Screening of microalgal isolates for pigments

DISSERTATION

Submitted by

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Master of Science in Biotechnology

Under the guidance of

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July 15, 2019

DECLARATION

I, hereby declare that the work presented in the dissertation entitled "**Screening of microalgal isolates for pigments**"in partial fulfilment of the requirement for the award of degree of Master of Science in Biotechnology, Department of Biotechnology, Thapar Institute of Engineering and Technology (TIET) Deemed to be University, Patiala, is an authentic record of my own work during the period from January 2019 to July 2019, under the supervision of Dr. Dinesh Goyal, Professor, Department of Biotechnology, TIET. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or abroad.

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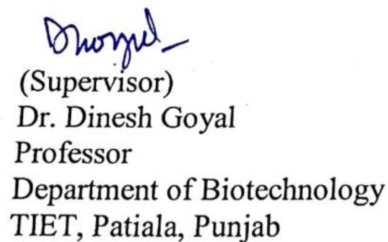
CERTIFICATE

This is certified that the thesis entitled "**Screening of microalgal isolates for pigments**" submitted by Ms. Babbanpreet Kaur (301701005),in partial fulfilment of the requirements for the award of degree of Master in Science in Biotechnology at Thapar Institute of Engineering and Technology (TIET), Deemed to be University, Patiala is a record of student bonafidework carried out by her under my supervision and guidance. The matter embodied in the thesishas not been submitted in part or full to any other university or institute for award of any other degree.



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List of abbreviations

CO_2	Carbon dioxide
HEP	Hetrocyst envelope polysaccharide
PS	Photosystem
PBP	Phycobiliprotein
RuBisCO	Ribulose-1, 5-bisphosphate carboxylase oxygenase
SC-CO_2	Supercritical carbon dioxide extraction
HCL	Hydrochloric acid
MPa	Megapascals
DME	Di methyl ether
W	Watt
kHz	Kilohertz
NaOH	Sodium hydroxide
DMF	Dimethyl formamide
SFE	Supercritical fluid extraction
T_c	Temperature
P_c	Pressure
K	Kelvin
rpm	rpm
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
CHAPS	3-[(3 cholamidopropyl) dimethylammonio] propane sulfonic acid
PC	Phycocyanin
PE	Phycoerythrin
DEAE	Diethylaminoethyl cellulose
UV	Ultraviolet
LDL	Low density lipids
HDL	High density lipids
ROS	Reactive oxygen species
NK Cells	Natural killer cells
PPAR	Peroxisome proliferator activated receptor
PGE2	Prostaglandins

LTB4	Leukotrienes
TNF-α	Tumour necrosis factor
ALT	Alanine amino transferase
MDA	Malondialdehyde
AST	Aspartate amino transferase
BBM	Bold basal medium
PAR	Photosynthetically Active Radiation
CCA	Complementary chromatic adaptation
LDL	Low density lipoprotein

List of symbols

Symbol	Meaning
nm	Nanometer
µm	Micrometer
°C	Degree Celsius
mg	Milligram
g	Gram
µg	Microgram
v/v	Volume by volume
%	Percentage
Kg	Kilogram

Abstract

Microalgae are source of various valuable pigments which have applications in pharmaceuticals, neutraceuticals and as live feed stock. In the present study native algae were isolated and screened for highest pigment content, followed by enhancement of the pigment production. Screening was done to find out the maximum pigment producing microalgae and it was found that *Chlorella* sp. produced maximum chlorophyll, *Anabaena* sp. produced maximum phycocyanin, *Nostoc* sp. produced maximum phycoerythrin whereas *Hematococcus pluvialis* was a good souce of astaxanthin. Increase in pigment production was observed by the inclusion of carbon dioxide sparging in growth media. Growth measurement showed increase in the absorbance with respect to the days and other studies for pigments such as astaxanthin, chlorophyll and phycobilins showed increase in the pigments comaparative to without carbon dioxide sparging. Amount of chlorophyll *a* obtained was maximum (11.87 mg/l) with CO_2 sparging and 11.27 mg/l without CO_2 sparging. Maximum phycocyanin obtained was 0.17 mg/ml by CO_2 sparging from *Anabaena* sp. (DGNB1) which was relatively high compared with 0.09 mg/ml without CO_2 sparging. Similarly astaxanthin content increased from 0.21 mg/l to 0.28mg/l by CO_2 sparging.

Introduction

Microalgae are photosynthetic microorganisms. They are ubiquitous as they can survive in almost every type of environment. Depending on the species, various bioactive compounds can be extracted from different microalgae, such as pigments, polysaccharides, triglycerides, fatty acids and vitamins. Use of microalgae is advantageous because they require water and small amount of nutrients for their growth and produces large biomass due to their short growth cycle. Microalgae can sequester CO_2 from the gases released by industries. Due to their medicinal properties they can also be used in pharmaceuticals (Bhalamurugan *et al.*, 2018).They are source of wide variety of metabolites such as chlorophyll, astaxanthin and phycobiliproteins.

Astaxanthin is a ketocarotenoid with structure 3, 3' –dihydroxy-β, β'-carotene- 4, 4'-dione and belongs to class of the terpenes. It has 2 carbonyl groups, 2 hydroxyl groups and 11 conjugated ethylenic double bonds. It exists as three different stereoisomers - (3S, 3'S), (3RS, 3'RS) and (3R, 3'R) out of which only former exists naturally whilst latter two are synthesized synthetically. In *Hematococcus pluvialis*, astaxanthin accumulation is observed only in encysted cells and this can be induced by giving stress in the form of nitrogen or phosphate starvation, elevated temperature or salt stress. Astaxanthin has wide applications as it can be used as nutraceutical, cosmeceutical, feed additive and as feed to livestock. To explore its possibilities as super antioxidant, studies have been undertaken in food, cosmetic and medical fields.

Phycobiliproteins are water-soluble chromoproteins assembled into phycobilisomes, which are macro molecular aggregates attached to the thylakoid membranes on its outer surface. They can be produced by eukaryotic microorganisms such as rhodophyta or prokaryotic microorganisms such as cyanobacteria. They are only found in microalgae and cannot be synthesized synthetically. The cyanobacteria contain c-phycocyanin, allophycocyanin, c-phycoerythrin and phycoerythrin. The covalently bound prosthetic group provides the colour to phycobiliproteins. The market value of these pigments depends upon their purity which is usually determined by taking absorbance. Phycobilins also have antioxidant properties and due to their colouring property they can also be used as alternative to synthetic food colouring agent (Sudhakar *et al.*, 2014) as well as in cosmetic industry (Sekar *et al.*, 2008).

Chlorophyll is another valuable microalgal derived compounds which can be of two types chlorophyll *a* and chlorophyll *b* (Hosikan *et al*., 2007). It has four pyrrole rings which comprises in the skeleton of porphyrin macromolecule. Each pyrrole ring has one nitrogen and four carbon atoms. Attachment of single isocyclic to one of pyrrole rings results in the formation of phorbin structure (Humphrey *et al*., 2004). The difference between chlorophyll *a* and chlorophyll *b* is that in chlorophyll, the methyl group in ring II of chlorophyll *b* is replaced with formyl group (Scheern *et al*., 2004). The structural difference results in change of absorbance spectra of chlorophyll *a* and *b* results in chlorophyll *a* being blue/green pigment with absorption from 660 to 665 nm whereas, of chlorophyll *b* being green/yellow with maximum absorbance from 642 to 652 nm (Humphrey *et al*., 2004). It has applications in the food industry as colouring agent as well as in pharmaceutical industries as antioxidant and antimutagen (Hosikian *et al*., 2010).

Several companies have commercialised production of these metabolites from microalgae, however their cost of production and including extraction and purification is high. Extensive work is required for cost effective biomass production and selection of suitable high yielding microalgal strains for the production of phycobilins, astaxanthin and chlorophyll. Present work is an attempt to screen different microalgal isolates from soil and waste water for pigments and their production.

Review of literature

Microalgae are photosynthetic microorganisms, having size of 2-20µm. Most microalgae have immense value as they are rich source of various metabolites such as pigments, fatty acids, amino acids, vitamins (Lee *et al*., 2019).

3.1 Characteristics of different microalgae

3.2.1 *Hematococcus pluvialis*

The *Hematococcus* sp. genus was created in 1828 by Agardh. *Hematococcus pluvialis* was first described in 1844 by Flotow. It is a fresh water species of the Chlorophyta. The first detailed analysis of nomenclature was done by Droop in 1956. The cells can be ovoid, ellipsoid or spherical having two flagella whose strands branch out towards wall in certain species. Also there is wide space between the protoplast and cell wall which is filled with slimy substance. There is large nucleus which is present along with nucleolus in the centre. In the halfway along the cell there is club shaped stigma. There is cup shaped chloroplast in periphery which reticulates with many pyrenoids. There are more than 60 contractile vacuoles which are usually scattered in the cytoplasm. The aging cell shows the deposit of astaxanthin near the nucleus. Aplanospores are formed in the adverse conditions by the deposition of thick wall close to the protoplast inside the outer cell wall. The species are 34 – 37.5 µm in diameter and up to 5 µm broad and 63 µm long. The aplanospores are spherical and can reach the diameter upto 3 times the vegetative cells (Bai *et al*., 2016).

Reproduction and life cycle

The life cycle of *Haematococcus pluvialis* consists of mainly four phases which are macrozooids (zoospores), microzooids, palmella and hematocysts (aplanospores) (Hazen *et al*., 1899; Elliot *et al*., 1934). The microzoids are derived from excessive division of hematocysts whereas macrozoids are larger than microzoids and more or less spherical with typical widely separated wall. Both the forms have ability to form resting cells with the deposition of new, thick and resistant cell wall around the cell membrane (Bai *et al*., 2016).

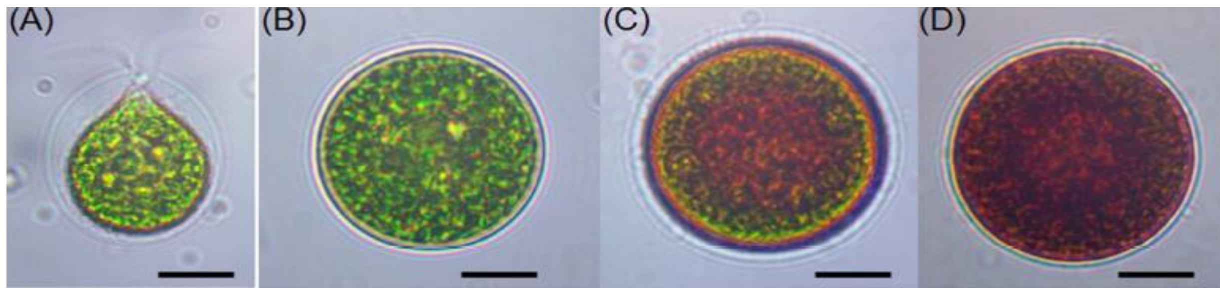

Figure 1: Light microscopic images of *H.pluvialis* cells in life cycle.

A. Green vegetative motile cells **B.** Green vegetative **C.** palmella cells
D. Aplanospore (Astaxanthin forming cell) Scalebar: 10µm
(Shah *et al*., 2016)

Production of motile cells takes place asexually by simple division with longitudinal plane. Divisions take place and the daughter cells called zoospores form fully in the parent cells. These remain motile until the flagella are left and daughter cells are released by the cell walls. *H.pluvialis* has very short motile phase as its flagella are dropped sooner. The young palmella cells enlarge and form the hematocysts and further undergo division to form aplanospores under adverse conditions. It was observed that zoospores are released by alkinetes when are transferred to fresh media and divisions are restricted to maximum of 4 doublings (Lee *et al*., 1994), it was also confirmed by Bai *et al* (2016). After enlargement by undergoing few divisions the palmella stage can revert back to motile stage. Gamete formation takes place the very next day with starvation of culture for at least one month along with the supply of fresh medium. There are usually 32 - 64 gametes formed with the size less than 10 µm (Bai *et al*., 2016).

3.2.2 *Anabaena* and *Nostoc*

Cyanobacteria are prokaryotic photosynthetic microorganisms which are distributed worldwide. According to the description of *Anabaena* sp. by Desikachary *et al* (1959) they can be identified by the presence of trichomes, absence of sheath or presence of sheath which is more or less diffluent and forming floccose or free mucilaginous thallus. They are filamentous cyanobacteria and fix nitrogen in the absence of nitrogen by differentiating into specialized cells called heterocyst. Nitrogen is fixed by the enzyme nitrogenase which is sensitive to oxygen thus is protected within the hetrocyst by outer covering made up of two layers of glycolipids. There is also homogenous layer of polysaccharide which is called HEP (Hetrocyst

envelope polysaccharide) which works in favour by inactivating oxygen generating complex of PS II (Photosystem II) and other factors such as physical damage (Yossef *et al.*, 2011).

Reproduction and life cycle

In cyanobacteria asexual reproduction takes place by the formation of hormogonia or baeocytes or colonies fragmentation. Hormogonia are characteristic of all the filamentous cyanobacteria. These are short pieces of trichome which gets detached from parent filament and move away with gliding which further gets developed into a separate filament. It can be distinguished on the basis of gliding motility also with the absence of heterocyst.

To fulfil the different function under different environmental conditions *Anabaena* sp. can have three distinct phases: the vegetative phase, the heterocyst, and the akinete phase. The most photosynthetic activity takes place in vegetative stage as it utilize both chlorophyll *a* (Chl *a*) and phycobiliproteins (PBP) for absorbing light. The heterocyst stage specializes in reducing atmospheric nitrogen and achieved in the absence of combined nitrogen (Herdman *et al.*, 1988). Akinates form under the adverse conditions such as nutrient stress and light quality (Fay *et al.*, 1984). They are larger than vegetative cells and granular due to accumulation of glycogen. They have greater resistance to cold which makes it most prominent property of them.

The heterocyst cells are larger than vegetative cells and they appear under microscope as empty cells. They do not perform photosynthesis. They neither fix CO_2 nor O_2. They show high rate of respiratory O_2 consumption (Lee *et al.*, 2008).

3.2.3 *Chlorella*

Chlorella sp. belongs to the chlorophyta division and was first discovered by Martinus Willem Beijerinck, as a microalga with an apparent nucleus in 1890 (Beijerinck *et al.*, 1890). They are 2-10µm, spherical microscopic cell. There is cell wall outside which preserve the integrity of the cell to protect cell from harsh environment and invaders. During the early development in its autosporangia, newly form cell wall form 2nm thin electron-dense unilaminar layer. They gradually become 17–21 nm in thickness after maturation, and a microfibrillar layer forms which shows chitosan like layer made up of glucosamine (Kapaun *et al.*, 1995; Yvonne *et al.*, 2000). The cell wall and its composition keeps on changing according to environment and growth condition (Atkinson *et al.*, 1972). The cytoplasm is made up of water, minerals and soluble proteins which keep the nucleus, vacuoles, golgi bodies along with the internal organelles (Kuchitsu *et al.*, 1987; Solomon *et al.*, 1999).

The single chloroplast is present with double enveloping membrane which is made up of phospholipids. The outer membrane chloroplast of *Chlorella* sp. is permeable to metabolites and ions whereas inner membrane is permeable to only specific type of proteins. Inside the chloroplast, there are granules made up of amylose and amylopectin which forms under adverse conditions. The pyrenoid is the hub of carbon fixation and contains high levels of an enzyme called ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCO). The dominant pigment is synthesized in the thylakoids which mask other pigments example lutein. Accumulation of globules which are made up of lipids can be found under the nitrogen stress conditions (Van den Hoek *et al*., 1995; Lee *et al*., 2008).

Reproduction and life cycle

C. vulgaris are non- motile and autospores. They reproduce rapidly asexually making them multiply within 24 hours by autosporulation when grown under optimal conditions (Yamamoto *et al*., 2004). In this type of reproduction four daughter cells simultaneously formed inside the mother cell with their own cell walls (Yamamoto *et al*., 2004). After maturation the daughter cells rupture and feed on the remaining debris of mother cell (Yamamoto *et al*., 2005).

3.2 Extraction methods of different pigments

3.2.1 Astaxanthin

Astaxanthin is a xanthophyll which contains carbon, hydrogen and oxygen. The molecule has polyene chain with two terminal rings joined on the both sides. The molecule has asymmetric carbon on ionone rings along with hydroxyl group on both sides of the molecule. These fatty acids react with esters forming mono-esters, whereas when both hydroxyl groups reacts, they result in di-ester formation (Higuera-Ciapara *et al*., 2006). Due to the lipophilic nature of astaxanthin it can be dissolved in various solvents and oils. The different methods have been used for extraction of astaxanthin such as different solvents, acids, edible oils microwave assisted, supercritical fluid extraction method (SC-CO_2) and enzyme based methods for astaxanthin extraction (Rangarao *et al*., 2013; Mahfuzur *et al*., 2016). In an extraction method 70% recovery was observed when, encysted cells were treated with 40% acetone for 2 minutes at 80°C followed by kitalase, cellulose, and abalone and acetone powder (Kobayashi *et al*., 1997). According to Sarada *et al* (2006) hydrochloric acid gave 80% recovery of astaxanthin, when *Hematococcus* sp. was extracted using different solvents. Increase in the yield was found with HCl treatment at different temperatures along with 15 and 30 minute sonication (Mendes *et al*., 2001). When vegetable oils such as soyabean, corn, olive and grapeseed oils were used

for extraction, highest recovery was found to be 93% with olive oil from *Phaffiar hodozyma* (Kang *et al.*, 2008). When microwave assisted extraction was performed at conditions 75°C for 5 min it resulted in 75% of astaxanthin (Ruen-nagm *et al.*, 2010). The astaxanthin content was found to be higher in acetone extract (Storebakken *et al.*, 2004). Super critical extraction is another method which can be used for extraction of astaxanthin and has been used widely for industrial scale extractions. The system can be operated at moderate temperature due to low critical temperature of CO_2 thus preventing the degradation of the compound (Machmudah *et al.*, 2006). The SC-CO_2 is the most favourable criteria, considering the quality of the astaxanthin. It provides shorter extraction time and limited use of toxic organic solvents thus making this method cheap, inert, stable and non-toxic (Guedes *et al.*, 2011). SC-CO_2 combined with vegetable oil and ethanol increased the extraction efficiency by 80-90% in *Hematococcus* sp. (Nobre *et al.*, 2006; Krichnavaruk *et al.*, 2008). Another method was pressurized fluid extraction method in which, firstly dry cells were pre-treated and frozen using liquid nitrogen followed by running in a column by taking isocratic mixture of acetone and water as mobile phase. Pressurized liquid extraction has many advantages such as less solvent, shorter time over traditional methods of extraction (Jamie *et al.*, 2009). Liquefied dimethyl ether can also be used to directly extract the astaxanthin from *Hematococcus* sp. (Boonnoun *et al.*, 2014). Another way of extracting astaxanthin is firstly extracting with solvents and evaporating by using rotary evaporator, then measuring the absorbance at 476-480nm by re-dissolving in solvent to estimate astaxanthin content (Rangarao *et al.*, 2009). The SC-CO_2 extraction with conditions 20 MPa, at 60°C and using 2ml of ethanol for 1 hour of treatment time gave the astaxanthin 2.45mg/g DW (Fuji *et al.*, 2012). The extraction done by SC-CO_2 at 20 MPa with 13% ethanol for 120 min at 55°C gave 83% recovery of astaxanthin whereas with 50% ethanol at 7MPa, 45°C for 120 min extraction time gave 124.2% recovery (Reyes *et al.*, 2014). Direct extraction by using liquefied di methyl ether (DME) at 25◦C and 0.59 MPa without drying, heating or cell disruption gave 1 mg/g cells (Boonnoun *et al.*, 2014) whereas using acetone:HCl in ratio 5:5 at 70◦C gave the recovery of 19.8mg/gm cell (Dong *et al.*, 2014). As an extraction method ultrasound also can be used along with the solvent Ethyl alcohol and Ethyl acetate (20:1) for 16 min at 41°C and frequency of 40 kHz along with 200W gave the recovery 28mg/g (Zou *et al.*, 2013). Another method for extraction is grinding (Jamie *et al.*, 2010) in which repetitions of grinding were done thrice with pressurized hexane at 10.3 MPa which gave recovery of 35mg/g cell. Enzyme treatment can also be used such as viscozyme and alcalase with temperature 50ºC for 2h, which gave astaxanthin of 2649 ± 359 μg/gm cell (In *et al.*, 2009)

Table 1. Extraction methods for extracting pigments from *H. pluvialis*

Method	Solvent	Extraction efficiency	References
Lyophilization	40%(v/v) acetone	87% recovery	Kobayashi *et al.*, 1997
Solvent extraction	NaOH and acetone	7mg/g cell	Mendes-Pinto *et al.*, 2001
SC-CO_2	Ethanol 9.4% (w/w)	97% recovery	Valderrama *et al.*, 2003
SC-CO_2	10% ethanol (v/v)	90% recovery	Nobre *et al.*, 2006
SC-CO_2	-	80% recovery	Machmudah *et al.*, 2006
Acid digestion (acetone extraction)	Acetone	87% efficiency	Sarada *et al.*, 2006
SC-CO_2	0-12% vegetable oils	51% recovery	Krichnavaruk *et al.*, 2006
Saponification with methanolic NaOH (0.02M)	Dodecane, methanolic NaOH (0.02M)	85% efficiency	Kang *et al.*, 2008
Enzyme treatment	Viscozyme and alcalase	2649 ± 359 µg/gcell	In *et al.*, 2009
Grinding	3 repetitions of grinding, pressurized hexane	35 mg/g cell	James *et al.*, 2010
SC-CO_2	Ethanol	2.45 mg/g DW	Fuji *et al.*, 2012
SC-CO_2	1.25-8.75% ethanol (v/v)	87.4% recovery	Wang *et al.*, 2012
SC-CO_2	0.154-1% ethanol (v/v)	74% recovery	Pan *et al.*, 2012
SC-CO_2	1.25-8.75% ethanol (v/v)	87.4% recovery	Wang *et al.*, 2012
Ultrasonication	Ethanol and ethyl acetate (20:1)	28 mg/g	Zou *et al.*, 2013
Solvent treatment	HCl: acetone (5:5)	1908 mg/g cells	Dong *et al.*, 2014
Solvent treatment	Dimethyl ether (DME)	1 mg/g cells	Boonnoun *et al.*, 2014
SC-CO_2	13% ethanol (w/w)	83% recovery	Reyes *et al.*, 2014
Solvent along with pressure pre-treatment	Solvent ethyl acetate	20 pg/cell	Praveen *et al.*, 2015
Homogenization along with solvent extraction	Solvents acetone, methanol, separately used along with HCl pre-treatment	95% with acetone and 10% with methanol	Singh *et al.*, 2018

Saponification with methanolic NaOH (0.02M) followed by sedimentation in darkness for 12 h at 4ºC after dodecane mixing gave 85% efficiency (Kang *et al*., 2008).

3.2.2 Chlorophyll

Chlorophyll is mainly of two types; Chlorophyll *a* and chlorophyll *b*. Chlorophyll molecule has skeleton of porphyrin macrocycle, having four pyrrole rings. There is formation of phorbin structure due to attachment of single isocyclic ring to pyrrole ring (Humphrey *et al*., 2004). Every pyrrole ring have four carbon along with one nitrogen atom and all the nitrogen atoms are inwards which creates space for Mg^{2+} metal to easily bind (Scheer *et al*., 2004). The difference between the chlorophyll *a* and *b* is that in chlorophyll *b* there is formyl group attached whereas in chlorophyll *a* there is attachment of the methyl group in ring II (Scheer *et al*., 2004; Cubas *et al*., 2008). This difference in the structure results in different absorbance spectra, chlorophyll *a* pigment absorbance from 660 to 665nm and chlorophyll *b* having maximum absorbance from 642 to 652nm.To quantify the amount of chlorophyll firstly the intracellular chlorophyll must be extracted. The organic solvent extraction method is traditional method that has been employed for extraction (Jeffery *et al*., 1997; Simon *et al*., 1998). The extraction process using the organic solvents infiltrates the cell membrane thus dissolving the major components of chloroplast membrane which are lipids as well as lipoproteins (Jeffery *et al*., 1997).The cell disruption can be achieved through sonication, homogenisation and grinding, which significantly improves the solvent extraction (DP *et al*., 1984; MD *et al*., 2009). Simon *et al* (1998) found that only one fourth of potential chlorophyll *a* was able to be removed without the cell disruption. They conducted their chlorophyll extraction in the dark and in ice to prevent from further degradation. In an experiment it was found that methanol was able to extract thrice more chlorophyll than the acetone taken in concentration of 90%. Storage conditions of filtered microalgae, organic solvent used and extraction steps also affect the competence of the solvent extraction (Schumann *et al*., 2005). Degradation happens when the molecule is exposed to additional high temperature, oxygen/air, light and different pH conditions. Methanol and ethanol are found to be superior to acetone (Jeffery *et al*., 1997; Sartory *et al*., 1984). It was found that methanol and 95% ethanol were better than 90% acetone (DP *et al*., 1984). However, it was observed that organic solvent methanol in extraction results in unstable solution which leads to chlorophyll *a* degradation (Jeffery *et al*., 1997). Even though 100% acetone was unable to extract high yield of chlorophyll but it still strongly prevented the degradation of product formation. It was also found that dimethyl formamide (DMF) was better

than methanol, 90% ethanol, 100% ethanol and 90% acetone. Also extraction with DMF saves from additional cell disruption steps as pigments can fully extracted after number of steps of soaking in DMF (MD *et al*., 2009). The amount of chlorophyll extraction also depends on the algal growth stage. Microalgae at stationary phase show significantly higher chlorophyll *a* compared to the different phase of growth cycle (Schumann *et al*., 2005). The other method for extraction is Supercritical fluid extraction (SFE) which can use as replacement of organic solvents (Herrero *et al*., 2010).The supercritical stage is achieved by exposing the substance to conditions beyond its critical temperature (T_c) and pressure (P_c). The solvent power of supercritical fluids is lowest for high molecular weight analytes whereas is high for slightly polar or non-polar solvents. The fluid can easily be removed through expansion to atmospheric pressure (Brunner *et al*., 2005). The most frequently used fluid for SFE is CO_2 as it is readily available, cheap, and non-flammable and slightly inert. The critical temperature and pressure of CO_2 is 304.1 K and is 7.38MPa respectively. It avoids excessive heating thus high quality yield is extracted. By using SC-CO_2 extraction, highly purity and solvent free extract can be achieved (Herrero *et al*., 2006).

Michael *et al* (2007) extracted chlorophyll by two methods using ultrasonication and homogenisation followed by filtration and centrifugation at 4°C. For homogenisation they did centrifugation at 20000 rpm for 15seconds. It was found that highest concentrations of Chl *a* were obtained in acetone and dimethyl formamide (DMF). The Ultrasonication as extractant method can be widely used due to its efficiency and shorter time requirement (Parniakov *et al*., 2015). The process of extraction was performed using ultrasonic bath at different temperature ranging from 30-40 ºC and time 60-120 min. The solvents used for extraction were such as ethanol, methanol and acetone and methanol:hexane (2:1 v/v). It was found that high temperature leads to degradation of the pigments. The mixture of methanol:hexane was found to be effective for extraction (Amin *et al*., 2018).

Table 2. Methods for extraction of Chlorophyll

Microalgal species	Treatment	Reference
Stichococcus sp.	Extraction was done using 90% acetone	Rhena *et al*., 2004
Chlorella sp.	Acetone, DMF and Grinding, Ultrasound	Schuman *et al*., 2005
Anabaena torulosa	Sonication	Michael *et al*., 2007
Anabaena torulosa	Acetone, methanol and Dimethyl formamide	Michael *et al*., 2007
Dunaliella salina	DMF Methanol and Ultrasound	Macıas-Sanchez *et al*., 2009
Chlorella sp.	Methanol: hexane (2:1)	Amin *et al*., 2018

3.2.3 Phycobiliproteins

Cyanobacteria possess major light harvesting pigments called phycobiliproteins. Due to presence of blue coloured protein, phycocyanin many of cyanobacteria looks blue in colour and have been called blue green algae despite being prokaryotic (Chakdar *et al*., 2016).There is no standard technique to extract phycobiliproteins from different cyanobacteria, however various methods are there for extraction (Wiltshire *et al*., 2000). Various methods have been used such as freezing and thawing, use of sonicators and french pressure cells (Abalde *et al*., 1998; Minkovete *et al*., 2003). Enzymes such as lysozyme can also be used to extract the phycobiliproteins as they break the cells (Vernet *et al*., 1990). The use of 3-[(3 cholamidopropyl) dimethylammonio] propane sulfonic acid (CHAPS) 0.3 % asolectin along with nitrogen cavitation can achieve extraction efficiencies of larger than 85 % (Viksar *et al*., 2003). Higher extraction efficiency was achieved by freezing–thawing and sonication in comparison with tissue grinding (Lawrenz *et al*., 2011). It was found that not only freezing and thawing is efficient method but also most economical way to extract C-PC (C-phycocyanin) from cyanobacteria (Abalde *et al*., 1998; Doke *et al*., 2005). The buffers can also be used such as tris buffer (Bhaskar *et al*., 2005), HEPES buffer (Gupta *et al*., 2009) etc. For purification, different steps are available; most of these steps include ammonium sulphate precipitation, dialysis, gel filtration chromatography, hydroxy apatite chromatography, anion exchange chromatography etc. Some also include additional steps such as membrane filtration or usage of solvents preferably organic. These strategies have been used to extract and purify PC and

PE from various cyanobacteria (Chakdar *et al*., 2016). When rivanol treatment was used it was able to extract purity of 46% (Minkova *et al*., 2003). Soni *et al* (2006) used ammonium sulfate precipitation along with other methods such as gel filtration chromatography, and DEAE-cellulose column chromatography and were able to acquire phycocyanin of purity above 68%.

Table 3. Methods for extraction of Phycobiliproteins

Microalgal species	Treatment	Type	Reference
Limnothrix sp.	Distilled water, activated carbon (1%) and Chitosan	Phycocyanin	Gantar *et al., 2012*
Spirulina platensis	100 mM phosphate buffer (pH 7.0) in ratio 1:100	Phycocyanin	Chaiklahan and collegues *et al.,2012*
Alvarezi kappaphycus	Lysozyme (2ug/ml) and sonication	Phycoerythrin	Sharmila *et al., 2016*
Rhodomonas salina	Phosphate buffer (0.1M, pH 6.7) freeze at -80 ˚C	Phycoerythrin	Christina *et al*., 2017
Spirulina platensis	20mM acetate buffer containing 50 mM NaCl and 0.002 M Sodium Azide (pH 5.10) followed by freeze thawing	Phycoerythrin	Kamble *et al., 2018*
Spirulina platensis	100mM phosphate buffer (pH 7.0)	Phycocyanin	Alam *et al*., 2019

Two phase extraction (ATPE) combined with exchange chromatography was reported to get recovery of 73%. This disabled the need of multiple steps (Patil *et al*., 2006) using ammonium sulfate precipitation and anion exchange chromatography using DEAE sepharose fast flow column. Rivanol treatment on cyanobacteria was able achieve the purity more than 5 (Tchernov *et al*., 1999). On treatment with 55% ammonium sulphate, it was able to get recovery of 85% with purity 2.89 (Ranjitha *et al*., 2005). Fractionation of this PE with Sephadex G-150 further increased the purity ratio to 3.9 and 3.6 for both young and old cultures. Another protocol for extraction involves using ammonium sulphate precipitation and dialysis in combination with anion chromatography by DEAE-cellulose-11 and acetate buffer (Chakdar *et al*., 2016).

3.3 Applications of various photosynthetic pigments

3.3.1 Astaxanthin

Astaxanthin as pigment

Astaxanthin is used in aquaculture and poultry industry as a pigmentation source (Lorenz *et al*., 2000). It has importance in salmon feed industry to provide pigment to salmon (Gracia *et al*., 2005). Natural Astaxanthin can be used to tint the yolks of eggs. When hens were fed the diet supplemented with different concentrations of astaxanthin for few weeks it was found that it helped in the enhancing their muscles (Lorenz *et al*., 2000).

Astaxanthin as nutraceutical, pharmaceuticals and cosmetics

Astaxanthin also gained applications in the field of nutraceuticals and pharmaceutical industries due to its superior antioxidant activity (Lorenz *et al*., 2000). It has wide applications in the pharmaceutical and cosmetic industry which makes it most widely used Xanthophyll (Capelli *et al*., 2007).

Astaxanthin as antioxidant

Free radicals and reactive forms of oxygen e.g. peroxyl, hydroxyl and singlet oxygen are produced by different processes and metabolisms inside body. Various factors can further trigger the process such as pollution, stress, exposure to ultraviolet rays and chemicals etc. These radicals cause DNA damage and membrane damage (Guerin *et al*., 2003).

Various studies have shown that the molecular structure of astaxanthin provides potential of quenching singlet oxygen as well as against free radicals thus making it extremely effective antioxidant (Lorenz *et al*., 2000).

Studies have shown that astaxanthin performs much better than other commercially available antioxidants that are widely popular among the consumers, which includes vitamin C and vitamin E. In terms of singlet oxygen quenching test, astaxanthin proved 550 times stronger than vitamin E (Shimidzu *et al*., 1996) and 64.9 times stronger than vitamin C (Bagchi *et al*., 2001).

Astaxanthin is found to be more effective antioxidant than β-carotene, zeaxanthin, canthaxanthin, vitamin C and vitamin E. It can protect the skin, thus, preventing from damage of ultraviolet radiation, ameliorate age-related macular degeneration, increase high-density lipoproteins, protect against certain type of cancers and improve the immune system. It protects the fatty acids and sensitive membranes of the cells by effectively scavenging the lipid radicals and destroying the peroxide chain reactions (Lorenz *et al*., 2000).

Zeaxanthin, lutein, canthaxanthin and β-carotene are 10 times less potent than astaxanthin in terms of antioxidant activity. Also, it's 500 times greater than alpha-tocopherol. (Jorgensen *et al*., 1993; Shimidzu *et al*., 1996). During normal metabolic processes, stress, pollution, tobacco smoke, contact to chemicals or ultraviolet (UV) light free radicals are generated and Astaxanthin absorbs the free radicals and singlet oxygen therefore preventing from the damage of tissues. (Mortensen *et al*., 1997; Beutner *et al*., 2001).

Astaxanthin against heart diseases

LDL cholesterol ('bad' cholesterol) is linked to increase the risk of atherosclerosis. Usually oxidation of LDL in plasma does not happen and it adds on to development of atherosclerosis. Thus to reduce risk, antioxidant supplement can be a way to reduce risk (Frei *et al*., 1995). Also, clinical data shows cardiovascular diseases can be prevented by adding antioxidant in diet (Kritchevsky *et al*., 1999).

Astaxanthin as anti-inflammatory agent

Inflammation related conditions are created when phagocytic leukocytes release toxic reactive oxygen species (ROS) at the location of inflammation. Also oxidative imbalance is created due to high concentration of neutrophils at the site (Aghdassi *et al*., 2000). ROS have been linked in aggravation in inflammation in the case of asthma (Greene *et al*., 1995) and muscle damage due to physical work like exercise (Dekkers *et al*., 1996).

Astaxanthin as anti-cancer

Astaxanthin in diet also found to be effective against the mammary cancer by reducing growth of induced tumours by 50% more than other carotenoids like β-carotene and canthaxanthin (B.P. *et al*., 1999). It also inhibits the suppression of tumour fighting NK cells (natural killer cells), when supplemented to rats (Kurihara *et al*., 2002).

Astaxanthin against neurodegenerative diseases

Astaxanthin is also found to be effective against neurodegenerative diseases. In nervous system there is intense metabolic aerobic activity making the tissues susceptible to oxidative damage (Facchinetti *et al*., 1997) and can be a factor in various neurodegenerative diseases such as alzheimer's, huntington's, parkinson's and amyotrophic lateral sclerosis. As astaxanthin can cross the barrier of blood and brain in mammals, thus can be further effective antioxidant for brain. (Grant *et al*., 1997; Rijk *et al*., 1997).

Astaxanthin against diabetes

Astaxanthin show great potential in the prevention and treatment of diabetes. When astaxanthin was tested on db/db mice (a well-known obesity model for T2DM) it showed decrease in the glucose tolerance, attenuates blood glucose level and enhances serum insulin level. It showed protective effect on pancreatic cell function due to anti-oxidative properties (Uchiyama *et al.*, 2002). The similar anti diabetic effect was also reported (Bhuvaneswari *et al.*, 2014) on high fat and high fructose diet mice. The studies show astaxanthin intake decrease triglyceride levels, blood glucose levels, as well as enhance serum level of adiponectin and HDL cholesterol (Hussein *et al.*, 2007). Some recent studies also report role of astaxanthin in carbohydrate metabolism by targeting the peroxisome proliferator-activated receptor (PPAR) (Inoue *et al.*, 2012).

3.3.2 Phycobiliproteins

Phycobiliproteins as natural dyes

Phycobilins have various applications in different fields. The primary application of phycobilins to be used as natural dyes and replacement for synthetic dyes. Research has shown its applications in the field of pharmaceuticals (Sekar *et al.*, 2008).

Phycobiliproteins in food items

Even though phycobilins are sensitive to heat and light than other pigments such as gardenia and indigo, still they are more versatile to be used in food items like candies (Lone *et al.*, 2005). Phycobilins can be used in various food items such as dairy and sweets (Spolaore *et al.*, 2006).

Phycobiliproteins in cosmetics

They can be used in cosmetics such as lipsticks and eyeliners (Spolaore *et al.*, 2006). These pigments also have been studied to stimulate synthesis of collagen which is crucial structural protein of the skin thus can have applications in the field of cosmetic industry (Christaki *et al.*, 2016).

Phycobiliproteins in pharmaceutical industry

Phycocyanin can be used as an antioxidant agent, neuroprotective, anti-inflammatory and hepatoprotective agent. In vitro studies have shown that it was able to scavenge radicals (alkoxyl, hydroxyl and peroxyl), and inhibits lipid peroxidation induced by the free radicals

initiators 2, 2′ Azobis (2-amidinopropane) dihydrochloride. It was able to reduce levels of prostaglandins (PGE_2) and leukotriens (LTB_4) in the inflamed tissues. It also reduced the TNF-α (tumour necrosis factor) in the mices blood serum treated with endotoxin. It has also shown to protect neurons in various studies (Bhatt *et al.*, 2000; Romay *et al.*, 2003).

Phycobiliproteins in health

It was shown in studies that due to addition of phycocyanin, haemolysis and lipid peroxidation in red blood cells generated due to aqueous peroxyl radical and APPH were significantly lowered (Sekar *et al.*, 2008). These findings about phycocyanin show its potential in disorders related to inflammation. Spirulina extracted c-phycocyanin showed influence on the serum cholesterol and also provided powerful activity in reducing cholesterol (Nagaoka *et al.*, 2005). On human hepatitis animal models c- phycocyanin showed reduced alanine amino transferase (ALT), malondialdehyde (MDA) and aspartate amino transferase (AST) in serum (Gonzalez *et al.*, 2003).

Phycobiliproteins as antioxidant

Phycobilins also exhibit antioxidant activity. Due to their chemical structure and chelating properties they can effectively neutralize reactive oxygen species (ROS), thus reduce the oxidative stress on the body (Rodriguez *et al.*, 2012).

Phycobiliproteins as fluorescent agent

Due to their fluorescent property they are used as biochemical tracers in immuno assays, especially phycoerythrin, due to its intense florescence, it is used as sensitive indicator in pharmaceutical industry (Christaki *et al.*, 2016). They are extensively used in immunological and clinical research as labels for various biological molecules like antibodies, receptors and in various assays for diagnosis (Spolaore *et al.*, 2006).

3.3.3 Chlorophyll

Chlorophyll as pigment

Chlorophyll is a bioactive compound which can be extracted from microalgae and have various uses in food, colouring and pharmaceutical industry (Amin *et al.*, 2018). Chlorophyll can be used as colouring agent but is unstable at different pH (Spears *et al.*, 1988). So in order to make it stable as food colouring agent, chemical modification was done to replace magnesium centre

with copper ion. This complex was stable and excreted from body thus considered to be safe but concentration of free ionisable copper should be kept below 220 ppm in colouring agent as per current regulations (Humphrey *et al*., 1980; Timberlake *et al*., 1986).

Chlorophyll in pharmaceuticals

Chlorophyll has healing properties which makes it suitable for use in pharmaceuticals. It has been found that chlorophyll increases wound healing about 25%. It stops the advancements of bacteria, accelerates the wound healing process and stimulates tissue growth (Smith *et al*., 1945). It stimulates tissue growth, predicted due to similar structure to haemoglobin thus works in similar way through the enablement of rapid carbon dioxide and interchange of oxygen (Horwitz *et al*., 1951).

Chlorophyll for healing

Chlorophyll also used for treatment of ulcers. The significant health problem, in society related to chronic ulcers, is the lengthy period of treatment. It has been found that healing process gets escalated by chlorophyll. The chlorophyll derivative when applied with an ointment on affected area was found not only removes pain but also improves the appearance of the tissue in several days. After few days, the discharge and characteristic odour from ulcers was also improved with treatment for few days (Cady *et al*., 1948).

Chlorophyll as antioxidant

Many studies reported the antioxidant properties of chlorophyll. The derivatives of chlorophyll- pheophorbide *b* and pheophytin *b* have always been known as strong antioxidants (Lanfer-Marquez *et al*., 2005). Chlorophyll and its derivatives have been shown to work in prevention of cancer due to their antimutagenic and antioxidant activities. In the gastrointestinal tract, it traps the mutagens thus works in preclusion of cancer (Ferruzzi *et al*., 2007). Chlorophylls derivative chlorophyllin is similar in structure with Hemoglobin. Major difference lies in the central atom, in chlorophyll its magnesium whereas in haemoglobin its iron. Thus it increases the stability and bioavailability of compound (Vinod *et al*., 2011).

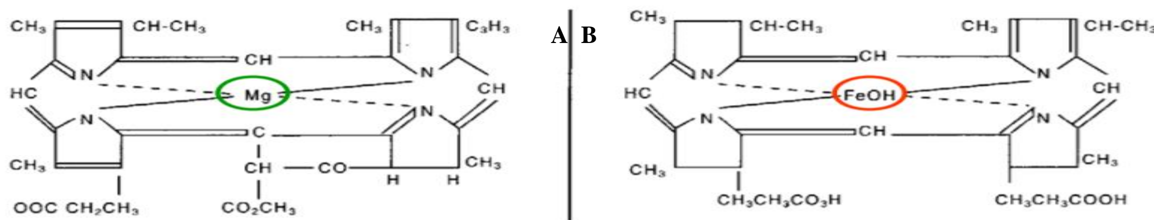

Figure 2. Molecular structure of **A.** Chlorophyll and **B.** Red blood cells (Vinod *et al.*, 2011)

3.4 Methods of isolation of microalgae

The water sample collected were diluted and plated on multiple agar media for 14 days at temperature from 20-25℃ and were further streaked until axenic uni-algal culture were obtained. In general, microalgae respond good to media with high nitrogen and phosphorus but for hetrocystous cyanobacteria the media recipes without nitrogen were more selective (Lee *et al.*, 2019).

The isolated samples were placed directly on BBM agar (Bold's Basal medium) plates and kept at 20 ± 2 ℃. After the growth the colonies were inoculated in Erlenmeyer flask having BBM medium and incubated at 20±2 °C along with shaking at 120 rpm (Ahmed *et al.*, 2014).

3.5 Parameters affecting the microalgal growth

3.5.1 Light

All forms of life depend directly or indirectly upon light for photosynthesis as source of energy making it one of the most important mechanisms of natural world. Oxygen dependent photosynthesis in microalgal cultivation can be stated as a reaction which is driven by the energy of light, in which compounds such as nutrients, water and carbon dioxide are transformed to oxygen and algal biomass which is mostly carbohydrate (Richmond *et al.*, 2004).

Sunlight is the major source for the cultivation of energy in photoautotrophic microalgae. Microalgae can use only portion of the very broad wavelength of solar irradiation and it is termed as photosynthetically active radiation (PAR). This PAR is between 400nm-750nm which is the visible light's spectral pattern and is about 40-45% of the light spectrum in total (Richmond *et al.*, 2004).

For phycobilins, light also plays an important role as both colour and light intensity can influence the synthesis of phycobilins. Complementary chromatic adaptation (CCA) is the phenomena in which relative amount of phycocyanin (PC) and phycoerythrin (PE) depends

upon light colour in cyanobacteria. This has been studied on *Calothrix* sp. strain 7601 but not on other species (Chakdar *et al.*, 2016).

Light intensity is the most important environmental factor which influences the light harvesting complex which are phycobilisomes. It is of particular importance as it varies largely in natural environment during outdoor cultivation. The impact of light has been reported in various microorganisms. It was reported by Lorimier *et al* (1992) that the ratio of PC: APC (Allophycocyanine) in *Agmenellum quadruplicatum* PR-6 during growth in the light intensity of 1260 µE as compared to that in 20 µE decresed by 1.8 folds. It was also reported that *Synechococcus NKBG 042902,* 25 µmol photons/m ^{2} /s is optimum light intensity (Takano *et al.*, 1995). Whereas, for *Nostoc UAM 206* and *N. Muscorum,* the optimum light intensity was found to be 12.5 µmol photons/m^{2}/s (Poza-Carrion *et al.*, 2001; Ranjitha and Kaushi, 2005). On the other hand, 150µmol photons/m^{2}/s was found to be optimal light intensity in *Arthronema africanum* for phycobiliprotein production (Chaneva *et al.*, 2007). Hemalata and Fatma (2009) reported that the most appropriate light intensity for maximum production of phycocyanin in *Anabaena sp.* NCCU9 is 1260 µE. Due to their pigment composition and low energy in maintenance cyanobacteria prefer low light intensities to stimulate phycobiliprotein synthesis (Chakdar *et al.*, 2016).

3.5.2 Temperature

Different strains of microalgae that exist in nature have high variations in the growth temperature. The optimum temperature for photosynthesis can vary from 15°C to 35°C (Arnold *et al.*, 2013). The optimal environmental conditions in terms of cell proliferation involve moderate light intensity (250µ mol $m^{-2}s^{-1}$) and temperature (20°C) (Giannelli *et al.*, 2015). While conducting research on astaxanthin both Evens *et al* (2008) and Giannelli *et al* (2015) have reported that increased temperature of 27°C along with nutrient starvation had increased the Astaxanthin production in *Haematococcus pluvialis.* According to most studies for accumulation of astaxanthin in *H.pluvialis*, the most suitable temperature is between 20-28°C (Fan *et al.*, 1994; Hata *et al.*, 2001; Lababpour *et al.*, 2005; Kang *et al.*, 2010; Yoo *et al.*, 2012; Wan *et al.*, 2014a). The transition of *H.pluvialis* from green vegetative stage to red stage can be induced within 2 days by providing temperature above 30°C. It was reported that there was an increase in astaxanthin about 2-3 times though this transition slowdowns the growth (Shah *et al.*, 2016). The increase in astaxanthin synthesis due to increase in temperature is because of oxygen radicals stimulation and high reactivity by them (Tjahjono *et al.*, 1994b). According to

Hata *et al* (2001), for allowing better acclimation to new condition it is preferred that change in temperature happen gradually.

3.5.3 Carbon dioxide

Microalgae, like all photosynthetic microorganisms need carbon dioxide as carbon source, which convert into chemical energy inside the algal cell (Wang *et al.*, 2008; Iersel *et al.*, 2009). In fact, microalgae can't grow in the absence of carbon dioxide and growth can be affected by the insufficient supply of CO_2. The Microalgae can get CO_2 mainly from three major sources: 1) Atmospheric CO_2 2) CO_2 emissions from industrial processes (e.g. flaring and ashes flue gases); 3) fixed CO_2 (e.g., $NaHCO_3$ and Na_2CO_3 in the form of soluble carbonates) (Brennan *et al.*, 2010). The most basic method of providing CO_2 in microalgae lifecycle is introduction of atmospheric CO_2 (Venkata *et al.*, 2013). However to harvest 1 ton of algal biomass about 1.8 tons of Carbon dioxide is needed. (Chisti *et al.*, 2007; Wang *et al.*, 2008; Iersel *et al.*, 2009).

3.5.4 Nutrients

Along with CO_2 as carbon source the inorganic nutrients are also very important in phototrophic cultivation. The combination of all these leads to production of metabolites through photosynthesis and further proliferation of microalgal cells (Iersel *et al.*, 2009). The nutrients can be divided into two categories, 1) Macronutrients and 2) Micronutrients. Macronutrients involve mainly carbon (if CO_2 supply is inadequate), nitrogen, phosphorus, hydrogen, oxygen, sulphur, calcium, magnesium, potassium and chlorine is essential for the growth of algae. Whereas, micronutrients constitute trace quantities of micro, nano or even picograms per litre and involve mainly iron, boron, manganese, copper, zinc, molybdenum, vanadium, cobalt, nickel, silicon and selenium (Suh *et al.*, 2003). Different type of media can be used for growth of *H.pluvialis.* The most frequently used medias for production of *H.pluvialis* are BG-11 (Rippka *et al.*,1979), BBM (Bischoff and Bold *et al.*, 1963), OHM (Fábregas *et al.*, 2000), KM1-basal medium with organic carbon sources in the form of sodium acetate (Kobayashi *et al.*, 1993) and their modifications. For nitrogen source, sodium nitrate was found to be most optimal but urea can also be used (Sarada *et al.*, 2002).

3.6 Mass production of microalgae

In order to grow the microalgae, efficient photobioreactors are required. Although many models have been proposed, only few of them have been practically used. Algae can either be

grown on open cultures or in closed systems (Photobioreactors) (Ugwu *et al*., 2007). Recently closed photobioreactors have gained popularity as algal growth conditions can be efficiently monitored in them. Higher biomass productivity can be obtained and contamination can be prevented with closed photobioreactors (Lee *et al*., 1995; Cogne *et al*., 2005). Algal cultures cultivation can be done both in open ponds as well under controlled conditions in photobioreactors.

3.6.1 Open pond system

Open pond system for algal cultivation has been extensively studied for last many years (Boussiba *et al*., 1988; Tredici *et al*., 1992; Hase *et al*., 2000). The categorisation of open pond can be done into natural waters such as lakes, ponds, lagoons, artificial ponds and containers. Shallow big ponds, circular ponds, tanks, and raceway ponds are most commonly used systems (Ugwu *et al*., 2007). The advantage of using open pond system is that they are easier to build and function than closed systems. However, poor light utilization by cells, evaporation losses, requirement of large land area, diffusion of CO_2 to the atmosphere are some of the major drawbacks of these systems. Furthermore, chances of contamination by other predators are higher in these systems (Ugwu *et al*., 2007). The open systems can be of many types. The most commonly used are raceway pond, circular ponds and unstirred ponds.

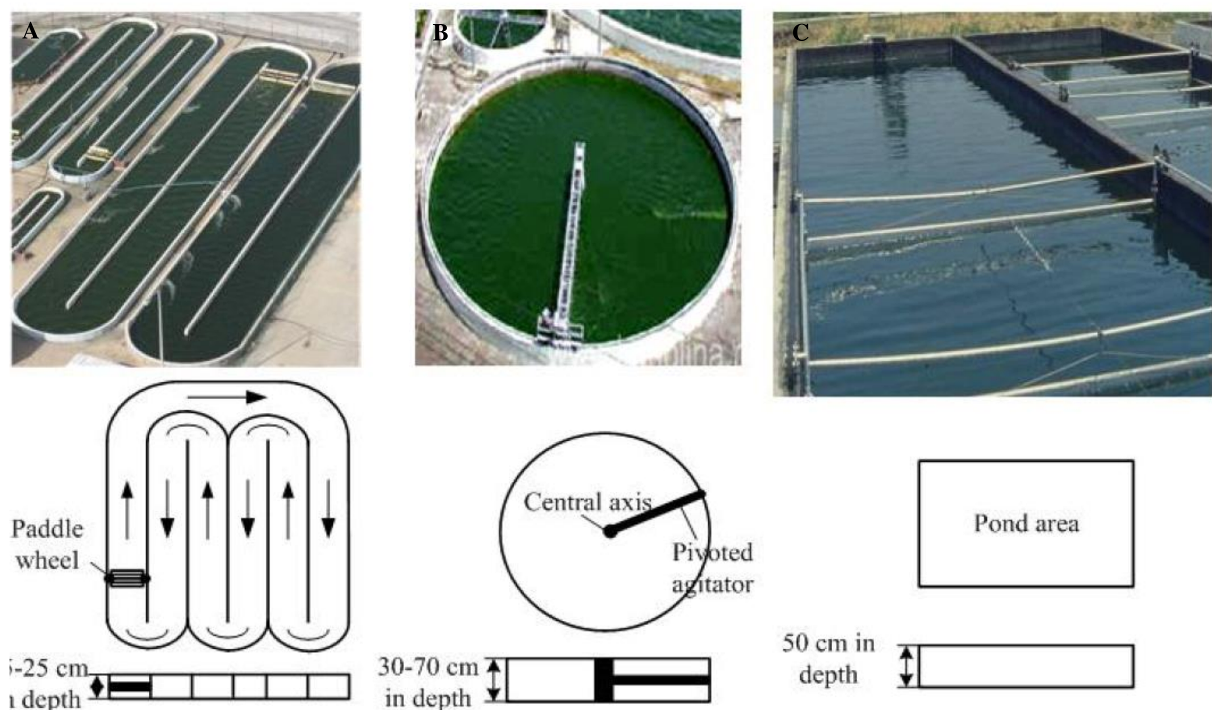

Figure 3. A. Raceway pond **B.** Circular pond **C.** Unstirred pond (Ugwu *et al*., 2007)

i) Raceway ponds

Raceway ponds are constructed as group of channels joined by individual raceway or in singlet. Channels can be built in various ways. They can be of concrete or lined with plastic or simply compacted earth. The depth of the ponds is usually around 15-30 cm and is accompanied by a paddlewheel to drive the continuous water circuit (Moheimani *et al.*, 2005; Schenk *et al.*, 2008). This allows the mixing, which exposes cells to sunlight and CO_2. This is one of the main features of basic raceway pond design. It was found by Sheehan *et al* (1998) that velocity of 10-20 cms^{-1} is effective but greater than 30 cms^{-1} is not viable as it will consume more energy.

ii) Circular ponds

Circular ponds are made like raceway ponds in design aspect and normally have diameter around 45m and depth around 30-70cm (Moheimani *et al.*, 2005). The ponds can be joined along with wastewater treatment (Garcia *et al.*, 2000). They are widely used for production of beta carotene (Lee *et al.*, 2001). Size can be a limiting factor for these ponds as the rotating arms gets too long resulting in poor mixing efficiency.

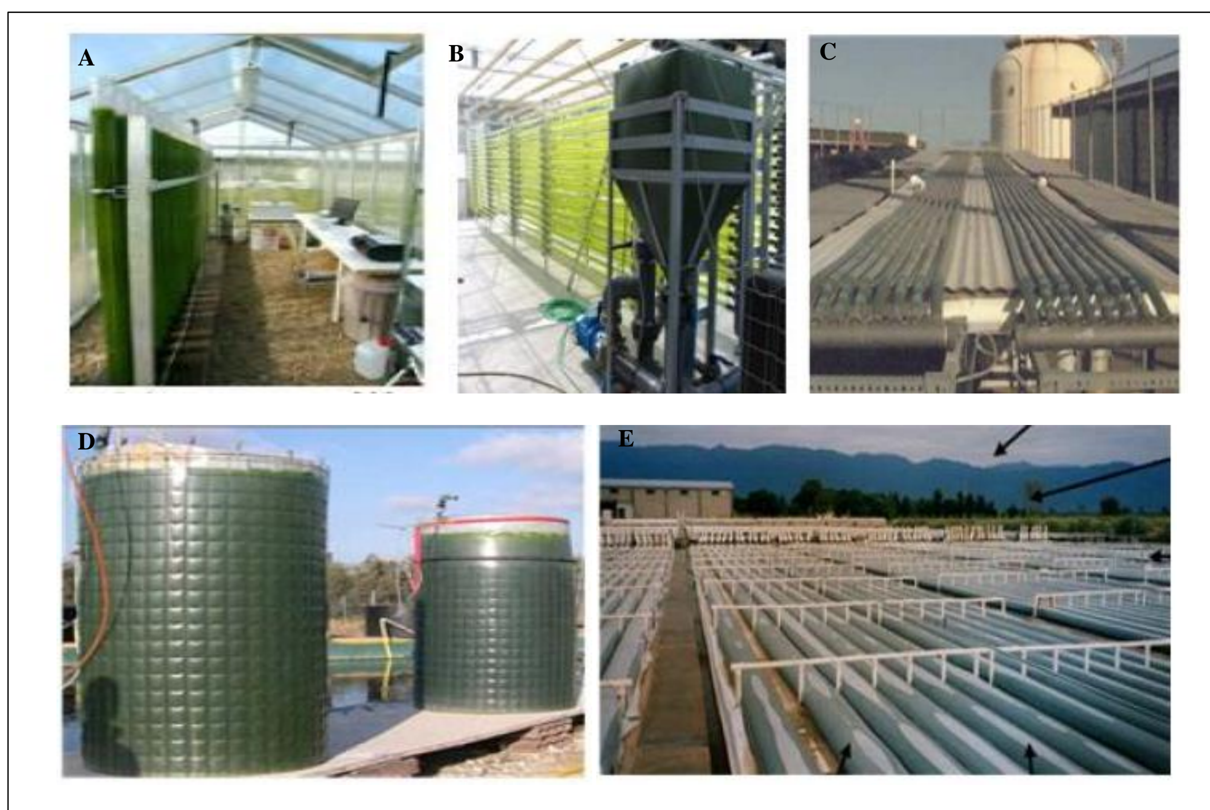

Figure 4: Different types of photobioreactors: **A.** Fence-like straight vertical **B.** Fence like straight horizontal **C.** Inclined **D.** Vertical helical **E.** Horizontal (Shen *et al.*, 2009)

(iii) Unstirred pond

The most economical and widely used type open pond system are unstirred ponds. Not much technical input is required in this system out of the all commercial culture methods. Natural lakes can be utilized for the purpose of making unstirred ponds or those can be constructed from natural ponds which are less than 50cm deep (Borowitzka *et al.*, 1990).

3.6.2 Closed photobioreactors

These type of culturing systems are covered with transparent materials to avoid any contact with the atmosphere. Most of the design elements of closed systems contain tubes of various sizes, shapes and lengths constructed with material like glass and/or plastic (Shen *et al.*, 2009). These can be illuminated by solar or artificial light; combination of both can also be used. The laboratory scale photobioreactors are generally illuminated with artificial light with lamps (Degen *et al.*, 2001; Ogbonna *et al.*, 2002)

Material and methods

4.1 Media composition

Table 4. Composition of BG-11 medium

For preparation of 100x stock of BG-11 (+N) medium, pH- 7.0 (Stainer *et al*., 1971)

Sl. No.	Component	Quantity(g/l)
1.	Sodium nitrate ($NaNO_3$)	1.5
2.	Di potassium hydrogen phosphate (K_2HPO_4)	0.04
3.	Magnesium sulphate ($MgSO_4.7H_2O$)	0.075
4.	Calcium Chloride ($CaCl_2.2H_2O$)	0.036
5.	Citric acid (Di-hydrate)	0.006
6.	Ammonium Ferric Citrate	0.006
7.	$Na_2EDTA.\ 2H_2O$	0.001
8.	Na_2CO_3	0.02
9.	Trace metals	1ml

Composition of trace metals

Sl. No.	Component	Quantity(g/l)
1.	Boric acid (H_3BO_4)	2.86
2.	Manganese chloride ($MnCl_2.4H_2O$)	1.81
3.	Zinc sulphate ($ZnSO_4.7H_2O$)	0.222
4.	Sodium molybdate ($Na_2MoO_4.\ 2H_2O$)	0.39
5.	Cupric sulphate ($CuSO_4.5H_2O$)	0.079
6.	Cobalt Nitrate ($Co(NO_3).6\ H_2O$)	0.0494

Note: For making BG-11(-N), all the components were added except Nitrogen source which is sodium nitrate ($NaNO_3$).

4.2 Isolation of microalgae by Skinner method

In order to isolate native microalgae, water samples were collected from various agricultural fields of Patiala district, Punjab. Further serial dilution was performed for diluting the samples.

Culture of *H.pluvialis* was obtained from ABCA biosolutions, Mohali, Punjab and was grown in BG-11 (-N) media at 25 ± 2ºC, with 2500-3000 lux light intensity and pH maintained at 7.0

Procedure

1. 9ml of distilled water was added into each of five test tubes.
2. The dilutions were prepared up to 10^{-5} by taking 1 ml algal sample and putting it in test tube containing 9 ml distilled water. All the dilutions were prepared similarly.
3. 1 ml of each dilution sample was poured in agar plates for pour plating
4. These plates were kept at 28±2ºC with 2500-3000 lux light intensity for 21 days
5. The colonies were observed after 21 days.

4.3 Screening of microalgae for highest pigment (Astaxanthin, Phycobilins & Chlorophyll)

4.3.1 Screening for Phycobilins

Phycobilin estimation was done by the method given by Bennet and Bogorad *et al.*, 1973

Procedure:

1. 10 ml samples of isolated algal cultures were taken in the centrifuge tubes.
2. The samples were centrifuged at 6000rpm for 10 minutes and the supernatant was discarded.
3. 3 ml of distilled water was added into each tube and was gently mixed.
4. The samples were kept for freezing in the refrigerator for 24 hours at -4ºC.
5. After 24 hours the samples were taken out from the refrigerator and kept at the room temperature for thawing and centrifugation was done at 6000 rpm for 10 minutes.
6. The readings were taken at three different wavelengths 562 nm, 615 nm and 652 nm respectively.

Formula:

$$PC\ (mg/ml) = [A_{615} - 0.474\ (A_{652})]/5.34$$

$$APC\ (mg/ml) = [A_{652} - 0.208\ (A_{615})/5.09$$

$$PE\ (mg/ml) = [A_{562} - 2.41\ (PC) - 0.849\ (APC)]/9.62$$

4.3.1 Estimation of Chlorophyll

Estimation of chlorophyll was done by the methods given by (Sukran *et al.*, 1997)

Procedure

1. 3ml homogenised culture were withdrawn from homogenously shaken culture in 10ml centrifuge tubes.

2. The samples were centrifuged at 6000 rpm for 10 minutes.
3. The supernatant was discarded and.3ml of methanol was added to the centrifuge tubes and kept for sonication for 10 minutes
4. After 10 minutes the sample were again centrifuged for 10 minutes at 6000 rpm
5. Then absorbance was noted at three different wavelengths of 470nm, 656nm and 666nm respectively.

Calculation

Chlorophyll $a = 15.65A_{666} - 7.340A_{653}$

Chlorophyll $b = 27.05A_{653} - 11.21A_{666}$

4.4 Growth kinetics

Growth curve

To study the growth kinetics, spectrophotometer analysis of microalgal growth, dry biomass estimation, chlorophyll estimation and astaxanthin estimation were performed.

Spectro-photometric analysis

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.

Dry biomass estimation

Procedure

1. Empty centrifuge tube were weighed (W_1).
2. 10 ml of homogenized culture, was taken and centrifuged the culture at 6000 rpm for 10 minutes. Supernatant was discarded.
3. Pellets were dried in hot air oven at 50˚C, until a constant weight was obtained.
4. The centrifuge tubes were weighed again with dried pellet (W_2).

Calculation for dry biomass estimation

Weigh of the biomass= $W_2 - W_1$

Astaxanthin estimation

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

Procedure

1. 10 ml culture was withdrawn from homogenously shaken culture in the 10 ml centrifuge tube.
2. The samples were centrifuged at 6000 rpm for 10 minutes. The supernatant obtained was discarded.
3. The pellet obtained was saponified with 5% KOH prepared in 30% methanol and incubated at 70ºC for 10min in water bath to degrade the chlorophyll.
4. The sample was allowed to cool and centrifuged at 6000rpm for 10 min. The supernatant was discarded.
5. The remaining pellet was extracted with DMSO and incubated in water bath for 5 min at 60 ºC.
6. The absorbance of the combined extract is obtained at 490nm.

The concentration of astaxanthin was calculated using the formula given below:

$$\text{Concentration of Astaxanthin (mg/L)} = \frac{(4.5 \times \text{A490}) \times \text{Va}}{\text{Vb}}$$

A_{490} = Absorbance of the extract at 490 nm
V_a = Volume of DMSO
V_b = Volume of microalgal culture

Everyday all the media were sparged with CO_2 to observe the change in the pigment concentration with and without CO_2 sparging.

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Results and Discussion

5.1 Isolation of microalgae by Skinner method

All dilutions during Skinner technique were prepared according to serial dilution 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} respectively. After incubating the plates for 21 days, microalgal colonies shown in (Figure 5) were picked under sterile conditions in laminar air flow chamber and inoculated into enrichment media (Figure 6) containing BG-11(+N) and BG-11(-N).

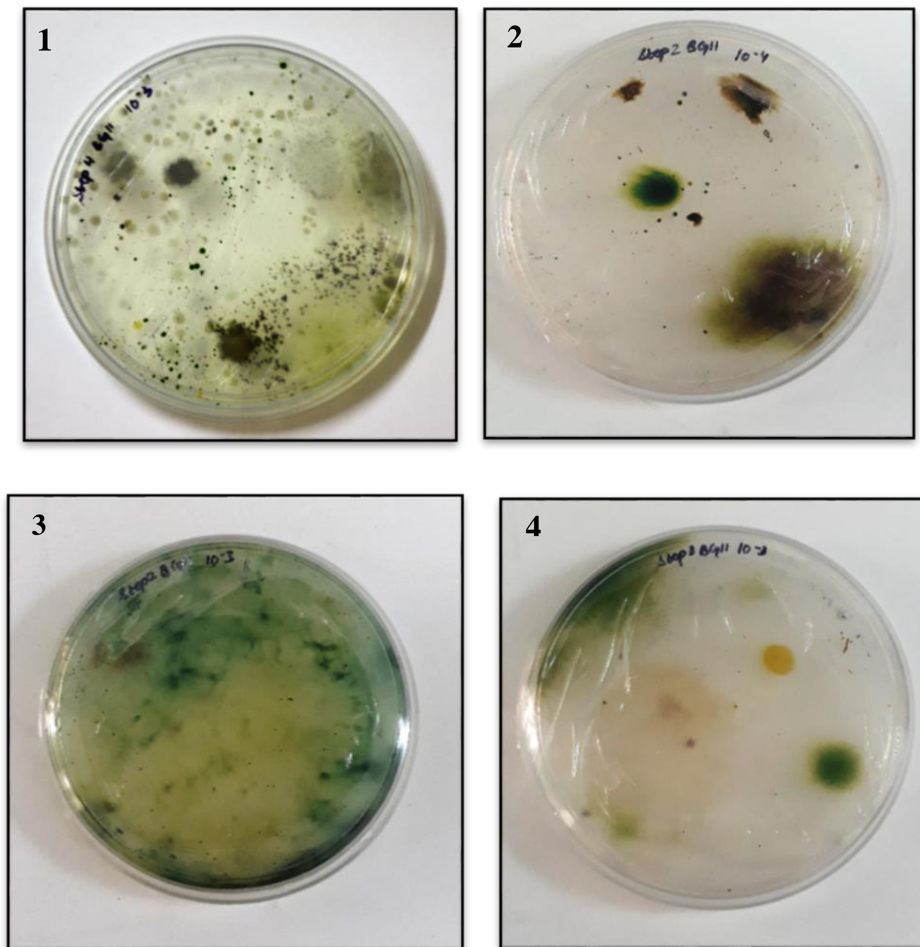

Figure 5. Algal colonies on agar plate containing BG-11(+N) and BG-11(-N) medium.

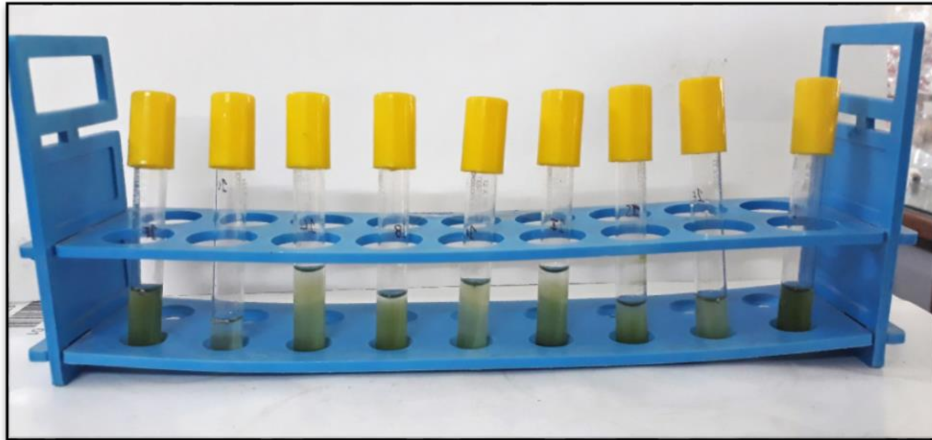

Figure 6. Enrichment in culture tubes containing BG-11(+N) and BG-11(-N)

5.2 Characterisation of algal isolates

After enrichment of microalgal isolates, the monoculture were identified using the compound microscope and the characteristics were noted down on the basis of morphology.

On the basis of morphology of algae they were identified as A (*Nostoc* sp.), B (*Nostoc* sp.), C (*Anabaena* sp.), D (*Anabaena* sp.), E (*Anabaena* sp.), F (*Chlorella* sp.), G (*Nostoc* sp.), H (*Anabaena* sp.), I (*H.pluvialis*), J (*Anabaena sp*), K (*Anabaena sp*), L (*Nostoc sp*).

Isolates were found to be *Anabaena sp.* (DGNB1), *Nostoc* sp. (DGNB2), *Chlorella* sp. (DGNB3) *Anabaena* sp. (DGNB4), *Nostoc* sp. (DGNB5), *Anabaena* sp. (DGNB6), *Nostoc* sp. (DGNB7), *Nostoc* sp. (DGNB8), along with previously isolated strains of *Anabaena* sp. (SG), *Anabaena* sp. (444), *Anabaena* sp. (443). The culture of *Hematococcus pluvialis* was obtained from ABCA biosolutions pvt ltd, Mohali, Punjab and was grown on BG-11(+N) medium.

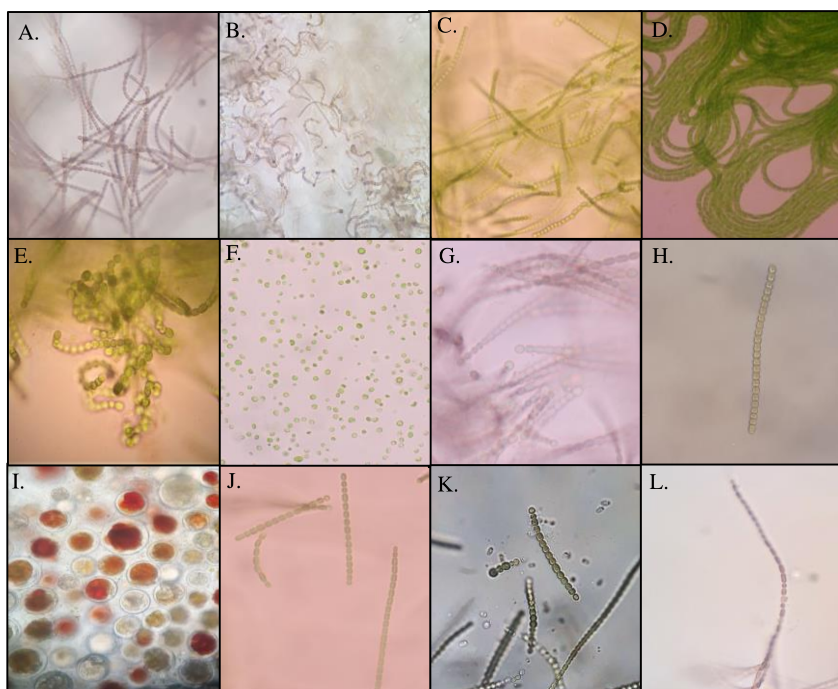

Figure 7. Photomicrographs of isolated microalgae

Table 5. Morphological characteristics of different microalgae

Sl. No.	Isolated algae	Characteristics
1.	DGNB 1	Filamentous cyanobacteria with strings of beaded cells
2.	DGNB 2	Filamentous cyanobacteria with strings of beaded cells along with mucilaginous sheath
3.	DGNB 3	Single cell green algae, spherical shape cells without flagella
4.	DGNB 4	Filamentous cells along with string of beaded cells
5.	DGNB 5	Filamentous microalgae
6.	DGNB 6	Filamentous cyanobacteria with beaded cells along with heterocyst
7.	DGNB 7	Filamentous cyanobacteria with strings of beaded cells along with mucilaginous sheath
8.	DGNB 8	Filamentous microalgae with beaded cells
9.	SG	Filamentous cyanobacteria with strings of beaded cells
10.	443	Filamentous cyanobacteria with strings of beaded cells
11.	444	Filamentous cyanobacteria with strings of beaded cells

5.3 Screening of algal isolates for photosynthetic pigments

The isolated microalgal cultures were inoculated in 250 ml conical flask containing BG-11(+N) and BG-11(-N) medium. After 21 days, 10 ml culture was harvested by centrifugation at 6000 rpm. In the harvested biomass 6 ml of distilled water was added and the samples were placed at -4ºC in the refrigerator. After 24 hours the frozen samples were thawed by keeping at room temperature. The absorbance was noted at three different wavelengths 562 nm, 615 nm, and 652 nm respectively.

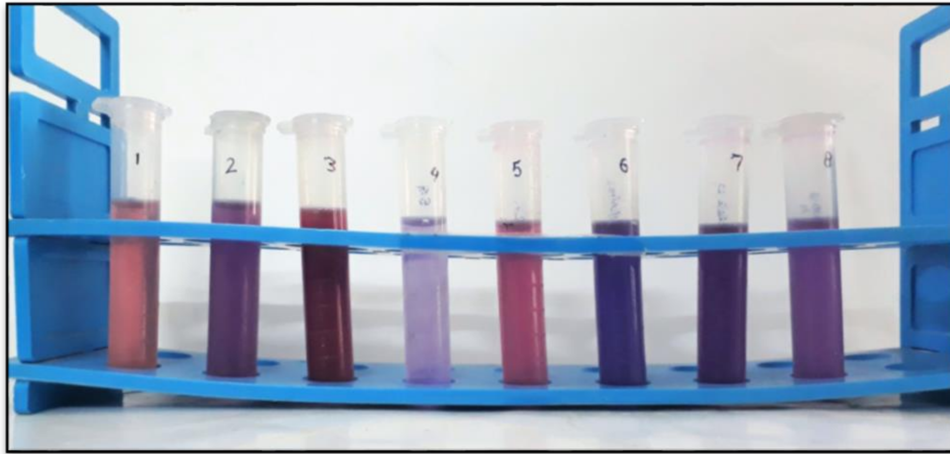

Figure 8. Phycobilins extracted from various isolated microalgae

Table 6. Concentration of phycobiliprotein in microalgal isolates

Isolate number	Phycocyanin	Allophycocyanin	Phycoerythrin
444	0.04±0.001	0.02±0.001	0.09±0.001
443	0.07±0.002	0.02±0.006	0.10±0.002
DGNB 1	0.16±0.001	0.06±0.001	0.13±0.003
DGNB 2	0.14±0.003	0.02±0.004	0.18±0.001
DGNB 3	0.02±0.002	0.03±0.002	0.02±0.003
DGNB 4	0.03±0.006	0.02±0.003	0.05±0.003
DGNB 5	0.03±0.002	0.02±0.003	0.08±0.001
DGNB 6	0.02±0.002	0.03±0.002	0.06±0.002
DGNB 7	0.09±0.002	0.09±0.002	0.15±0.001
DGNB 8	0.03±0.001	0.03±0.003	0.07±0.002
H.pluvialis	0.02±0.003	0.08±0.003	0.02±0.004
SG	0.04±0.002	0.01±0.001	0.03±0.002

*values given are average of three replications

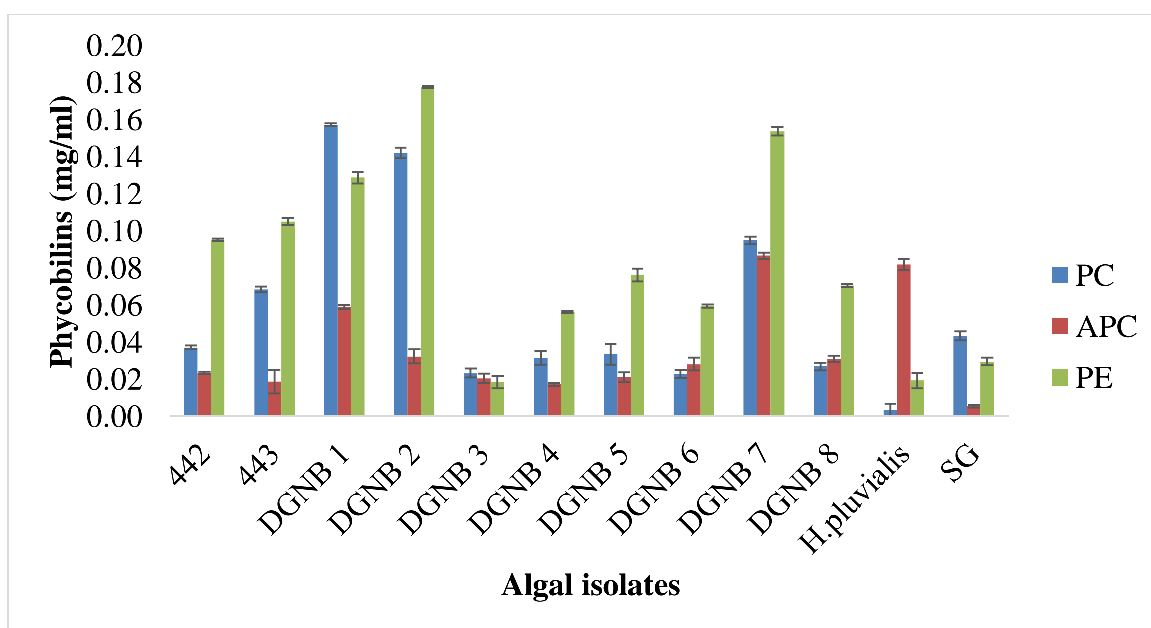

Figure 9. Concentration of phycobiliproteins in microalgal isolates

The maximum phycocyanin content was obtained from DGNB1 which was identified as *Anabaena* sp. isolate. Whereas maximum phycoerythrin was obtained from DGNB2 which was identified as *Nostoc* sp.

Table 7. Concentration of chlorophyll in microalgal isolates

Algal isolates	Chlorophyll *a* (mg/L)	Chlorophyll *b* (mg/L)
444	1.1±0.01	1.3±0.10
443	0.9±0.05	1.5±0.02
DGNB 1	5.5±0.28	1.0±0.00
DGNB 2	3.0±0.04	1.3±0.06
DGNB 3	8.8±0.13	4.1±0.12
DGNB 4	3.0±0.03	3.8±0.01
DGNB 5	6.2±0.17	13.3±0.51
DGNB 6	7.6±0.13	10.2±0.15
DGNB 7	3.7±0.12	4.8±0.14
DGNB 8	2.1±0.13	9.1±0.03
H.pluvialis	0.4±0.00	0.4±0.01
SG	3.4±0.10	2.6±0.01

*values given are average of three replications

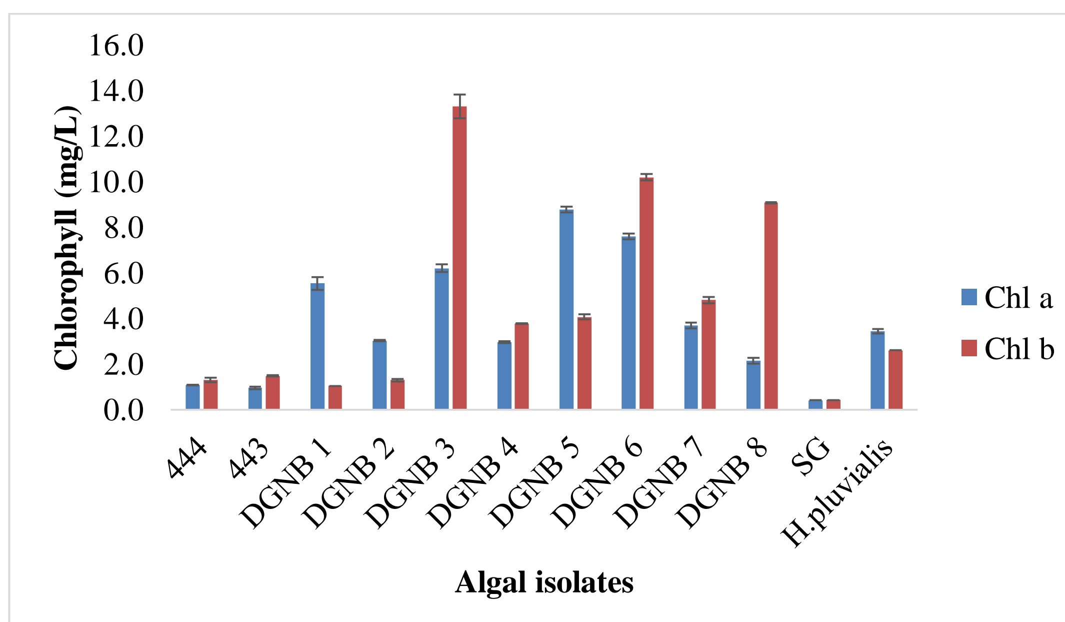

Figure 10. Concentrations of chlorophyll in microalgal isolates

On the basis of high chlorophyll content *Chlorella* sp. (DGNB3) was selected for further studies.

5.4 Growth Kinetics

(i) Absorbance at 680 nm (Growth curve with CO_2 sparging)

Table 8. Absorbance obtained at 680nm with CO_2 sparging

Days	DGNB1	DGNB2	*H.pluvialis*	DGNB3
0	0.03±0.01	0.03±0.00	0.03±0.00	0.03±0.01
4	0.08±0.09	0.01±0.01	0.09±0.01	0.09±0.02
8	0.11±0.01	0.15±0.04	0.12±0.00	0.13±0.01
12	0.15±0.01	0.19±0.06	0.36±0.01	0.39±0.03
16	0.35±0.07	0.29±0.03	0.36±0.03	0.51±0.04
20	0.36±0.04	0.51±0.02	0.35±0.02	0.92±0.05
24	0.77±0.01	1.19±0.03	1.19±0.01	1.92±0.03
28	1.34±0.06	0.81±0.03	1.16±0.04	1.30±0.06
32	1.61±0.03	0.62±0.02	1.70±0.07	1.05±0.05
36	0.82±0.02	0.52±0.03	1.20±0.00	0.95±0.04

*values given are average of three replications

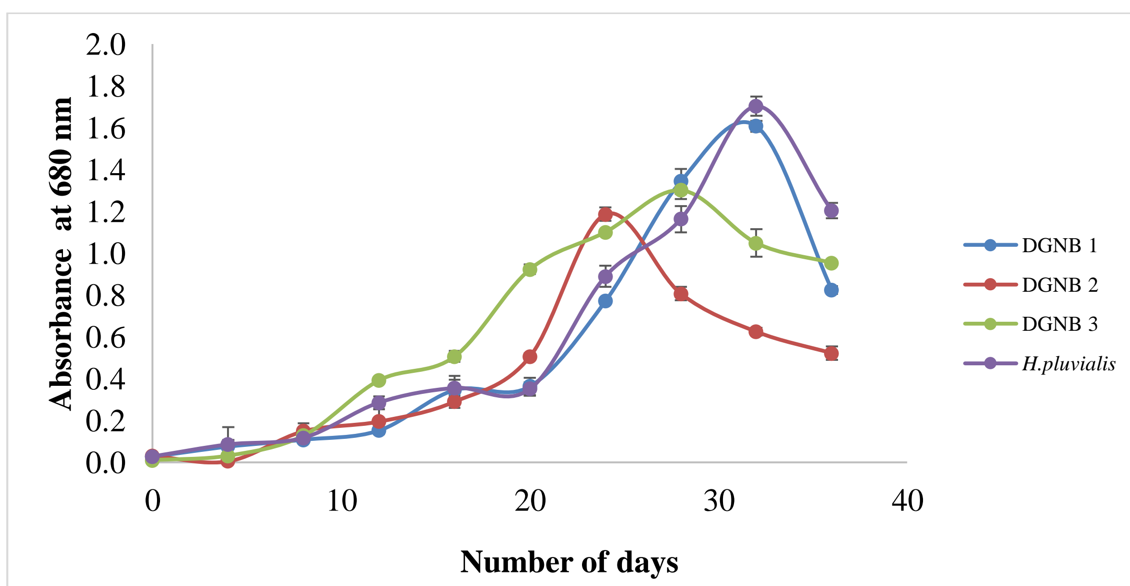

Figure 11: Absorbance at 680 nm of different microalgae with CO_2 sparging

Absorbance of all the microalgae and cyanobacteria was taken at 680nm. Absorbance of *Anabaena* sp. (DGNB1) was 0.027 on the 0th day at 680 nm which became 0.825 on 36th day. The maximum absorbance was obtained as 1.607 on the 32nd day. The absorbance of *Nostoc*

sp. (DGNB2) was 0.030 on the initial day which became 0.522 on the 36th day. The maximum absorbance for it was noted on the 24th day, which was 1.186. The absorbance for *H.pluvialis* was 0.011 on the 0th day and became 0.954 on the 36th day. Maximum absorbance was obtained as 1.049 on the 32nd day. The absorbance of *Anabaena* sp. (DGNB1) was found to be 0.028 on the initial day and increased to 1.204 on the 36th day. The maximum absorbance was 1.703 on the 32nd day.

All the algal species were aerated with CO_2 which acted as carbon source for the production of the biomass. The CO_2 is utilized by them in the process of photosynthesis. Moreover, it helped to maintain the pH of the culture mediums which rises up as the growth takes place (Fei *et al*., 2007).

(ii) Absorbance at 680 nm (Growth curve without CO_2 sparging)

Table 9. Absorbance at 680 nm without CO_2 sparging

Days	DGNB 1	DGNB 2	*H.pluvialis*	DGNB 3
4	0.02±0.01	0.02±0.00	0.02±0.01	0.03±0.01
8	0.01±0.004	0.01±0.01	0.02±0.02	0.08±0.01
12	0.02±0.01	0.06±0.01	0.11±0.04	0.26±0.03
16	0.05±0.002	0.10±0.00	0.19±0.06	0.28±0.10
20	0.07±0.01	0.16±0.01	0.34±0.09	0.57±0.13
24	0.14±0.02	0.22±0.00	0.36±0.08	0.93±0.12
28	0.72±0.07	0.55±0.03	0.70±0.03	1.19±0.16
32	1.10±0.07	1.07±0.06	0.99±0.005	1.46±0.14
36	1.27±0.02	0.63±0.06	1.25±0.01	1.70±0.08

*values given are average of three replications

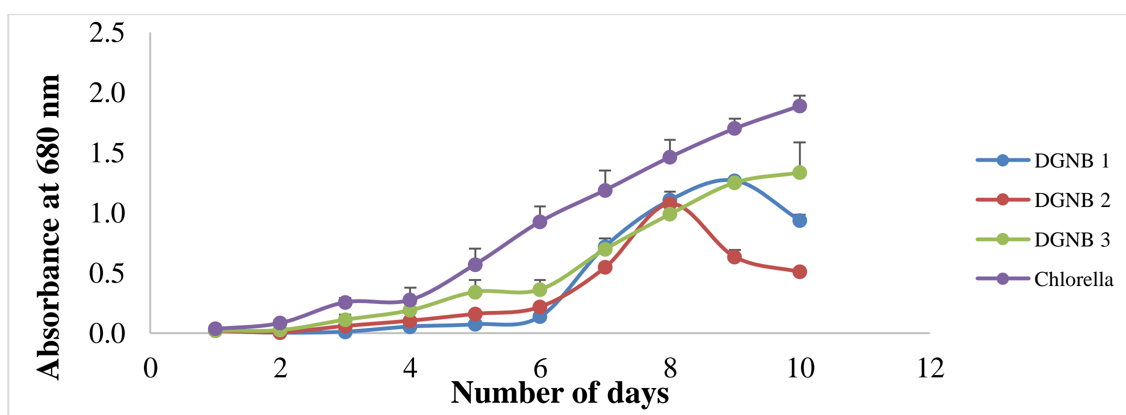

Figure 12. Absorbance at 680 nm of different microalgae without CO_2 sparging

Absorbance of all the microalgae and cyanobacteria was taken at 680nm. Absorbance of *Anabaena* sp. (DGNB1) was 0.02 on the 0th day at 680 nm which became 0.94 on 36th day. The maximum absorbance was obtained as 1.27 on the 32nd day. The absorbance for *H.pluvialis* was 0.02 on the 0th day and became 1.33 on the 36th day. Maximum absorbance was obtained as 1.33 on the 36nd day. The absorbance of *Chlorella* sp. (DGNB3) was found to be 0.03 on the initial day and increased to 1.89 on the 36th day. The maximum absorbance was 1.89 on the 36nd day.

(iii) Dry biomass estimation with CO_2 sparging

Table 10. Biomass (mg/ml) with CO_2 sparging

Days	DGNB1 (mg/ml)	DGNB2 (mg/ml)	*H.pluvialis* (mg/ml)	DGNB3 (mg/ml)
4	0.001±0.0005	0.001±0.0005	0.001±0.00	0.001±0.00
8	0.001±0.00	0.001±0.00	0.001±0.00	0.001±0.00
12	0.001±0.00	0.001±0.00	0.001±0.00	0.001±0.00
16	0.002±0.0011	0.002±0.0005	0.002±0.00	0.002±0.0005
20	0.002±0.0011	0.003±0.0011	0.003±0.0005	0.002±0.0005
24	0.005±0.0005	0.005±0.0017	0.004±0.001	0.006±0.0015
28	0.005±0.001	0.003±0.0015	0.004±0.001	0.006±0.00
32	0.005±0.00	0.002±0.0005	0.003±0.0017	0.006±0.0011
36	0.004±0.001	0.002±0.0005	0.003±0.0011	0.004±0.0005

*values given are average of three replications

It was found at initial stage of growth cycle the biomass content was low but as algae started growing the biomass content started increasing. In the estimation of dry biomass it was found that *Chlorella* sp. (DGNB3) gave the maximum biomass after the period of 30 days which is higher than *Anabaena* sp. (DGNB1), *Nostoc* sp. (DGNB2) and *Hematococcus* sp. (Singh *et al*., 2014) observed that sparging CO_2 increase the biomass of *Chlorella* sp. (DGNB3) at 30ºC.

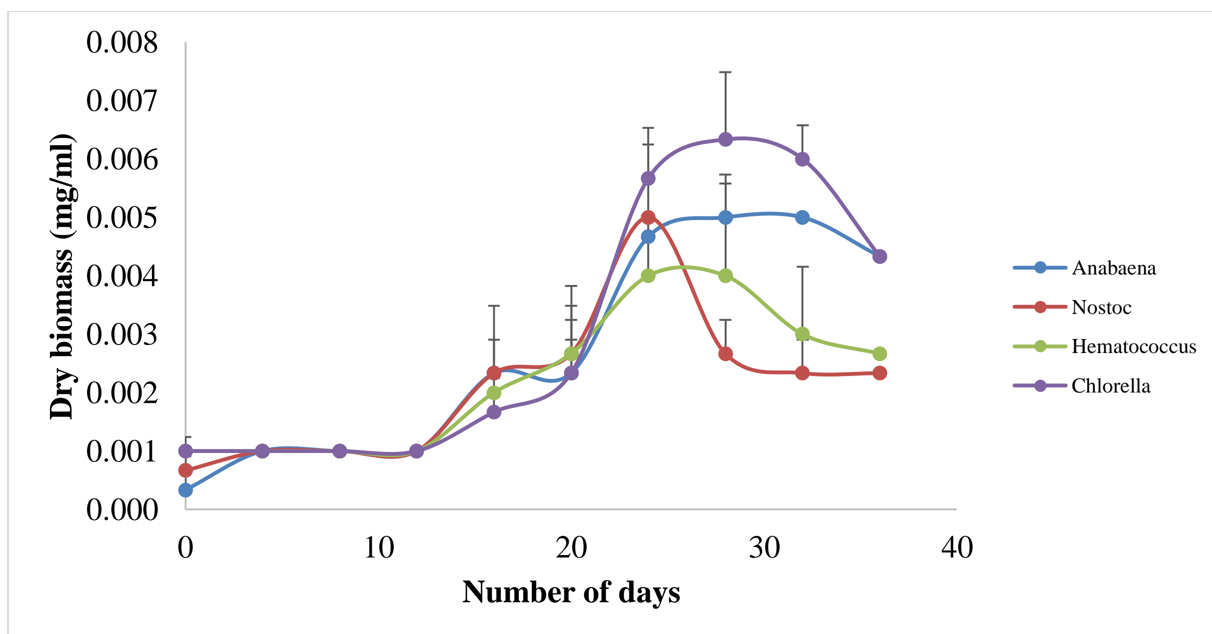

Figure 13: Biomass (mg/ml) in different microalgae with CO_2 sparging

(iv) Dry biomass estimation without CO_2 sparging

Table11. Dry biomass (mg/ml) without CO_2 sparging

Days	DGNB1 (mg/ml)	DGNB2 (mg/ml)	*H.pluvialis* (mg/ml)	DGNB3 (mg/ml)
4	0.001±0.001	0.001±0.001	0.001±0.001	0.001±0.001
8	0.001±0.000	0.001±0.001	0.001±0.001	0.001±0.001
12	0.001±0.001	0.001±0.001	0.001±0.001	0.002±0.002
16	0.002±0.001	0.001±0.001	0.002±0.002	0.003±0.001
20	0.003±0.001	0.002±0.001	0.003±0.001	0.003±0.001
24	0.004±0.001	0.003±0.001	0.003±0.001	0.003±0.001
28	0.004±0.001	0.005±0.001	0.004±0.002	0.004±0.001
32	0.004±0.002	0.002±0.001	0.003±0.001	0.004±0.001
36	0.004±0.001	0.002±0.000	0.003±0.001	0.004±0.001

*values given are average of three replications

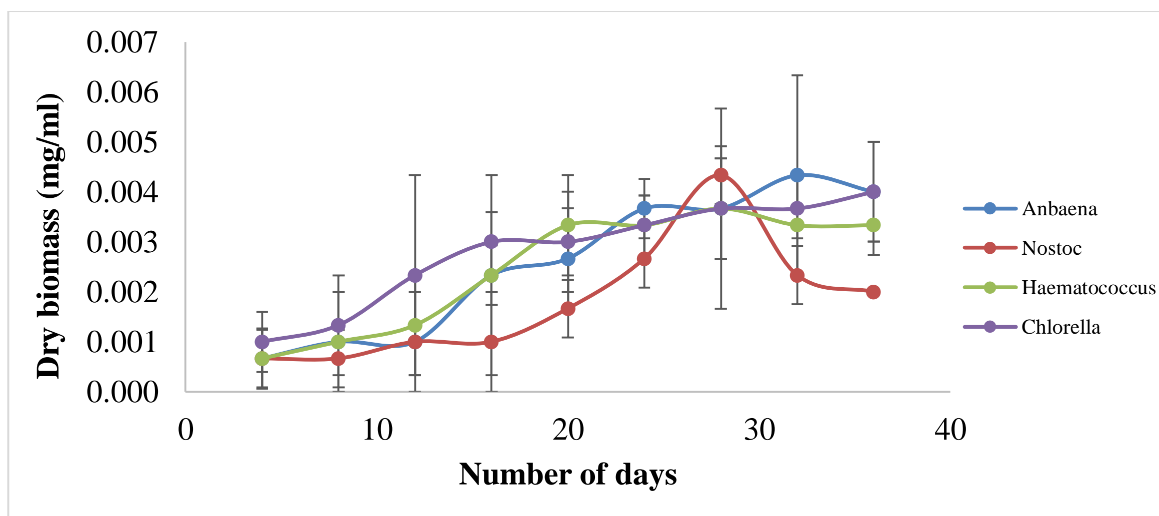

Figure 14. Biomass (mg/ml) in different microalgae without CO_2 sparging

It was found that initial biomass of microalgae was 0.001 for all the algae but as the algae started utilizing the nutrients, exponential increase was seen in the growth. The maximum biomass in *Anabaena* sp. (DGNB1) was found on 32nd day which was 0.0043mg/ml. In *H.pluvialis* maximum biomass was found on 28th day which was 0.00367mg/ml whereas, in *Chlorella* sp. (DGNB3) the maximum biomass found was 0.004 mg/ml on the 36th day of the growth cycle.

(v) Chlorophyll *a* and *b* estimation

The increase in the chlorophyll *a* and *b* content of cell indicated the increase in the growth. Chlorophyll *a* is the primary pigment for photosynthesis inside the cell and plays significant role in the growth of cell. Chlorophyll content decreases when there is decrease in the cells metabolic activity. Thus chlorophyll content varies in different stages of growth.

Table 12. Chlorophyll *a* (mg/L) in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

Days	Chlorophyll *a* (mg/L) with CO_2 sparging	Chlorophyll *a* (mg/L) without CO_2 sparging
4	0.47±0.20	0.08±0.01
8	0.25±0.13	0.06±0.03
12	1.37±0.19	0.65±0.13
16	1.35±0.42	0.73±0.04
20	1.56±0.47	1.06±0.20
24	3.73±0.12	2.73±0.31
28	4.15±0.17	2.14±0.07
32	6.34±0.44	5.87±0.06
36	2.71±0.92	6.04±0.53

*values given are average of three replications

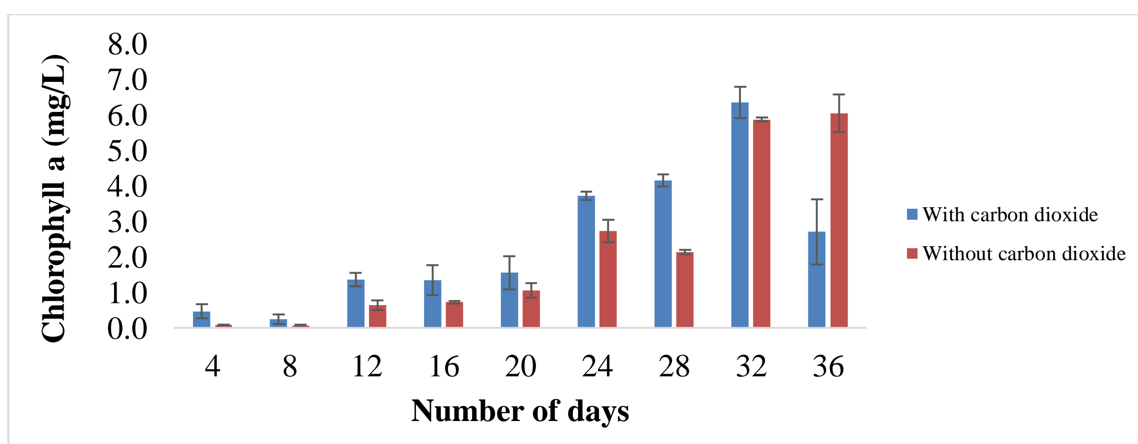

Figure 15. Chlorophyll *a* (mg/L) in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

The chlorophyll *a* in *Anabaena* sp. (DGNB1) was 0.47 mg/L on the 4th day with CO_2 sparging and 0.08 mg/L in without CO_2 sparging. The highest concentration in algae with CO_2 sparging was 6.34 mg/L and in without CO_2 sparging it was 6.04 mg/L. The increase in the pigment was observed with the increase in the growth. According to Gracia-Malea *et al* (2006) aeration of medium with carbon dioxide causes increase in chlorophyll and other carotenoids but above the optimal range it can also lead to cessation of growth.

Table 13. Chlorophyll *a* (mg/L) in *Nostoc* sp. (DGNB2) with and without CO_2 sparging

Days	Chlorophyll *a* (mg/L) with CO_2 sparging	Chlorophyll *a* (mg/L) without CO_2 sparging
4	0.09±0.03	0.11±0.03
8	0.34±0.32	0.23±0.03
12	1.50±0.38	1.23±0.50
16	1.72±0.30	1.47±0.05
20	1.91±0.49	1.64±0.04
24	3.38±0.13	1.95±0.03
28	3.67±0.84	2.84±0.07
32	2.20±0.93	3.52±0.03
36	1.6±0.86	2.85±0.05

*values given are average of three replications

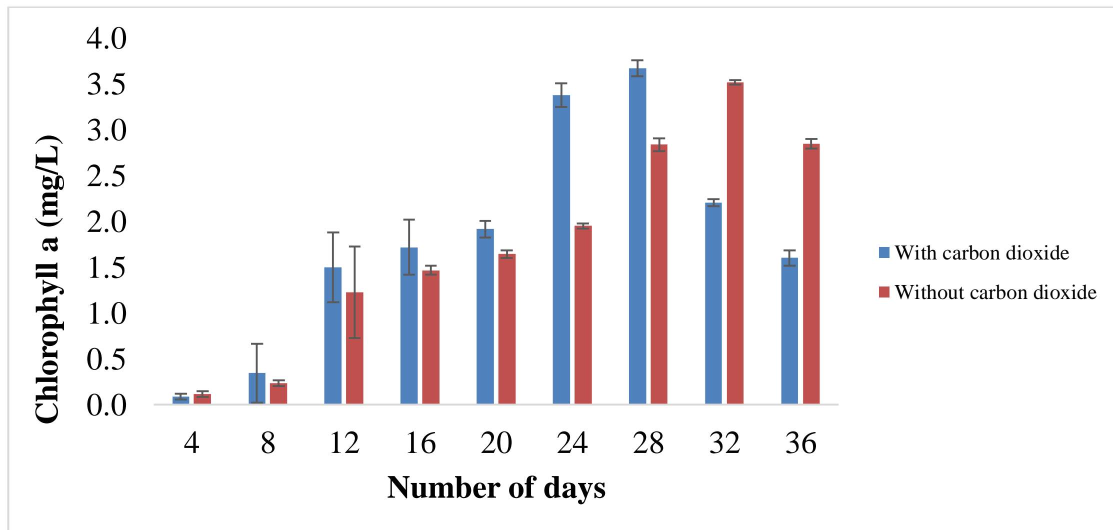

Figure 16. Chlorophyll *a* (mg/L) in *Nostoc* sp. (DGNB2) with and without CO_2 sparging

In *Nostoc* sp. (DGNB2) the chlorophyll *a* was 0.09 mg/L in algae with CO_2 sparging and 0.11 mg/l in the without CO_2 sparging. The maximum chlorophyll *a* was 3.67 mg/L on the 28th day with CO_2 sparging and was 3.52 mg/L without CO_2 sparging on 32nd day of the growth cycle. CO_2 sparging enhanced the growth thus increased the chlorophyll *a* concentration as the suitable percentage of CO_2 can increase the chlorophyll *a* concentration (Kin-Chung *et al.*, 2013)

Table 14. Chlorophyll *a* (mg/L) in *Chlorella* sp. (DGNB3) with and without CO_2 sparging

Days	Chlorophyll *a* (mg/L) with CO_2 sparging	Chlorophyll *a* (mg/L) without CO_2 sparging
4	1.27±0.54	0.47±0.11
8	0.29±0.38	0.30±0.07
12	1.22±0.06	0.64±0.14
16	2.36±0.38	1.65±0.30
20	3.96±0.04	3.78±0.18
24	5.65±0.29	5.33±0.09
28	6.92±0.05	5.04±0.52
32	8.28±0.09	7.16±0.08
36	11.87±0.14	11.27±0.50

*values given are average of three replications

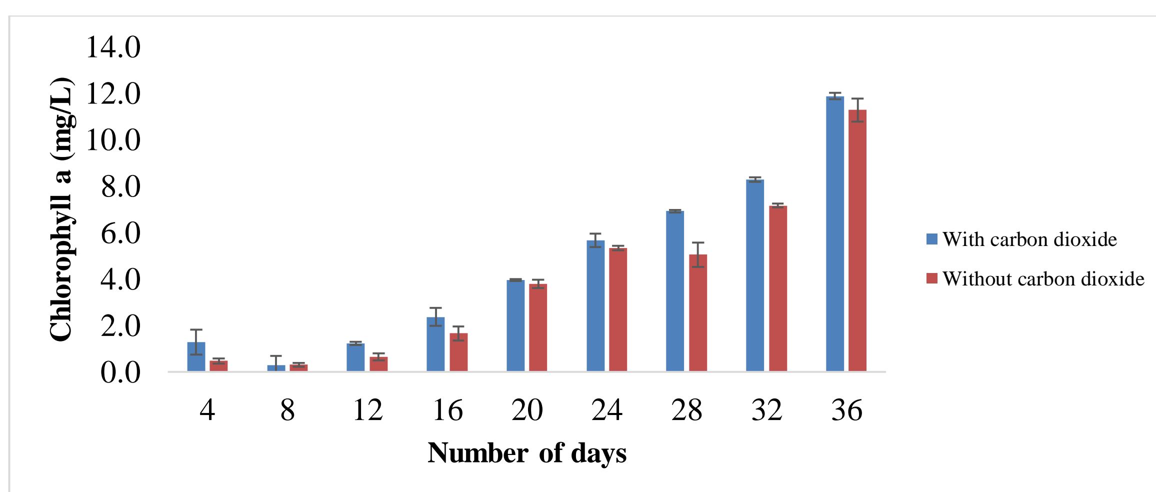

Figure 17. Chlorophyll *a* (mg/L) in *Chlorella* sp. (DGNB3) with and without CO_2 sparging

In *Chlorella* sp. (DGNB3) the chlorophyll *a* concentration was initially 1.27 mg/L with CO_2 sparging and 0.47 mg/L without CO_2 sparging. There was observed exponential increase in the chlorophyll concentration with the passage of days. The maximum chlorophyll *a* was observed 11.87 mg/L on 36th day of the growth cycle with CO_2 sparging and 11.27 mg/L without CO_2 sparging.

Table 15. Chlorophyll *a* (mg/L) in *H.pluvialis* with and without CO_2 sparging

Days	Chlorophyll *a* (mg/L) with CO_2 sparging	Chlorophyll *a* (mg/L) without CO_2 sparging
4	0.32±0.06	0.44±11
8	0.56±0.08	0.20±0.19
12	0.97±0.16	0.61±0.15
16	2.10±0.09	1.38±0.05
20	1.57±0.15	1.54±0.32
24	1.73±0.69	2.48±0.14
28	2.36±0.59	2.75±0.08
32	2.56±0.52	1.78±0.09
36	2.87±0.26	2.75±0.40

*values given are average of three replications

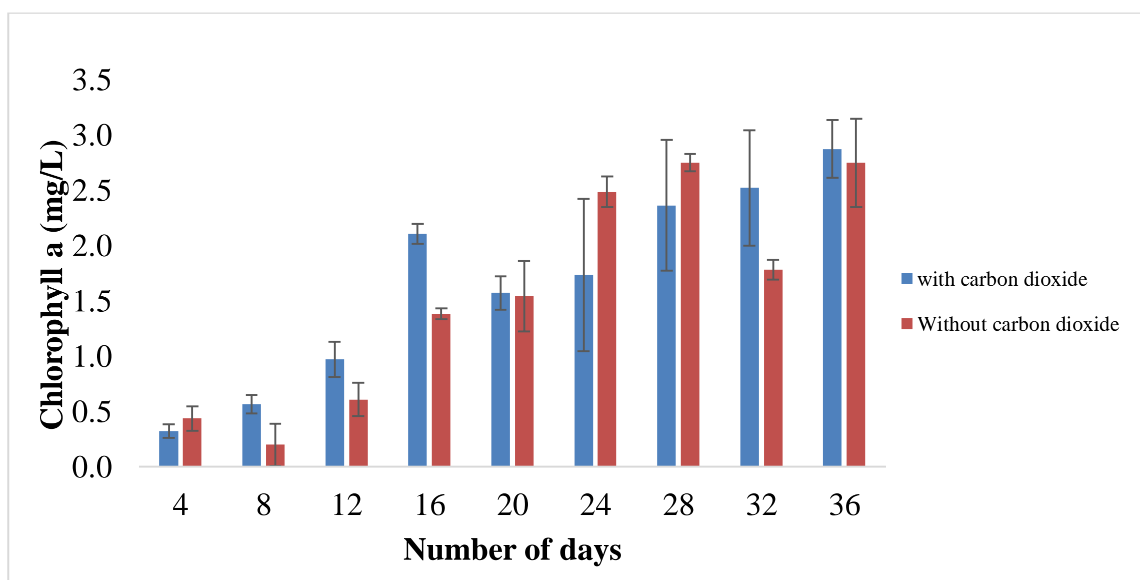

Figure 18. Chlorophyll *a* (mg/L) in *H.pluvialis* with and without CO_2 sparging

In *H.pluvialis* the chlorophyll *a* was 0.32 mg/L with CO_2 sparging on the 4th day of the growth cycle whereas was 0.44 mg/l without CO_2 sparging. The maximum chlorophyll *a* was obtained on the 36th day of growth cycle which was 2.87 mg/L with CO_2 sparging and 2.75 mg/L without CO_2 sparging. Carbon dioxide lead to increase in the enzymes such as Rubisco and carbonic

anhydrase which play crucial role in carbon metabolism thus increase the chlorophyll (Gordillo *et al*., 1999).

Table 16. Chlorophyll *b* (mg/L) in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

Days	Chlorophyll *b* (mg/L) with CO_2 sparging	Chlorophyll *b* (mg/L) without CO_2 sparging
4	0.36±0.01	0.10±0.13
8	0.13±0.03	0.12±0.13
12	0.71±0.013	0.65±0.32
16	0.57±0.04	0.88±0.22
20	0.71±0.12	0.91±0.09
24	3.18±0.03	0.79±0.34
28	3.57±0.07	1.36±0.10
32	2.29±0.07	1.66±0.18
36	0.62±0.03	1.77±0.27

*values given are average of three replications

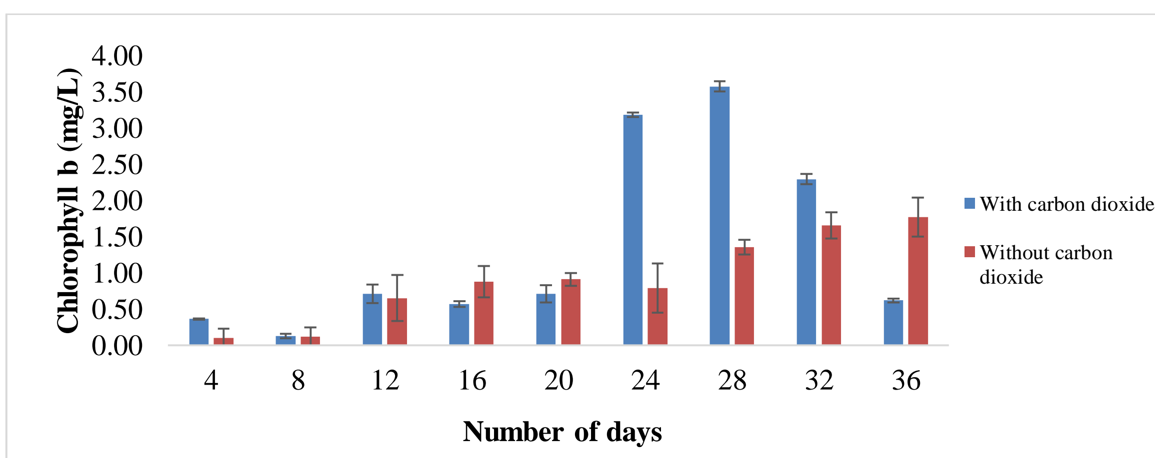

Figure 19. Chlorophyll *b* (mg/L) in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

The chlorophyll *b* content in *Anabaena* sp. (DGNB1) with and without CO_2 sparging was 0.36 mg/l and 0.10 mg/L respectively on the 4th day of the growth cycle. In the algae with CO_2 sparging the maximum chlorophyll *b* was 3.57 mg/l on the 28th day and in the without CO_2 sparging it was 1.77 mg/L on 36th day of the growth cycle. The significant difference in the chlorophyll *b* was obtained by sparging CO_2.

Table 17. Chlorophyll *b* (mg/L) in *Nostoc* sp. (DGNB2) with and without CO_2 sparging

Days	Chlorophyll *b* (mg/L) with CO_2 sparging	Chlorophyll *b* (mg/L) without CO_2 sparging
4	0.14±0.04	0.15±0.03
8	0.32±0.00	0.24±0.34
12	0.76±0.04	0.44±0.07
16	1.02±0.04	0.71±0.07
20	1.46±0.04	0.76±0.05
24	3.80±0.01	1.25±0.21
28	1.29±0.00	3.65±0.08
32	0.52±0.01	1.19±0.20
36	0.11±0.04	0.63±0.02

*values given are average of three replications

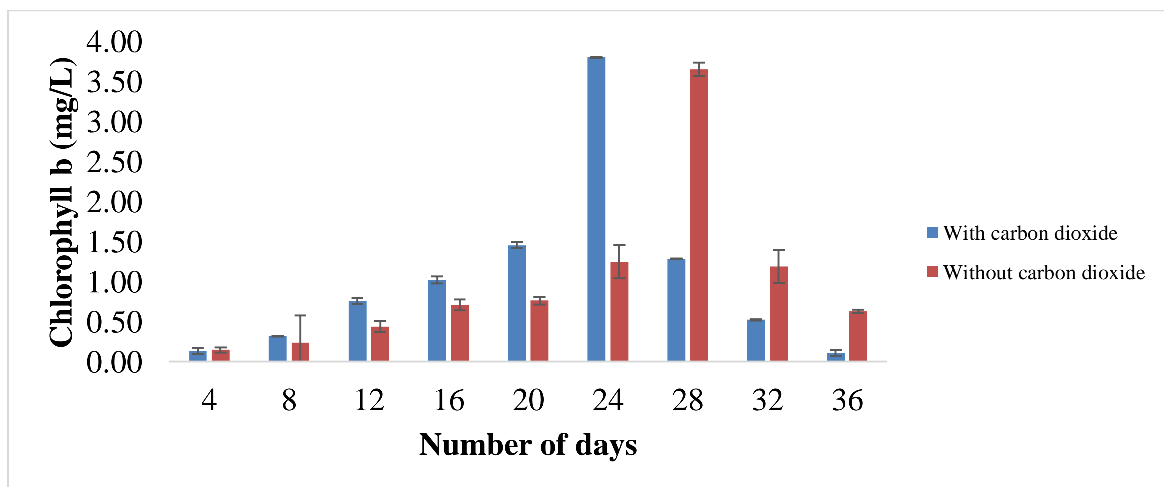

Figure 20. Chlorophyll *b* (mg/L) in *Nostoc* sp. (DGNB2) with and without CO_2 sparging

In *Nostoc* sp. (DGNB2) with CO_2 sparging, chlorophyll *b* on the fourth day was 0.14 mg/L and in without CO_2 sparging it was 0.15 mg/L. The chlorophyll *b* increased exponentially along with the growth of algae and reached 3.80 mg/L on the 24th day of the growth cycle in the algae that was sparged CO_2 and in the without CO_2 it reached the maximum 3.65 mg/L on the 28th day of the growth cycle. On the 36th day, it became 0.11 mg/ml for CO_2 sparged and 0.63 mg/L for without CO_2 sparged.

Table 18. Chlorophyll *b* (mg/L) in *Chlorella* sp. (DGNB3) with and without CO_2 sparging

Days	Chlorophyll *b* (mg/L) with CO_2 sparging	Chlorophyll *b* (mg/L) without CO_2 sparging
4	1.36±0.11	1.06±0.09
8	0.38±0.07	0.29±0.06
12	0.77±0.14	0.65±0.15
16	1.62±0.30	1.28±0.19
20	2.01±0.18	2.81±0.33
24	1.88±0.09	2.85±0.53
28	2.61±0.05	3.40±0.35
32	4.90±0.08	5.44±0.57
36	7.67±0.05	4.92±0.09

*values given are average of three replications

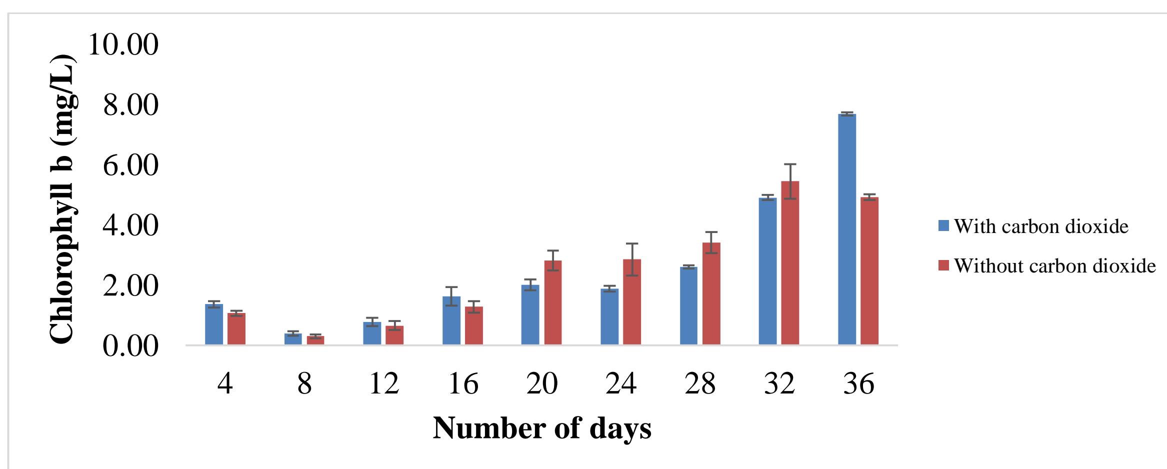

Figure 21. Chlorophyll *b* (mg/L) in DGNB3 with and without CO_2 sparging

In *Chlorella* sp. (DGNB3) the chlorophyll b on the 4th day was 1.36 mg/L with CO_2 sparging and 1.06 mg/L without CO_2 sparging. There was exponential increase in the chlorophyll concentration with the passage of days. The maximum chlorophyll b was observed 7.67 mg/L on 36th day with CO_2 sparging and 4.92 mg/L without sparging.

Table 19. Chlorophyll *b* (mg/L) in *H.pluvialis* with and without CO_2 sparging

Days	Chlorophyll *b* (mg/L) with CO_2 sparging	Chlorophyll *b* (mg/L) without CO_2 sparging
4	0.63±0.11	0.53±0.09
8	0.84±0.07	0.27±0.06
12	1.32±0.14	0.60±0.15
16	1.01±0.30	1.53±0.19
20	2.19±0.18	1.80±0.33
24	2.00±0.09	1.77±0.53
28	1.62±0.05	2.30±0.35
32	3.92±0.08	3.21±0.57
36	2.61±0.05	2.12±0.09

*values given are average of three replications

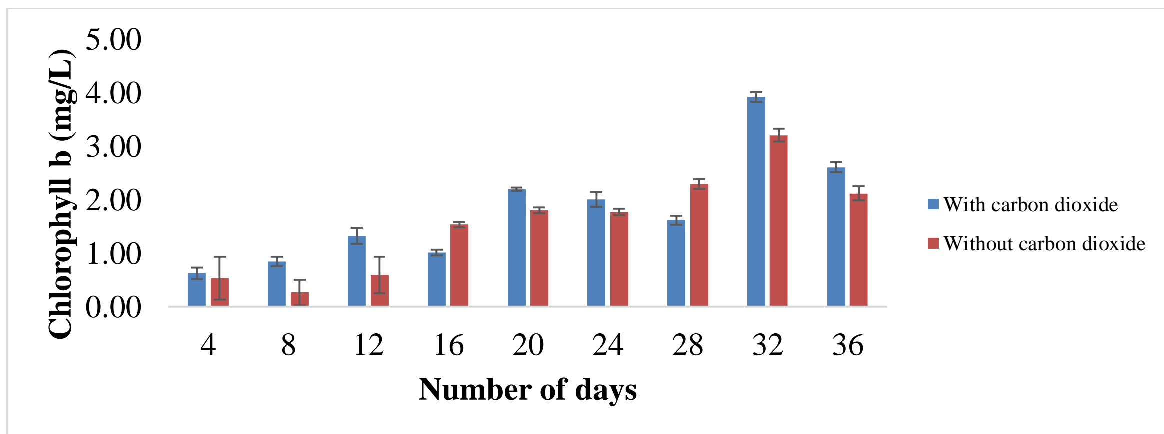

Figure 22. Chlorophyll *b* (mg/L) in *H.pluvialis* with and without CO_2 sparging

In *H.pluvialis* chlorophyll *b* was 0.63 mg/L on the 4th day with CO_2 sparging and 0.53 mg/L in without CO_2 sparging. There was observed increase in the chlorophyll *b* concentration with the days. The maximum chlorophyll *b* was observed on 3.92 mg/L with CO_2 sparging and 3.21 mg/L without CO_2 sparging on the 32nd day of the growth cycle.

(vi) Phycobilin estimation

Table 20. Phycocyanin in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

Days	Phycocyanin with CO_2	Phycocyanin without CO_2
4	0.00±0.00	0.01±0.00
8	0.02±0.00	0.02±0.00
12	0.05±0.00	0.04±0.00
16	0.07±0.00	0.04±0.00
20	0.06±0.00	0.05±0.00
24	0.16±0.00	0.09±0.00
28	0.15±0.00	0.09±0.00
32	0.17±0.00	0.09±0.00
36	0.16±0.00	0.08±0.00

*values given are average of three replications

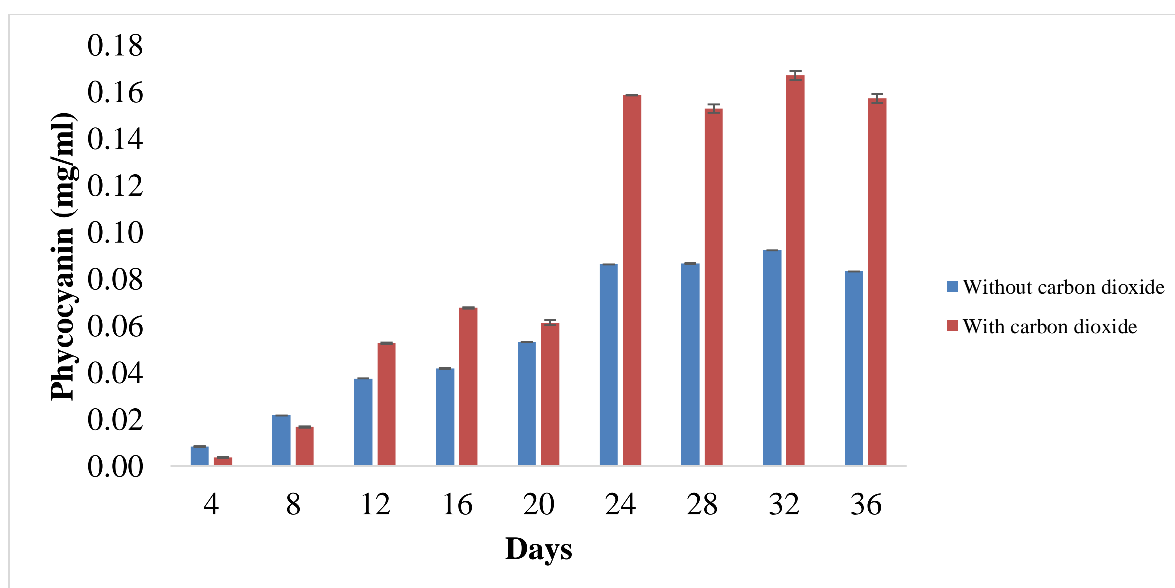

Figure 23. Phycocyanin in *Anabaena* sp. (DGNB1) with and without CO_2sparging

The phycocyanin content in *Anabaena* sp. (DGNB1) was 0.004 mg/ml with CO_2 sparging on 4th day of the growth cycle whereas in the culture without CO_2 sparging it was 0.01 mg/ml on the same day. The maximum phycocyanin was obtained 0.17 mg/ml on the 32nd day with CO_2 sparging whereas in the culture without CO_2 sparging it was only 0.09 mg/ml on the 32nd day of growth cycle. As the biomass increased the phycocyanin content also increased in the algae. Carbon dioxide lead to increase in the enzymes such as Rubisco and carbonic anhydrase which

play crucial role in carbon metabolism (Gordillo *et al.*, 1999). According to Fei et al (2007) elevated CO_2 increase phycoerythrin and phycocyanin content significantly due to increase in number of phycobilisomes.

Table 21. Allophycocyanin in *Anabaena* sp. (DGNB1) with and without CO_2sparging

Days	Allophycocyanin with CO_2	Allophycocyanin without CO_2
4	0.00±0.00	0.00±0.00
8	0.01±0.00	0.01±0.00
12	0.03±0.00	0.01±0.00
16	0.04±0.00	0.03±0.003
20	0.02±0.03	0.03±0.00
24	0.08±0.00	0.04±0.005
28	0.07±0.03	0.04±0.00
32	0.08±0.00	0.02±0.00
36	0.06±0.00	0.00±0.00

*values given are average of three replications

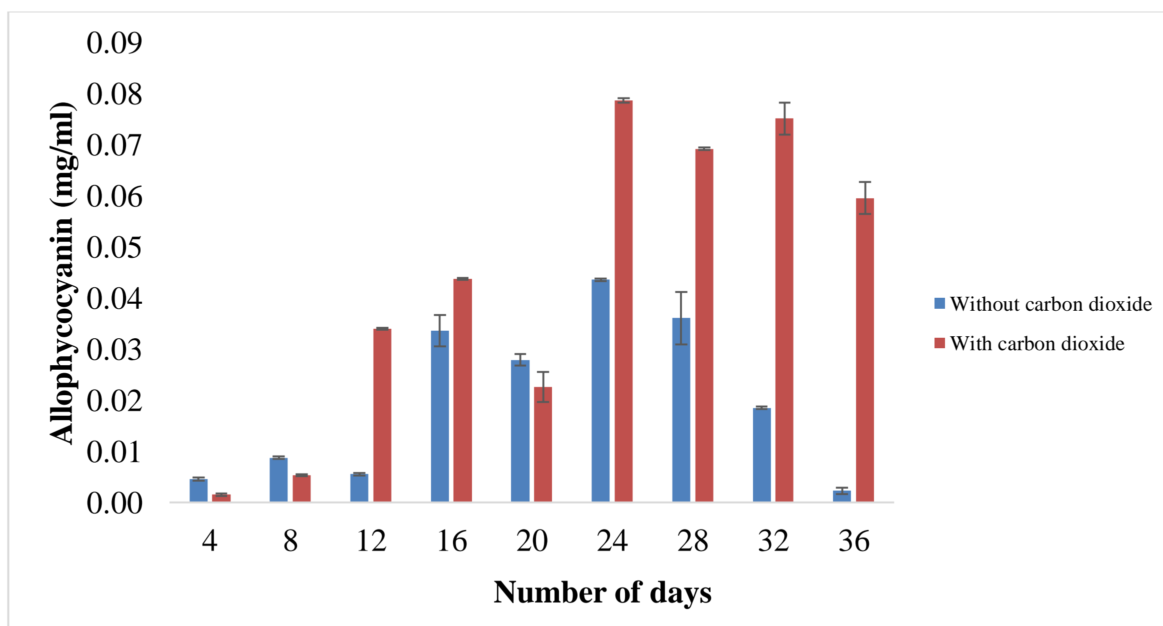

Figure 24. Allophycocyanin in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

The allophycocyanin content in the *Anabaena* sp. (DGNB1) was initially 0.002mg/ml with the CO_2 sparging on the 4th day of the cycle whereas in without CO_2 sparging it was 0.005 mg/ml on the similar day. As the algal growth increased with the passage of days so does increased

the allophycocyanin content in the algae. CO_2 sparging increased the algal growth thus resulting in the enhancement of the pigment. The maximum pigment was obtained with CO_2 sparging was 0.08 mg/ml on the 32nd day of the growth cycle whereas in the withoutCO_2 sparging it was 0.04 mg/ml on the 32nd day.

Table 22. Phycoerythrin in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

Days	Phycoerythrin with CO_2	Phycoerythrin without CO_2
4	0.00±0.00	0.01±0.00
8	0.02±0.00	0.02±0.00
12	0.07±0.00	0.02±0.00
16	0.04±0.03	0.03±0.00
20	0.04±0.00	0.05±0.00
24	0.12±0.00	0.07±0.00
28	0.11±0.001	0.06±0.00
32	0.10±0.00	0.04±0.00
36	0.10±0.00	0.03±0.00

*values given are average of three replications

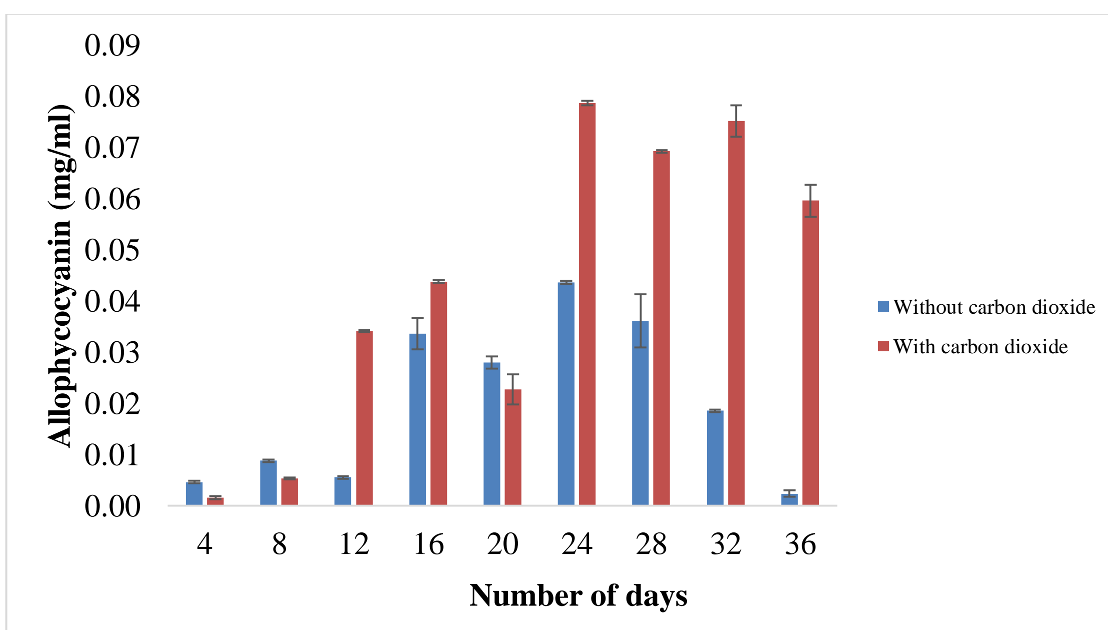

Figure 25. Phycoerythrin in *Anabaena* sp. (DGNB1) with and without CO_2 sparging

The phycoerythrin content in *Anabaena* sp. (DGNB1) was 0.003 mg/ml with CO_2 sparging on the 4th day of growth whereas in without CO_2 sparging it was 0.01 mg/ml on the similar day. The maximum phycoerythrin was obtained on the 24th day with CO_2 sparging which was 0.12

mg/ml whereas in the without CO_2 sparging it was 0.07 mg/ml on the 24th day. There was observed exponential increase in the phycoerythrin concentration. The CO_2 sparging resulted in the increase in the biomass thus increasing the pigment concentration.

Table 23. Phycocyanin in *Nostoc* sp. (DGNB2) with and without CO_2 sparging

Days	Phycocyanin with CO_2	Phycocyanin without CO_2
4	0.00±0.00	0.01±0.00
8	0.02±0.00	0.01±0.00
12	0.04±0.00	0.03±0.00
16	0.05±0.00	0.03±0.00
20	0.09±0.00	0.05±0.00
24	0.11±0.00	0.08±0.00
28	0.20±0.00	0.19±0.00
32	0.16±0.003	0.07±0.00
36	0.15±0.00	0.06±0.00

*values given are average of three replications

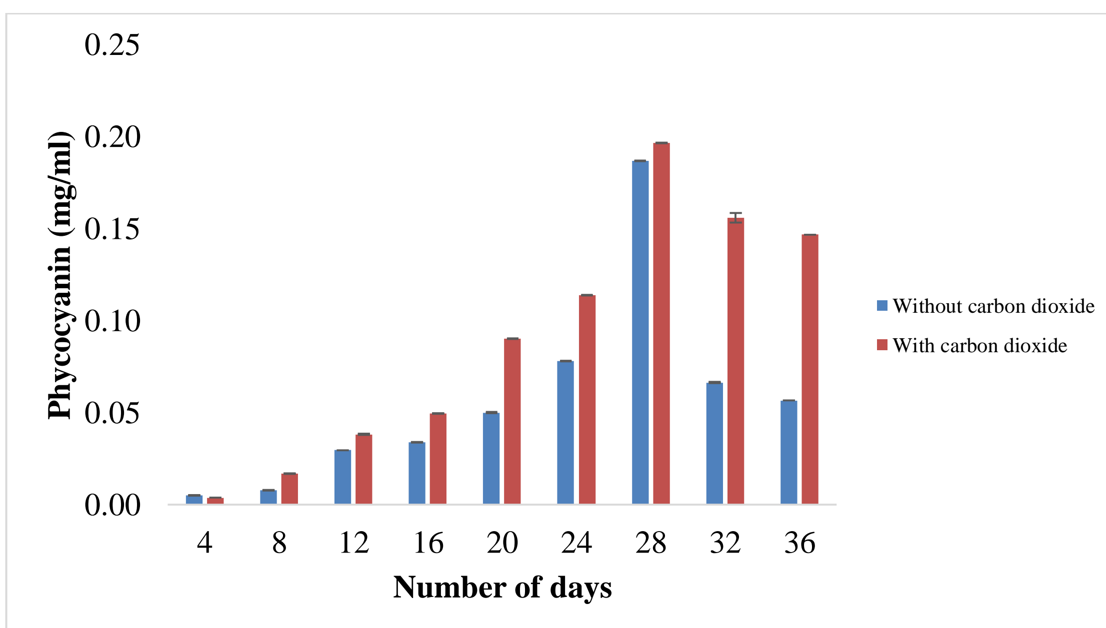

Figure 26. Phycocyanin in *Nostoc* sp. (DGNB 2) with and without CO_2 sparging

The phycocyanin concentration in *Nostoc* sp. (DGNB 2) on the 4th day was 0.001 mg/ml with CO_2 sparging whereas in the without CO_2 sparging it was 0.01 mg/ml on the same day. There was observed exponential increase in the pigment concentration with the passage of the days.

The maximum phycocyanin was observed on the 28th day of the growth cycle with CO_2 sparging and in without CO_2 sparging it was observed on the similar day, 0.19 mg/ml.

Table 24. Allophycocyanin in *Nostoc* sp. (DGNB2) with and without CO_2 sparging

Days	Allophycocyanin with CO_2	Allophycocyanin without CO_2
4	0.00±0.00	0.00±0.00
8	0.00±0.00	0.01±0.00
12	0.02±0.00	0.02±0.00
16	0.03±0.00	0.02±0.00
20	0.05±0.00	0.02±0.00
24	0.07±0.00	0.06±0.00
28	0.11±0.00	0.10±0.00
32	0.05±0.00	0.04±0.00
36	0.03±0.00	0.02±0.00

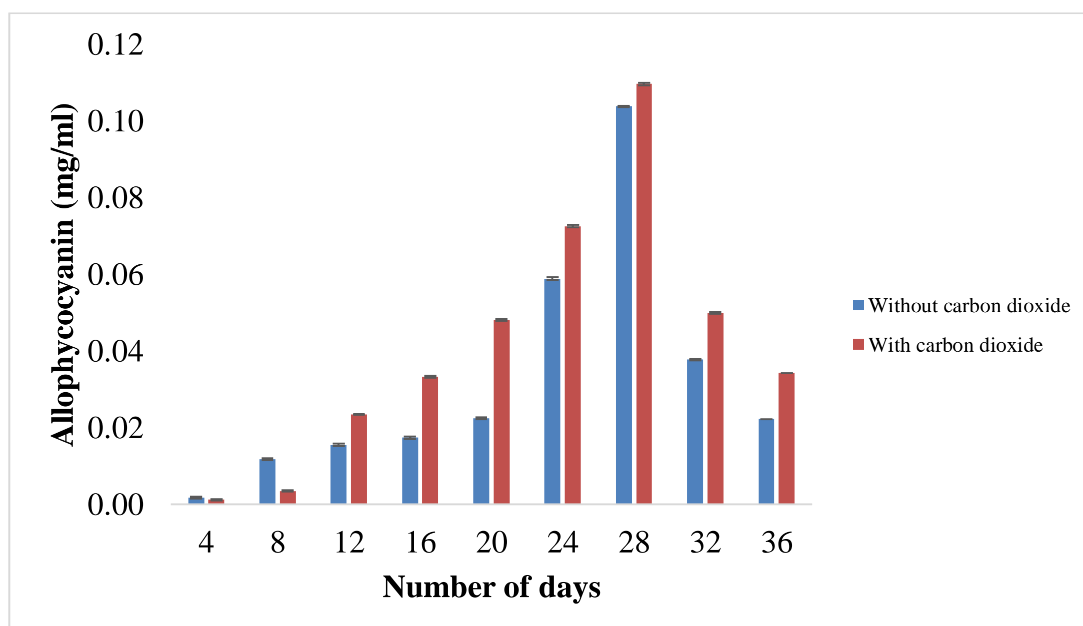

Figure 27. Allophycocyanin in *Nostoc* sp. (DGNB 2) with and without CO_2 sparging

The allophycocyanin concentration in *Nostoc* sp. (DGNB 2) was 0.001 mg/ml with CO_2 sparging and 0.002 mg/ml in the algae culture without CO_2 sparging. There was observed exponential increase in the allophycocyanin concentration with the days. The maximum concentration was observed on 28th day in both with and without CO_2, having 0.11 mg/ml for

the former and 0.10 mg/ml for the latter one. CO_2 sparging resulted in increase in the biomass as it further lead to increase in the pigment.

Table 25. Phycoerythrin in *Nostoc* sp. (DGNB 2) with and without CO_2 sparging

Days	Phycoerythrin with CO_2	Phycoerythrin without CO_2
4	0.01±0.00	0.01±0.00
8	0.03±0.00	0.02±0.00
12	0.07±0.00	0.05±0.00
16	0.09±0.00	0.06±0.00
20	0.17±0.00	0.10±0.00
24	0.21±0.00	0.16±0.00
28	0.18±0.00	0.21±0.00
32	0.19±0.00	0.14±0.00
36	0.18±0.00	0.13±0.00

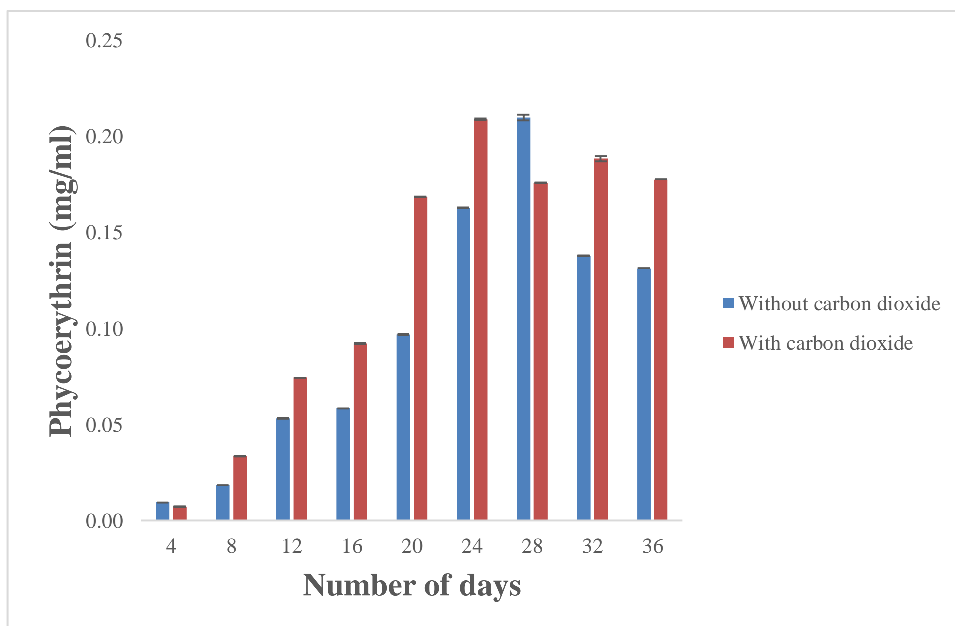

Figure 28. Phycoerythrin in *Nostoc* sp. (DGNB2) with and without CO_2 sparging

In *Nostoc* sp. (DGNB2) the phycoerythrin content on 4th day was 0.01 (mg/ml) both with and without CO_2 sparging. The maximum phycoerythrin was noted on 21st day in with CO_2 whereas for without CO_2 it was calculated on 28th day 0.21 (mg/ml). The pigment concentration

increased due to increase in the biomass concentration. According to Fei et al (2007) elevated CO_2 increase phycoerythrin and phycocyanin content significantly due to increase in number of phycobilisomes.

Table 26. Astaxanthin in *H.pluvialis* with and without CO_2 sparging

Days	With CO_2 sparging	Without CO_2 sparging
4	0.01±0.00	0.01±0.00
8	0.02±0.00	0.02±0.00
12	0.03±0.01	0.03±0.00
16	0.05±0.00	0.05±0.00
20	0.07±0.00	0.06±0.00
24	0.10±0.03	0.09±0.00
28	0.13±0.00	0.10±0.01
32	0.20±0.02	0.17±0.01
36	0.28±0.03	0.21±0.00

*values given are average of three replications

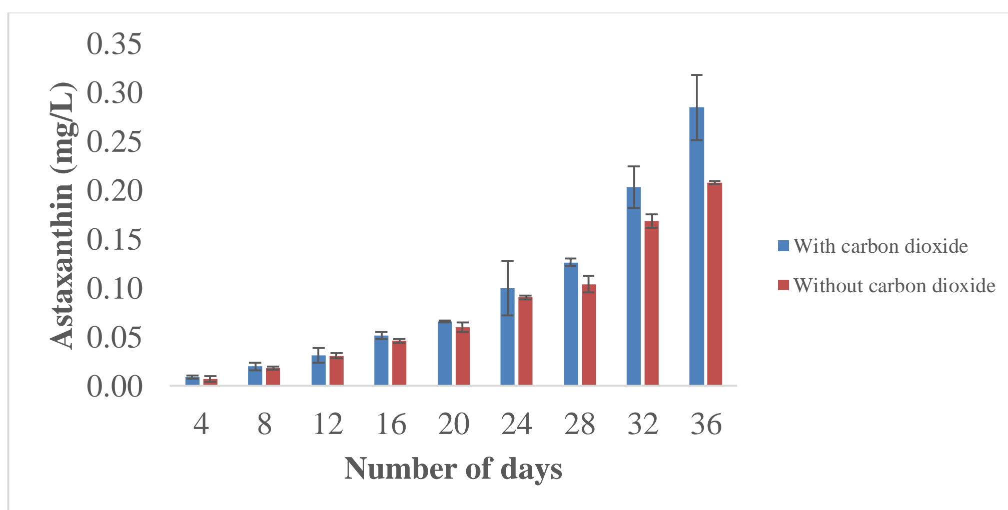

Figure 29. Astaxanthin in *H.pluvialis* with and without CO_2 sparging

Astaxanthin is a carotenoid and synthesized during the unfavourable conditions. As the days passes, the growth increases by utilization of nutrients present in the medium. When conditions turn unfavourable they result in increase in the astaxanthin production.

Astaxanthin concentration was 0.01 (mg/L) in both with and without CO_2 sparging on the fourth day of the growth cycle. There was observed exponential increase in the astaxanthin concentration and maximum astaxanthin was observed on 36th day of the growth cycle which was 0.28 (mg/L) with CO_2 sparging and 0.21 (mg/L) without CO_2 sparging.

Aeration in the medium leads to increase in the amount of pigment (Chen *et al*., 1997). Moderate carbon dioxide level are helpful as minimize the limitation of inorganic carbon and favour the fixation of it. However, very high levels of CO_2 can be deleterious for the growth and astaxanthin accumulation by the algae.

Conclusion

1. Native microalgae were isolated and screened for pigment content and further carbon dioxide sparging was done in media to enhance the pigment production.
2. The highest chlorophyll content was found in isolate *Chlorella* sp. (DGNB3) whereas, phycocyanin and phycoerythrin were found to be maximum in *Anabaena* sp. (DGNB1) and *Nostoc* sp. (DGNB2), respectively.
3. Growth measurement studies showed that highest biomass production was achieved in the carbon dioxide sparged cultures.
4. Significant amount of astaxanthin by *H.pluvialis,* 0.28 mg/L, was observed in the culture with the CO_2 sparging as compared to the culture without CO_2 sparging which was 0.21mg/L.
5. Similarly, the chlorophyll content was highest, 11.9 mg/L, in the culture with CO_2 sparged culture than without CO_2 sparging (11.27mg/L).
6. The CO_2 also increased the pigments-phycocyanin, allophycocyanin and phycoerythrin. Maximum phycocyanin obtained was 0.17 mg/ml by CO_2 sparging from *Anabaena* sp. (DGNB1) which was relatively high compared to 0.09 mg/ml without CO_2 sparging.

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