E shows lesser decrease of 17% as, only live cells shows the transition from green to red stage. Change in the protein profile of the culture is a confirmation of cell undergoing stress. Graph 12 B gives bar representation protein estimation in *Haematococcus* sp. in mg/mL. Cell undergoing transition from green stage to the red aplanospore stage shows decrease in protein content of the cell. Protein content of the cell decreases to up to 21% (Kim *et al*., 2015). Lorenz *et a*l., 1999, observed slightly higher percentage that is 23% of *Haematococcus* cells during red stage. Amino acids such as glutamic acid, aspartic acid, leucine and alanine forms the major part of this protein content (Shah *et al*., 2016).

Figure 20: Protein content (mg/mL) of treated culture of *Haematococcus*



v) Astaxanthin estimation

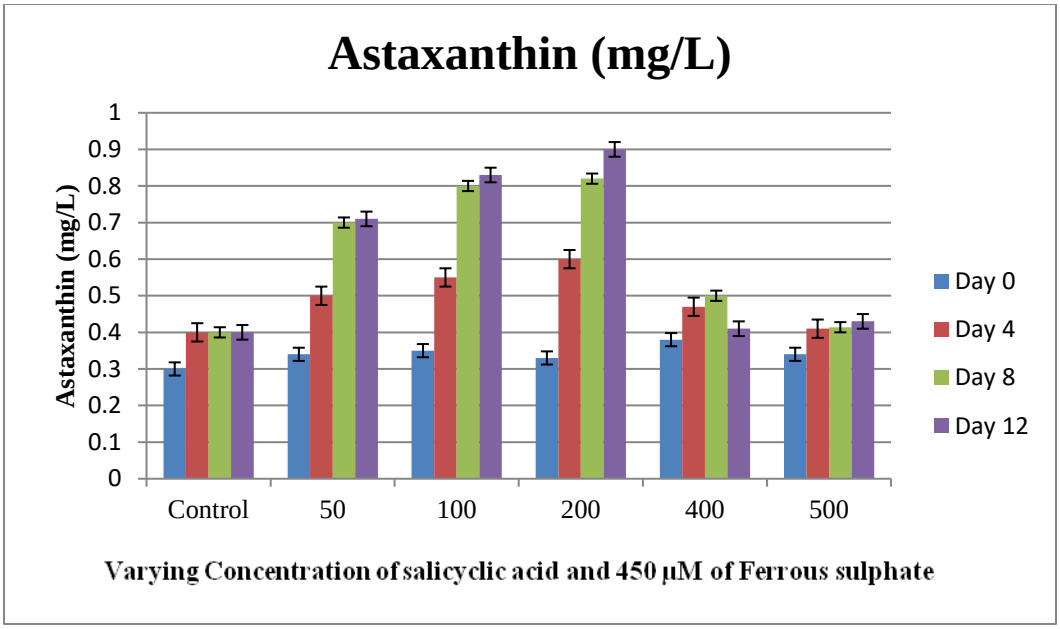
Astaxanthin, is accumulated only during unfavourable conditions. Normally, cell accumulates astaxanthin, over a period of time. There are various ways of inducing the cell to produce astaxanthin. This greatly reduces the time period required to accumulate astaxanthin. Figure 3 shows the microscopic images of treated and untreated *Haematococcus pluvialis.* Raman et al (2011) showed that salicylic acid causes secondary carotenoid formation in *Haematococcus pluvialis*. The astaxanthin accumulation in treated culture was determined (Table 20; Figure 21)

Table 20: Astaxanthin (mg/L) accumulation in treated culture in *Haematococcus*

Concentration of salicyclic acid (µM)	0^{th} day (mg/L)	4^{th} day (mg/L)	8^{th} day (mg/L)	12^{th} day (mg/L)
Control (0)	0.30±0.020	0.40±0.015	0.40±0.016	0.40±0.018
A (50)	0.34±0.014	0.50±0.04	0.70±0.025	0.71±0.025
B (100)	0.35±0.018	0.55±0.026	0.80±0.024	0.83±0.019
C (200)	0.33±0.020	0.60±0.014	0.82±0.024	0.90±0.015
D (400)	0.38±0.024	0.47±0.025	0.50±0.012	0.41±0.014
E (500)	0.34±0.020	0.41±0.020	0.41±0.018	0.43±0.015

*values given are average of three replicates

Figure 21: Astaxanthin accumulation (mg/L) in treated culture of *Haematococcus*



Highest astaxanthin content was obtained on 12th day of treatment. Culture A, B, C, D and E showed 61, 63, 64 and 10 and 20 % respectively, increase in astaxanthin content after 12 day treatment. Culture D and E showed least astaxanthin content, whereas culture C showed highest accumulation and highest accumulation rate of astaxanthin (Table 21).

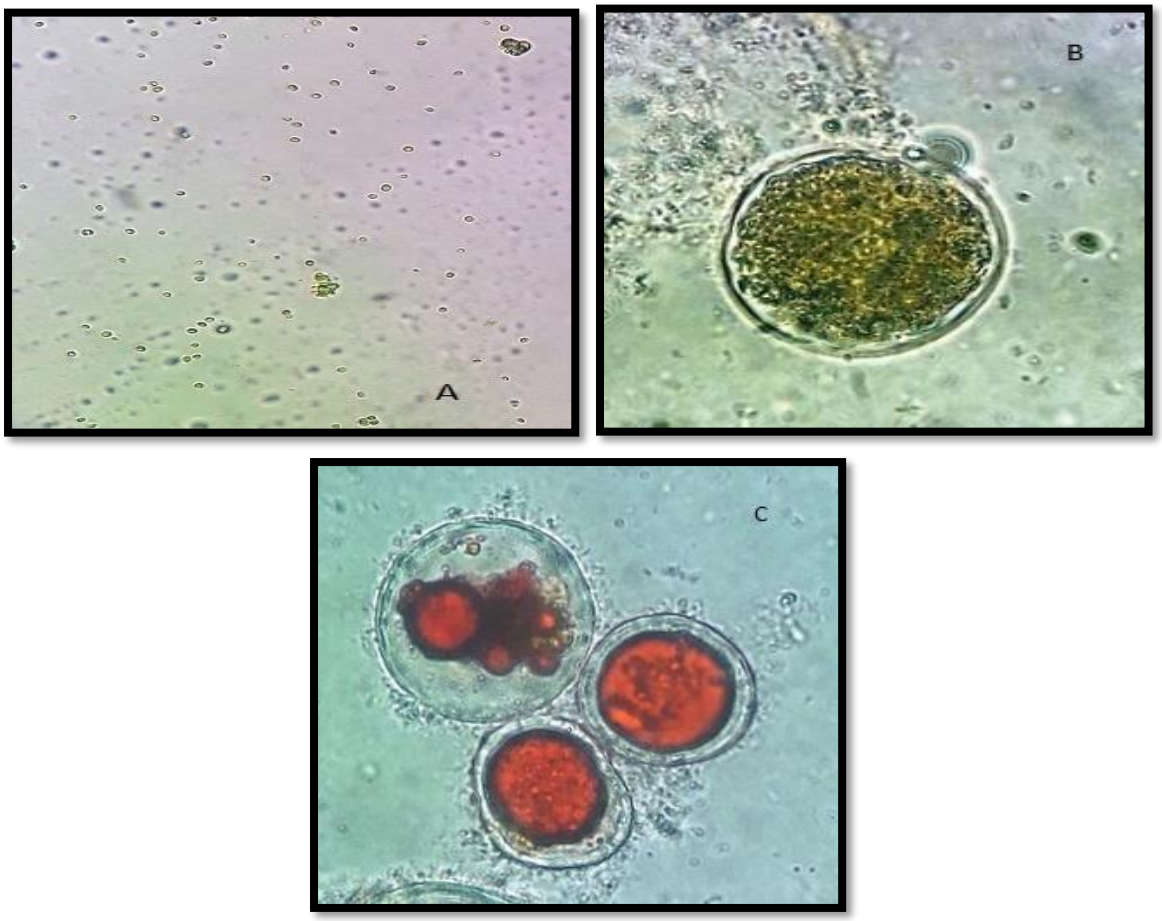
Table 21: Astaxanthin accumulation rate (mg/L/day) of treated culture of *Haematococcus*.

Concentration of salicylic acid (µM)	Astaxanthin accumulation rate (mg/L/day)
Control (0)	0.008
A (50)	0.030
B (100)	0.040
C (200)	0.0450
D (400)	0.0035
E (500)	0.0073

Figure 22: A) Photomicrograph of untreated culture of *H. pluvialis.*

B) Photomicrograph treated cell undergoing change from green stage to red stage cell.

C) Photomicrograph treated cells accumulating astaxanthin.



(Images taken at magnification of 40x)

Conclusion

1. Growth measurements showed that highest biomass production was achieved in indoor culture condition coupled with the carbon dioxide sparging. Dry biomass obtained in indoor culture with carbon dioxide sparging was 0.387 mg/mL, cell count obtained was of 10.46 x 10^6 cells/mL and absorbance obtained was 1.181 at 680 nm.
2. Chlorophyll a and b was highest in the indoor culture with carbon dioxide sparging. Chlorophyll a was 3.46 mg/L and chlorophyll b was 6.44 mg/L, on 24^{th} day.
3. Nitrate and phosphate estimation of culture filtrate showed that at the end of cultivation period, phosphate concentration in the medium became negligible and lowest nitrate concentration of 0.738 mg/mL, was observed in the culture with indoor conditions with carbon dioxide sparging.
4. Highest astaxanthin of 1.263 mg/L was observed in outdoor culture with carbon dioxide sparging under no stress.
5. Growth measurements of cells under stress conditions such as treatment with salicylic acid and ferrous sulphate, indicated growth retardation.
6. Chlorophyll *a* and *b*, in *Haematococcus* was also lowered and chlorophyll b was severely affected by the presence of salicylic acid and ferrous sulphate in the medium.
7. Effect of stress on the cell, was confirmed by protein and total carbohydrate estimation and it was observed that total carbohydrate concentration of the cell increased whereas, protein content decreased, with treatment.
8. 0.868 mg/L of astaxanthin was produced under stress conditions, when cells were treated with 200µM of salicylic acid and 450 µM of ferrous sulphate.

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