STUDY ON VARIOUS PHYSICO-CHEMICAL AND PHYSIOLOGICAL FACTORS REGULATING CARBON SEQUESTRATION, BIOMASS AND LIPID PRODUCTION IN MICROALGAE

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Submitted By

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Under the supervision of

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Dedicated to

Late Prof. D.P. Singh

CANDIDATE'S DECLARATION

I, **Nisha Yadav**, solemnly declare that the research work embodied in this thesis entitled **"STUDY ON VARIOUS PHYSICO-CHEMICAL AND PHYSIOLOGICAL FACTORS REGULATING CARBON SEQUESTRATION, BIOMASS AND LIPID PRODUCTION IN MICROALGAE"** carried out by me under the guidance and supervision of **Prof. Naveen Kumar Arora, Professor, Department of Environmental Science, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, India** is original and is also approved by Departmental Research Committee (RDC).

I further declare that to the best of my knowledge, this thesis does not contain part of any work submitted for the award of any degree either in this University or any other University around the globe. It is further undertaken that the thesis is essentially free from all kinds of plagiarism.

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Place: Lucknow

CERTIFICATE

This is to certify that the thesis titled **“STUDY ON VARIOUS PHYSICO-CHEMICAL AND PHYSIOLOGICAL FACTORS REGULATING CARBON SEQUESTRATION, BIOMASS AND LIPID PRODUCTION IN MICROALGAE”** submitted by **Ms. Nisha Yadav** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

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Nisha Yadav

PREFACE

Globally, carbon sequestration is attaining considerable attention to alleviate the impact of increasing CO_2 in the atmosphere. Carbon sequestration is a process consisting of carbon capture from the point source and long-term storage and isolation from the atmosphere. Carbon in the environment exists in inorganic, organic, and gaseous forms. Gaseous carbon is in the form of CO_2 gas while other forms of carbon are inter-convertible as seen in the carbon cycle. The fixed form of organic and inorganic carbon, if transformed into gaseous CO_2, may enhance the intensity of global warming. One important strategy to reduce atmospheric CO_2 involves carbon fixation by photosynthetic organisms such as plants, algae, and cyanobacteria. Over several decades microalgae have attained huge impetus from both academia and industry; however, their cultivation and further processing are techno-economically demanding. Microalgae are important organisms due to multiple benefits from a short life cycle to high growth rate and CO_2 fixation capacity as compared to plants. Microalgae in addition to CO_2 capture can produce biomass, which can further be utilized in generating industrially important products. The cultivation of microalgae for carbon capture and utilizing biomass for extracting valuable products give algal bio-sequestration an advantage over other methods. CO_2 mitigation strategy through useful biomass generation should not only focus on the atmospheric CO_2, but also on other organic and inorganic forms of carbon having potential to get converted into CO_2 through the natural carbon cycle. There are several physiological and physico-chemical factors such as type of microalgal species, pH, salinity, light, temperature, and nutrients which can be very helpful in determining the success of biological fixation of CO_2. The major drawback of microalgae cultivation under stress conditions

is low biomass production. This is considered a major bottleneck in the large-scale exploitation of microalgae. The continuous supplementation of optimum CO_2 can enhance the biomass as well as lipid yield of microalgae. Therefore, the present study entitled "**Study on various physico-chemical and physiological factors regulating carbon sequestration, biomass and lipid production in microalgae"** was carried out to give scientific knowledge about the ability of microalgae *Chlorella vulgaris* for utilization of carbon fixation (organic, inorganic, and gaseous) and lipid production. Further, the present investigation is an attempt to study the photosynthetic efficiency, biomass, and lipid production of the microalgae *C. vulgaris.* The changes in growth rate, biochemical constituents, and photosynthetic efficiency of *C. vulgaris* were measured by using UV-Visible spectrometry, fourier transform infrared spectroscopy (FTIR), Chlorophyll fluorescence induction kinetics (OJIP) curve, and Scanning Electron Microscopy-Energy Dispersive Spectroscopy (SEM-EDS) analysis. The research presented in this thesis was tailored towards improving the potential of microalgae for carbon-capturing under various stress conditions combined with enhancement in lipid, thereby addressing two vital aspects of a subsequent biorefinery concept. The present thesis is divided into nine chapters.

Chapter I '**Introduction**' presents the outline of the work and attempts to provide the rationale of the research work along with the list of objectives to be accomplished in this thesis.

Chapter II '**Review of Literature**' provids an overview of the work done by investigators in the field of microalgae, environmental stress conditions, carbon sequestration, nutritional alterations, and lipid production.

Chapter III '**Materials & Methods**' is exclusively assigned to the general methodology used during the present investigation.

Chapter IV Antagonistic effect of bicarbonate on salinity-induced changes in microalga *Chlorella vulgaris* deals with the role of sodium bicarbonate in the regulation of salinity stress-induced alterations in the growth, nutrient status of the cells, photosynthetic performance, and level of cell constituents including lipid in the microalga *C. vulgaris*.

Chapter V Effect of pH conditions on photosynthetic efficiency and macro-molecules of microalgae *Chlorella vulgaris* is assigned to study the effect of varying pH conditions on algal growth and cell constituents in the *C. vulgaris*. In addition, it was also attempted to see the interactive effect of bicarbonate and pH conditions on the photochemistry of PSII and cell constituents like protein, carbohydrates, total organic carbon, and lipids.

Chapter VI Effect of excess CO_2 and high light intensity on microalga *Chlorella vulgaris* deals with the study of the combined role of CO_2 and light intensity in the regulation of photochemistry of PS II, which has been elucidated by studying the photosynthetic performance parameters of microalga under varying light intensities.

Chapter VII Role of phosphate and organic carbon in the regulation of mixotrophic growth of *Chlorella vulgaris* was an attempt to analyze the impact of both mixotrophic (different C/P ratio) cultivation as well as phosphate nutrition on the growth, macromolecular content, and photosynthetic performance of microalga *C. vulgaris*.

Chapter VIII Effect of C:N ratio on mixotrophic cultivation of *Chlorella vulgaris* sheds light on the mechanism of cellular adaptation to nitrogen limiting conditions,

which is crucial for achieving higher lipid productivity. It deals with the biomass, cell composition, nutrient status, and photosynthetic attributes of unicellular microalga *C. vulgaris* in response to varying C:N ratios.

Chapter IX 'Summary and Conclusion summarises the outcome of the present research work.

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ABBREVIATIONS

µg	Microgram
µm	Micromolar
µl	Microliter
%	Percent
C	Degree Celsius
C:P	Carbon:Phosphorous
C:N	Carbon:Nitrogen
ABS/RC	Absorption of energy flux per active reaction center
Chl ‘a’	Chlorophyll ‘a’
cm	Centimeter
C:N	Carbon:Nitrogen
Conc.	Concentration
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
etc.	Et cetera (other similar things)
DNP	2,4- dinitrophenol
ET_0/RC	Trapping of energy flux beyond QA in the photosystem II
FAME	Fatty acid methyl ester
Fig.	Figure
Fo	Fluorescence intensity at 50 µs
Fm	Maximum chlorophyll fluorescence in the dark adapted state
Fm/Fo	Actual quantum yield of PSII photochemistry
FTIR	Fourier Transform Infra-red
Fv/F_m	Maximum quantum yield of PSII (dark-adapted state)
F_v/F_0	Maximum quantum yield of PSII (more sensitive than F_v/F_m)
G	Gram
H	Hour
HCl	Hydrochloric acid
HL	High light
H_2SO_4	Sulphuric acid
HNO_3	Nitric acid

I_{50}	50% inhibitory concentration
IR	Infra-Red
KBr	Potassium bromide
L	Liter
LED	Light–emitting diode
LI	Light intensity
LL	Low Light
M	Molar
Mo	Initial slope of chlorophyll fluorescence induction curve,
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
Nm	Nanometre
MV	Methyl viologen
NPQ	Non-photochemical quenching
OJIP	Chlorophyll fluorescence induction kinetics
OD	Optical density
PAR	Photosynthetically active radiation
PBS	Phosphate Buffer Saline
PI_{ABS}	Performance index on absorption basis
PI	Propidium Iodide
PMS	Phenazinemethosulphate
psi	Per square inch
PS I	Photosystem I
PS II	Photosystem II
PUFA	Poly-unsaturated fatty acid
QY	Quantum yield
qE	Energy dependent quenching
RC	Reaction centre
rETR	Relative electron transport rate
Rfd	Relative flouroscence decrease ratio
rpm	Rotation per minute

SEM	Scanning Electron microscope
SFA	Saturated fatty acids
SGR	Specific growth rate
SH	Sulfhydryl
TAG	Triacylglycerol
TOC	Total organic carbon
TR_0/RC	Efficiency of electron transport leading to reduction of QA
UPLC	Ultra-performance liquid chromatography
UV	Ultra Violet radiations
UV-Vis	Ultra Violet-Visible
Vj	Relative variable florescence at 2 ms.
v/v	volume/volume
Viz.	videlicet
w/v	weight/volume

Chapter I

General Introduction

Introduction

Microalgae belong to a broad group of eukaryotic autotrophic organisms, but they show a vast difference from the land plants. Algae can be defined as prokaryotic blue-green algae and eukaryotic green algae belonging to highly diverse phylogenetic clades. Eukaryotic microalgae are polyphyletic in origin and have an extensive ecological niche and evolutionary history. Microalgae are found in fresh water and marine ecosystems and are microscopic. They are generally unicellular species that exist either individually or in groups. Microalgae are photosynthetic organisms, use the atmospheric carbon dioxide (CO_2) and grow photoautotrophically. They are well known to produce almost half of the oxygen found in the atmosphere. The microalgal biodiversity is huge and they represent an almost untapped resource. Most microalgal species produce valuable products like carotenoids, fatty acids, toxins, antioxidants, peptides, and sterols. Microalgae store all the fixed carbons in the cell as proteins, starch, and lipids. Hence, microalgae are considered excellent candidates for the production of biofuel.

Algal species fix atmospheric CO_2 via the Calvin–Benson cycle with the help of the enzyme Rubisco and convert CO_2 to complex organic compounds. Microalgal species have a good potential for CO_2 sequestration as 1.0 kg of cultivated microalgae can fix 1.83 kg of CO_2. Microalgae species such as *Chlorella vulgaris* and *Anabaena sp*. fix CO_2 at the rate of 6.24 and 1.45 g/L/d, respectively (Mistry et al., 2019). The atmosphere is comprised of CO_2 at a concentration ranging from 0.03% to 0.06% (v/v), while flue gas has a CO_2 concentration ranging from 6% to 15% (v/v) (Rahaman et al., 2011). These are the major sources of CO_2, which can be utilized for the mass cultivation of microalgae. Moreover, microalgae cultivation near high CO_2 emission sources such as power plants and refineries is found to be beneficial and

cost-effective. The bio-fixation of CO_2 and algal biomass production varies greatly depending on the characteristics of microalgae species, cultivation conditions, and other physic-chemical conditions. The success of bioconversion of CO_2 into biomass production relies upon the choice of suitable microalgae species to be cultivated. An ideal algal species should have a high carbon sequestration rate, higher tolerance to extreme environmental conditions such as CO_2 concentration, nutrient limitation, temperature, pH, and pollutants. The most capable microalgal species reported for CO_2 sequestration, biomass production, and biofuel application are *C. vulgaris, Scenedesmus obliquus*, and *Nannochloropsis oculata, Botryococcus braunii* (Maheshwari et al., 2019).

C. vulgaris is a green microalga in the genus *Chlorella*, which has been found on earth since the Precambrian period (Safi et al., 2014). This eukaryotic unicellular alga was discovered in 1890 by Martinus Willem Beijerinck as the first microalga to have a well-defined nucleus. *C. vulgaris* is considered to be a good source of bioenergy and substitute for biofuel crops, like corn, soybean, or rapeseed. The microalga *C. vulgaris* as a biofuel source is very productive and does not compete with the production of food. *C. vulgaris* has been reported to produce a large number of lipids, approximately 20 times more than traditional crops which have a potential source of biodiesel production. This microalga also contains high amounts of starch, which can be used for the production of bioethanol. However, microalgal biofuels are unable to compete with fossil fuels, due to their high costs of production and controversial sustainability.

1.1 Benefits of microalgae

1.1.1 Wastewater treatment and biomass generation

Wastewater is a by-product of several industrial processes due to intensive water use The domestic wastewater is second to none and discharged in the form of sewage

wastewater. Wastewater produced from different industrial and domestic operations has a large amount of nitrogen, phosphorous, metals, organic matter, and other elements in large quantities (Gomez-Serrano et al., 2015). The seamless discharge of nutrient-rich wastewater is a serious environmental problem. Different physicochemical methods of wastewater treatment are found to be technically flawed and costly. Among the various biological treatment methods, phycoremediation of nutrient-rich wastewater by using microalgae is being preferred due to the unique composition of different wastewater which is very much similar to the culture media usually used to produce algal biomass. Microalgae exhibit the ability to fix CO_2 by using light and water as the only source of energy. Microalgae are outstanding cell factories to produce bioenergy-rich products and thus, microalgal cells can be termed as bio-refineries. Along with the photoautotrophic mode of nutrition, several microalgal species are capable of using a heterotrophic or mixotrophic mode of nutrition. The mixotrophic mode of microalgal cultivation can be of great interest as it can be employed to harness energy and biomass from nutrient-rich wastewater with a high organic load. At the same time, the microalgae-treated wastewater would be fit for either tertiary treatment or irrigation purposes. The utilization of microalgae for the treatment of different wastewater was for the first time reported by Oswald in 1960, but now several reports have been reviewed (Park and Craggs, 2010; Mehrabadi et al., 2015; Munoz et al., 2006; Olguín et al., 2012). However, there is still insufficient work in the direction of successful implementation and cost-effective phycoremediation technology. A serious effort has to be made to cultivate locally suitable algal strain which shows efficiency in utilization of wastewater, tolerance towards the wastewater toxicants, lipid and biomass production for clean and green energy. C. *vulgaris* is among the most widely studied strains for biomass production.

It has been examined for its potential of biomass production from food waste compost, cheese whey, and vinasse, sludge extracts, corn steep liquor, textile waste effluent, and industrial dairy effluents (Chen et al., 2019). Zhai et al., (2017) also demonstrated that the high N (81.51%) and P (80.52%) removal efficiency by the cyanobacterium, *Arthrospira platensis* using modified wastewater.

The five-decade history of research on wastewater treatment systems based on algae is a testimony to the contribution of algae in the management of the environment, particularly the freshwater aquatic ecosystem (Hoffmann, 1998). Most of the laboratory-scale experiments and treatment of small volumes of contaminated water by using algal biomass require upscaling. To achieve the target of implementing the technology at a large scale, professionals from various fields such as biologists, chemists, engineers, and environmentalists are required to be brought to a single platform. Since microalgae exhibit great potential to remove the nutrients from wastewater streams (Rawat et al., 2011), it provides an environmentally friendly approach to reduce the threat of water eutrophication at the point source of discharge of domestic wastewater (Hoffmann, 1998).

The disposal of wastewater after secondary treatment is one important challenge for sustainable wastewater treatment and management (Buys et al., 2008). Thus, the use of such wastewater for large-scale production of algal biomass could yield high-value products (Mallick, 2002) such as bioenergy & biogas, fertilizers, soil conditioners, cosmetics, pharmaceuticals, and other valuable chemicals (Mallick, 2002). In addition, algal treatment (Fig.1.1) replaces conventional tertiary treatment and also generates an oxygenated effluent that is environmentally safe, cost-effective, and fit for irrigation and other domestic use (Hoffmann, 1998). Furthermore microalgae,

such as *Chlorella sp.*, have a high photosynthetic rate, so they could be utilized for bioconversion of CO_2 into valuable microalgal biomass (Ruiz et al., 2011).

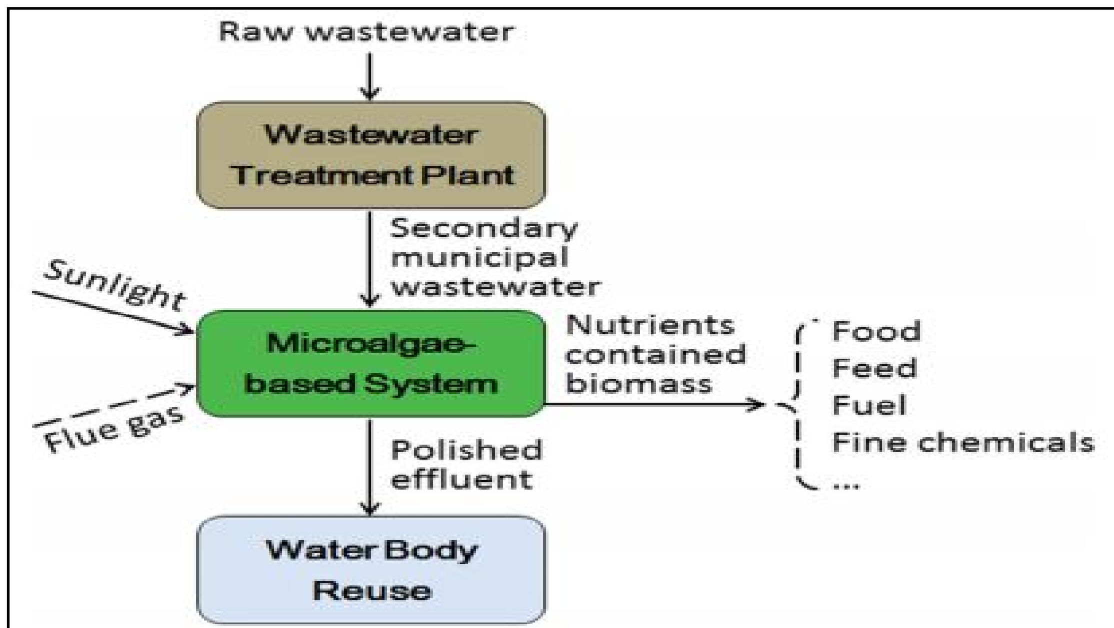

Fig. 1.1 Schematic diagram on microalgae-based advanced municipal wastewater treatment for water body reuse. Dashed arrows and braces indicate optional conditions and products. Source: (Wang et al., 2017).

1.1.2 Removal of heavy metals

Heavy metals are generally released into the water body as a result of various industrial activities. Since heavy metals are mostly toxic, non-biodegradable, and persistent, they pose a serious environmental problem to the biota. Therefore, their treatment is of special importance. If the heavily loaded wastewater is untreated, the heavy metals present in the discharged stream of wastewater remain in the soil and sediments, which are slowly, in course of time, released into the food chain. Among the treatment methods, biological treatment can be employed only for few toxic substances. Microalgae are very sensitive to changes in their environment. In response to trace level contamination in water, the microalgal metabolism is seldom changed. This attribute of microalgae has been exploited for detecting various organic and

inorganic pollutants including heavy metals (Torres et al., 2011; Savariraj et al., 2020). Several prokaryotic and eukaryotic microalgal strains as well as their dead cell biomass have been used for remediation of metal-polluted sites. Keeping in view the potential use of microalgal biomass for commercial application, a deep understanding of algal metabolism and its metal detoxification efficiency upon exposure to various metal-contaminated effluents would be essential. The toxic nature of effluents loaded with a mixture of various inorganic and organic compounds can seriously affect the metal speciation and metal sequestration and thereby, overall microalgal efficiency of metal detoxification processes.

1.1.3 Industrial application for commercial products

Microalgae are also considered as a promising food or feed ingredient, owing to their nutraceutical features (Batista et al., 2017) which are mainly dependent on the microalgal strain and their cell composition. Furthermore, the quantum of neutraceuticals also depends upon the cultivation conditions viz., quality and intensity of light, temperature, pH, and nutrient profile. A wide spectrum of bioactive compounds have been obtained from the microalgal biomass in the form of proteins, polyunsaturated fatty acids (PUFAs), pigments, vitamins, and minerals or as exogenous oligosaccharides (Sousa et al., 2008; Vidanarachchi et al., 2012). The efficiency of biosynthesis of these value-added products is greater in microalgae than that observed in many terrestrial crop plants. Some studies have demonstrated several health benefits from the extracted microalgal compounds, which are known to act as anti-cancer, anti-inflammatory, antioxidant, antimicrobial, anti-obesity, and anti-hyper cholesterol compounds. Hence, it is expected that the market prices of nutraceuticals (Vidanarachchi et al., 2012) and other valuable commercial products from microalgae

would shoot up in the long run as they are considered green products without adverse environmental and health issues (Priyadarshani et al., 2012). Microalgae have also been increasingly used as fish feed in aquaculture or as immune boosters for marine animals. Unfortunately, the commercial use of microalgae is facing hardships as the production costs are too high due to less production of biomass and lack of technology to enhance the product synthesis at a suitable cost (Spolaore et al., 2006). Additionally, industrial use of microalgae as prebiotics in feed formulation is also facing challenges as an improvised processing technology is still required to reduce the production costs (Gupta et al., 2017).

1.2 CO_2 as a greenhouse gas: Role in climate change

Excessive CO_2 emission due to anthropogenic activities has reached an alarming level and it is the primary cause of global warming and climate change, which may have devastating effects such as frequent recurrence of drought, torrential rains, floods, tornados, and wildfires, etc. across the globe (Arora, 2018). Such unforeseen happenings are responsible for thousands of deaths, loss of livelihood, and economic growth, particularly in many developing countries. The amount of greenhouse gases, mainly carbon dioxide, has been increasing at a very fast pace in the atmosphere as a result of different anthropogenic activities. As per the Intergovernmental Panel on Climate Change, the contribution of carbon dioxide as a greenhouse gas is estimated to be 76.7% (v/v) and its concentration has drastically increased since the beginning of industrialization (Mistry et al., 2019). The major contributors to increased atmospheric CO_2 levels are excessive use of fossil fuels and deforestation. The excessive use of fossil fuels such as oil, coal, and natural gas is contributing up to 87% of the total anthropogenic production of CO_2, while 9% increase in CO_2 is due

to deforestation and change in the land use pattern. The remaining 4% of CO_2 is produced by industrial processes (Pires et al., 2013). The highest amount of CO_2 is produced from the burning of coal as compared to other types of fossil fuel, which is approximately 2.5 tonnes of CO_2 produced from every tone of coal burned. Industry, Energy, and transportation are the three major economic sectors that use fossil fuels and lead to CO_2 emission. In the year 2010, it was observed that nearly two-thirds of the global CO_2 emission was contributed by the energy and transportation sectors. Carbon dioxide is one of the major greenhouse gases and its increase in atmospheric concentration is leading to serious negative environmental effects such as global warming leading to climate change. Some of the observed phenomena are (i) increased frequency and duration of heatwaves; (ii) rise in temperature of ocean and atmosphere; (iii) sea-level rise; and (iv) decreased mass of Greenland and Antarctic ice caps. The above-mentioned phenomena are known to adversely affect human health as there would be an increase in the frequency of heatwaves and other heat-related issues. The temperature variation will change the pattern of precipitation, leading to the spread of certain unknown diseases (Fouque et al., 2019). To manage atmospheric CO_2 levels, different approaches like enhancing the efficiency of energy conversion and use of energy sources with less carbon content or free from carbon are required.

1.3 Carbon cycle

Carbon serves as the chemical backbone of all life forms existing on Earth. The amount of carbon currently on Earth is the same as earlier. Carbon forms the backbone of key molecules such as protein and DNA, of newly formed life. It is also present in atmosphere in the form of CO_2. The carbon cycle is a complex chain of

processes through which all the atoms of carbon on the earth get recycled (Fig.1.2). The carbon cycle is a topic of interest in basic and applied ecology (Chapin et al., 2006). Most of the biological and anthropogenic activities are stimulated by a biochemical transformation of carbon either as CO_2 or in the form of organic compounds, which are eventually oxidized to release energy and CO_2 by the process of biological respiration or combustion.

Although the transfer of CO_2 to the atmosphere by natural means is approximately 20 times higher than those occurring as a result of human activity, they are normally in a balanced position. The supplementary CO_2 resulting from human activities; primarily the combustion of fossil fuels by the industries and by change in land use, have led to emissions of various greenhouse gases and have altered the global carbon cycle (IPCC 2007b). Folger, (2009) reported that nearly 7.2 GtC/year is being added to the atmosphere through the burning of fossil fuels, whereas land and ocean vegetation is capable of capturing (including microalgae) about 2.8 GtC/year. Thus, resulting in imbalanced carbon cycling and leaves a big share of carbon in the atmosphere. The carbon cycle is a process where nature reuses carbon atoms, which circulate from the atmosphere into organisms and then again come back to the atmosphere over and over again. The major part of carbon is stored in rocks and sediments, while the remaining carbon is preserved in the ocean, atmosphere, and living beings. These are the carbon sink, through which carbon is cycled. Oceans are the biggest sink of carbon that absorb and fix carbon. Marine organisms including marsh plants, fishes, seaweed, and birds, also produce carbon through living and dying. Sometimes these dead organisms become fossil fuels and give off carbon dioxide through combustion and carbon cycle goes on. Carbon exists only in the form of CO_2 in atmosphere. Carbon helps in maintaining the Earth’s temperature, makes it possible for all life forms to survive, is

a major ingredient of the food chain, and provides a major source of energy on Earth. Since our planet and its atmosphere form a closed environment, the amount of carbon in this system remains the same. Whether the carbon is present in the atmosphere or on the Earth is persistently in flux.

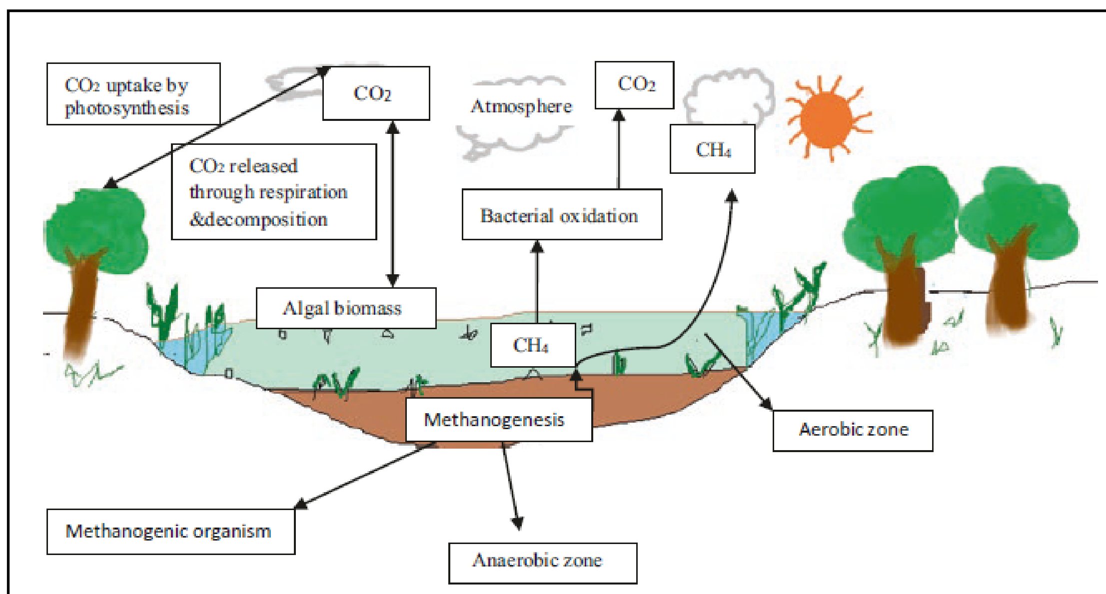

Fig. 1.2: Schematic diagram showing cycling of carbon in the aquatic ecosystem.

1.4 Methods of CO_2 capture and storage

Presently there are several physico-chemical methods of carbon trapping and carbon sequestration which are linked to carbon capture and storage (CCS) strategies. The CCS works over 3 major steps: CO_2 capture, CO_2 transportation, and CO_2 storage. Power plants and cement manufacturing plants are the point source of CO_2 from where carbon capture is done. The separation and capture of CO_2 from other exhaust sources are generally done via the following methods (Fig. 1.3):

(a) Chemical absorption

(b) Membrane separation

(c) Physical adsorption

(d) Cryogenic distillation (Figueroa et al., 2008; Pires et al., 2012).

The extremely concentrated CO_2 is compressed and transported to storage locations via pipelines or ships (Singh et al., 2019). In the next stage, the captured CO_2 is dumped into reservoirs viz. oceanic storage, geological storage, wherein the reserved CO_2 is directly inoculated deep into the ocean, aquifers or depleted oil/gas wells, saline formations, (Lackner, 2002). Despite good storage potential in these CCS methods, there are still considerable drawbacks including cost-effectiveness and environmental threat of CO_2 leakage (De Silva et al., 2015). Moreover, the CCS methods are practically efficient only for capturing CO_2 from a point source that produces remarkable concentrations, whereas capture from diffused non-point sources with fewer concentrations of CO_2 is not feasible (Nouha et al., 2015). Besides numerous CCS methods for CO_2 capture, the biological methods of capturing CO_2 such as forestation, reforestation, crop farming, and livestock can be considered better (Farrelly et al., 2013). The ocean fertilization with iron and other nutrients may increase the carbon dioxide uptake by certain phytoplanktons (Williamson et al., 2012). Among these biological methods of CO_2 sequestration, the cultivation of microalgae is regarded as an easier and efficient method (Lam et al., 2012; Zhou et al., 2017; Yadav and Sen, 2017).

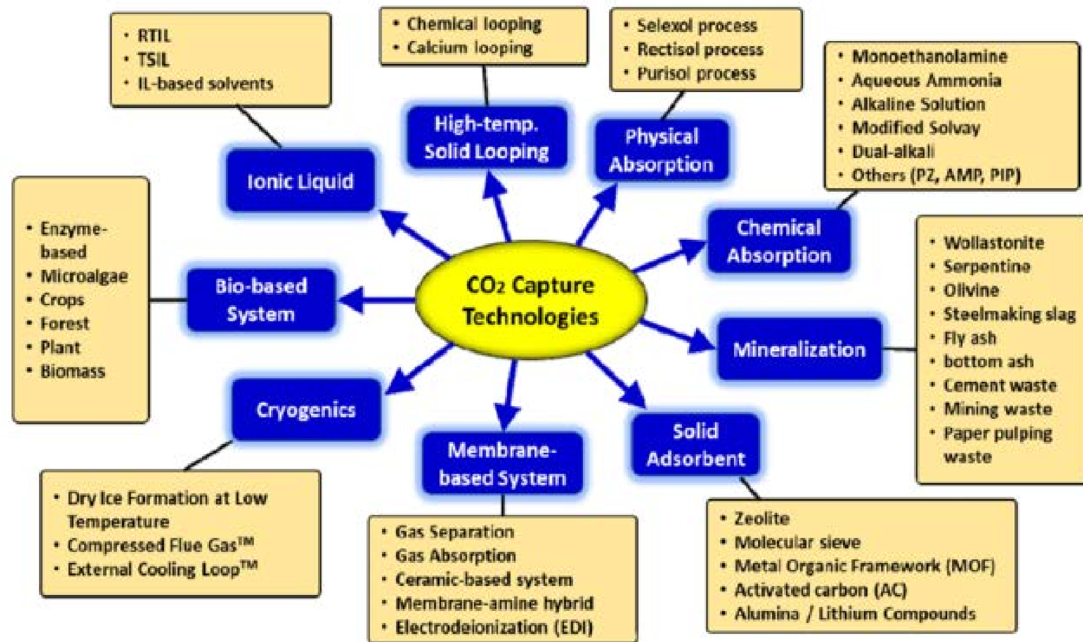

Fig: 1.3: Different technologies for CO_2 capture. Sources: (Li et al., 2016)

1.5 Use of algae for carbon sequestration and storage

Microalgae are microscopic organisms that frequently grow in fresh water and their growth is governed by the photosynthetic process, which takes place in the presence of light and CO_2, which is similar to that in the higher plants (Haneltet al., 2007). However, unlike higher plants, microalgae do not have a vascular system for nutrient transport, as every algal cell is photoautotrophic with minimal requirement of nutrients that are directly absorbed by the algae. Microalgae can convert CO_2 into raw materials for the production of biofuels (e.g., biohydrogen, biodiesel, and bioethanol), feedstock, animal food for biofuel production, and other value-added bioactive compounds (e.g., docosahexaenoic acid (DHA)) (Razzak et al., 2013). In particular, the efficiency of these cells to absorb CO_2 suggests biomass cultivation of microalgae is a very attractive alternative method for CO_2 sequestration that can be applied to power plant gas effluents to aid the reduction of CO_2 emissions (Yun et al., 1997).

The CO_2 fixation via microalgae can be considered as a potential and promising tool for its capture and storage (Razzak et al., 2013; Zhao and Su, 2014), which can transform water and CO_2 into organic compounds without the need for extra energy and secondary pollution. In comparison to other (CCS) methods, carbon fixation via microalgae is preferred due to high rate of photosynthesis (e.g., 6.9 × 104 cells/ml/h) (Suali and Sarbatly, 2012) and very rapid growth rate (0.7–3.2 day^{-1} (Ryu et al., 2009), good adaptability to the environment and low operation cost. The rate of CO_2 fixation via microalgae and biomass production relies on the cultivation conditions such as temperature, light, pH, and availability of nutrients, microalgal species, and CO_2 concentration (Zhao and Su, 2014). Flue gases and wastewater from other industrial processes can be used for algal growth providing ecological advantages and cutting down the cost of biomass production.

The atmospheric CO_2 gets bio-fixed during the process of photosynthesis performed by microalgae to produce food. There are two pathways by which green plants fix CO_2 from the atmosphere viz., C3 and C4 pathways. C3 pathway (Calvin Cycle) is used by most of the algae for CO_2 fixation. In this pathway, CO_2 is incorporated in the 5-carbon skeleton to produce 3-carbon compounds. Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo) is the enzyme that catalyzes the reaction. Most microalgae are photoautotrophic, which means that they can meet their energy requirements from the sunlight and preserve it in the form of a simple organic compound.

In recent years several studies have shed light on the need to determine the capability of microalgae cultivation systems to minimize CO_2 emissions (Stewart and Hessami, 2005; Wang et al., 2008). It has been estimated that approximately half of the

atmospheric oxygen is produced by microalgae and simultaneously CO_2 is being used to grow photoautotrophically (Tabatabaei et al., 2011). Microalgae have higher growth rates and CO_2 fixation in comparison to conventional forestry, aquatic plants, and agriculture, (Li et al., 2008). These qualities make them suitable candidates for removing the CO_2 present in the atmosphere. The microalgae own the ability to fix CO_2 using solar energy with efficiency which is approximately 10 times greater than the plants due to their energy-conserving metabolic system. The phototropic microalgae can be grown in open ponds and photobioreactors (Patil et al., 2005). Algae would not only help in the bio-fixation of CO_2, but the biomass obtained by algal cultivation can yield value-added products from biomass such as proteins, fatty acids, vitamin A, minerals, pigments. They are utilized as dietary supplements of humans as well as animals (Lopez et al., 2009). Microalgae-mediated carbon sequestration can be rendered more profitable and environmentally sustainable by the integration of microalgal biomass production along with wastewater treatment and commercial application (Kumar et al., 2010).

The development of sustainable and cost-effective technology for CO_2 capture, storage, and sequestration is the need of the hour, to minimize the adverse effects of CO_2 emissions on the environment, and on the climate (Shahbazi et al., 2016). Managing the levels of CO_2 is one of the sustainable and cost-effective approaches to carbon sequestration. The term "carbon sequestration" includes both natural as well as planned processes by which CO_2 is either captured and vanished from the atmosphere or diverted from a point source and stored in the terrestrial environments, geologic formations, and oceans. The natural sequestration of CO_2, especially by photosynthetic organisms, seems to be a promising tool to help in maintaining the global carbon cycle. Direct air capturing (DAC) is one of the

production techniques for absorbing carbon dioxide from the atmosphere. DAC converts it into numerous useful energy resources like methane. Though, these methods are effectively used to tackle the issues of climate change, water security, and energy crises, the efficient and cost-effective strategy needs further research to make these conversion methods more cost-effective and commercially viable.

1.6 Carbon concentrating mechanisms in algae

Microalgae are capable of utilizing both CO_2 and bicarbonate for their photosynthesis, but not carbonate ion (CO_3^{2-}) as a carbon source. They are inbound form at high pH. Microalgae can utilize both CO_2 and the charged anion HCO_3, but they use the HCO_3^- ions as the main carbon source as the rate of CO_2 diffusion in water is lower than that in the air (Beardall and Raven, 2016). Along with diffusive CO_2 uptake, microalgae have well-developed CO_2 concentration mechanisms (CCMs) for facilitating photosynthesis in response to the low CO_2 availability**.**

Inorganic carbon uptake strategies may be a factor in determining macroalgal response to elevated pCO_2 because individual species have different mechanisms of inorganic carbon uptake. The use of HCO_3^- can be advantageous as the marine macroalgae at ocean pH (8.1) maintain low CO_2 in seawater (13 µmol kg^{-1}) as compared to HCO_3^- (1867 µmol kg^{-1}). Furthermore, the diffusion of CO_2 is 10,000 times slower in water as compared to air. Thus, CO_2 availability is a limiting factor in marine macroalgal photosynthesis. Many marine macroalgae depend on HCO_3^- to supplement the CO_2 requirement as a source of inorganic carbon for photosynthesis to overcome the CO_2 limitation. Carbon concentrating mechanisms (CCMs) in macroalgae are characterized by the efficiency of providing CO_2 to RuBisco enzyme for photosynthetic CO_2 fixation. One of the important CCM mechanisms is the

secretion of external carbonic anhydrase (CA_{ext}) into the cell wall where it catalyzes the dehydration of HCO_3^- to CO_2 after which it passively diffuses into the cell. A second CCM mechanism is the use of ATPase supported H^+ pump that lowers the pH at the membrane surface and shifts the carbonate equilibrium towards CO_2. This mechanism involves the generation of proton-motive force for active transport of CO_2 or HCO_3^-. So far, CCM mechanism involving H^+ pump has been presented in the case of only a few temperate marine macroalgal species. Another CCM mechanism involves an anion exchange (AE) protein that facilitates the active uptake of HCO_3^-. It is a known fact that species using HCO_3^- as an inorganic carbon source may not confront carbon limitation under the current pCO_2 than the species that rely solely on CO_2. It has been hypothesized that algae that use HCO_3^- and possess CCMs will be less responsive to an increase in pCO_2 than the species using only CO_2 and inorganic carbon sources for photosynthesis. This has been experimentally proved that temperate macroalgae that rely exclusively on CO_2 have increased growth and photosynthesis under elevated pCO_2, whereas macroalgae that utilize HCO_3^- elicit no photosynthetic response to elevated pCO_2. Counter to these results, isotopic signature, as an indicator of greater CO_2 use, showed that macroalgae closer to high CO_2 seeps in the field utilize greater CO_2 and are competitively more dominant, even though they possess CCMs. These results indicated that potential facultative HCO_3^- using strains may be selected for growth in a high CO_2 condition if CCMs are down-regulated, which may confer competitive dominance. While facultative HCO_3^- use may be an optional carbon use strategy in microalgae in a low-light environment which is a non-CCM strategy. Thus, for establishing the role of C-use mechanisms and light in modulating the photosynthetic response, there is a need to understand the photosynthetic attributes under greater ocean CO_2 availability.

1.7 Role of temperature in algal biomass production

The cellular composition, nutrients uptake, CO_2 fixation, and growth rate of algal species are greatly influenced by the ambient temperature for every species of algae. Below optimal growth temperatures, a temperature rise is known to have a positive effect on photosynthesis and cell division. This trend is explained by the increase of enzymatic activities related to the calvin cycle (Falkowski, 1980). The relation between growth rate and below optimal temperatures has been widely studied, mostly by using the Arrhenius equation (Ahlgren 1987). The temperature coefficient Q10 (growth rate increase by a rise of 10° C) is usually one important parameter and is expected to present a value near 2. In other words, for an increase of each 10° C, photosynthesis, cell division, and growth rate is doubled until favourable temperature optima are attained. For temperature exceeding the optimal temperature, microalgae growth rate sharply decreases, which is generally elucidated by heat stress affecting the cellular enzyme reactions (inactivation, denaturation) or modify the proteins engaged in the photosynnnthetic process (Salvucci and Crafts-Brandnerrr, 2004) and thereby, restricting the growth. A bell-shaped growth curve is generally found for describing the temperature-dependent growth rate of microalgae. It appears that individual's shapes vary greatly from species to species or even amongst the variants of the same species.

Recent studies have projected a general trend between the lipid profile of microalgae and temperature. It has been observed in most of the microalgae that polar lipid content is enhanced with decreasing temperature, whereas a rise in temperature leads to the higher accumulation of non-polar lipids (TAG) (Renaud et al., 2002). Temperature at 35°C leads to an increase in the lipid content (22.7 %), particularly the

accumulation of neutral lipid (59 % of total lipids) as in *Acutodesmusdimorphus* (Chokshi et al., 2015). Long-chain polyunsaturated fatty acids (LC-PUFAs) play a crucial role in maintaining the cell membrane fluidity (Nishida and Murata, 1996). Thus, the fatty acid profile of microalgae grown in relatively low-temperature conditions must be composed of a good amount of polyunsaturated fatty acids (PUFA), suggesting the requirement of those long-chain PUFAs for survival under unfavourable conditions. In a recent study on five polar and cold-temperate microalgae, enhanced eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) production was achieved under low temperature and low light irradiance conditions (Boelen et al., 2013). With a decrease in the temperature from 25^oC to 10^oC, a significant increase in the alpha-linolenic acid (ALA) and DHA content was observed in *Isochrysis galbana* (Zhu et al., 1997). EPA content in *Pavlova lutheri* was enhanced from 20.3 to 30.3% when the temperature was reduced from 25 to 15^oC (Tatsuzawa and Takizawa, 1995). Similarly, when the growth temperature was shifted from 25^oC to 10^oC for 12 h, a significant increase in the EPA content was observed in *Phaeodactylum tricornutum* (Jiang and Gao, 2004). Mitra et al., (2015) observed a 3.4-fold increase in the EPA content (%) of *Nannochloropsis sp*., when cultivated using a two-stage cultivation process under the combined effect of low temperature and irradiance.

The feasibility of sequestering CO_2 directly from the flue gas depends on the use of thermotolerant algal strains as flue gas emitted from power plants and other sources has a very high temperature of around 120^oC. Several species can survive in temperatures up to 60^oC. When the unicellular cyanobacterium *Synechococcus elongatus* was allowed to grow at different concentrations of CO_2 at varying temperatures, a drop in pH at 52° C with 60% CO_2 which was comparable to drop in

pH at 25°C with 20% CO_2, suggested that the solubility of CO_2 depends on temperature and it provides an advantage to thermophilic algae to bear a very high concentration of CO_2 (Miyairi, 1995). On increasing the temperature ratio of O_2 to CO_2 solubility decreases, causing a good amount of O_2 fixation by RuBisco oxygenase activity. Vice-versa, the RuBisCO affinity for CO_2 decreases with rising temperature.

1.8 Role of light quality and light intensity in algal growth

Optimum light intensity is necessary for biomass production and CO_2 fixation. Low light intensity becomes a limiting factor for biomass production via microalgae. The long term exposure of cells with high light intensity leads to photoinhibition of photosystem II, especially down-regulation of repair mechanism of PS II, which further leads to inactivation of other systems including the oxygen-evolving complex, loss of D1/D2 proteins, and electron transport (Rubio et al., 2003). Highly reduced light intensity is observed at the bottom of dense algal suspension because of the absorption and scattering of light. The wavelength, cell concentration, and penetration distance of light are some factors on which attenuation of light intensity depends. The geometry of the algal photobioreactor can minimize the attenuation of light in microalgal cultures. Fernandes et al., (2010) worked on the effect of circular and plan geometry in the penetration of light. The circular design of the photobioreactor allows light to penetrate better, than the plane geometry which allows a higher volume fraction of the reactor to receive the required amounts of light. Photosynthesis saturating light intensity is a very important parameter that determines the light utilization efficiency and overall photosynthetic efficiency. Pigments present within the photosystems get oversaturated with the incoming light and lead to the production

of reactive oxygen species (ROS) which causes photooxidative death and/or photoinhibition (Torzillo et al., 2003). Saturation light intensity nearly varies from 30 to 45 W/m^2 (140–210 lE m^2 s^1) with a good estimation. Saturation light intensity of *Scendesmus sp.* and *Chlorella sp.* is the major factor in determining productivity. Excess light imposes serious problems for the growth of algae during mid-day light hours when the solar irradiance exceeds 2000 µmol photons m^2 s^{-1} (Cuaresma et al., 2011). Two factors affect the light utilization efficiency and overall photosynthetic efficiency (a) the ratio of incident light intensity (Io) and (b) saturation light intensity (Is) (Torzillo et al., 2003). It is worth noting that productivity does not in every case depend upon the light utilization efficiency. Biomass productivity increases proportionally with the light conversion efficiency of the strain.

The relative amount of metabolic products such as proteins, carbohydrates, and lipids are tightly linked with nutritional conditions, light quality and intensity of sunlight, CO_2 concentration, pH of the medium and temperature. In the previous studies, it has been found that green algae show better growth in blue and red light irradiance because of the presence of chlorophyll a and b, which are light-harvesting pigments and have sensitivity to these wavelengths. Normally, microalgae prefer blue light (around 450 nm) and red (around 650 nm) light better than other colours due to high pigment absorption in these two wavelength ranges. Since the red and blue light are mostly consumed by the microalgae, it penetrates less in microalgae suspension but more than the green light. This effect is well observed in the dense culture. A previous study has shown that red or blue lights lead to a significantly higher growth rate as compared to yellow, orange, green, and white light in *Coscinodiscus granii* (Su et al., 2015). Similarly, nutrient removal efficiencies under red light are significantly higher than those under white, yellow, purple, blue, and green light in *C. vulgaris* (Yan et al.,

2013). Under blue light, a higher growth rate and higher N and COD removal efficiencies were observed as compared to white, red, and green light for *C. vulgaris, Chlorella sp*, and *Scenedesmus sp*. (Kang et al., 2015). When comparing the effect of a single red, single blue, and combination of red and blue, the results showed an increase in the growth rate of *Scenedesmus sp.* and *Chlorella sp.* and their nutrient removal efficiencies when compared with white, single blue, and single red light irradiances (Kim et al., 2013; Yan and Zheng, 2014). The advantage of the combination of red and blue to C, N, and P removal from activated sludge was also found in co-cultures of microalgae with fungi (Wang et al., 2017).

1.9 Role of nutrients in algal growth and biomass

The physiological availability of macro and micronutrients greatly affects the biochemical composition of microalgae. Major nutrients include nitrogen, phosphorus, sulfur, potassium, and magnesium, and micronutrients such as iron, zinc, molybdenum, copper, nickel, cobalt, and manganese are required in smaller amounts (2.5-30 ppm). Other factors such as pH light, CO_2 concentration, and temperature are also major factors influencing the algal growth rate and metabolism. The inorganic phosphorus (Pi) is a necessary element for all living beings. It is a component of the backbone of RNA and DNA and also plays an important role in the transmission of chemical energy in the form of ATP. Furthermore, P is also a component of phospholipids and a constituent of the cell membranes. The main source of bioavailable Pi in aquatic environments is inorganic phosphate (PO_3^{4-}), which is released by weathering of the continental rocks. The phytoplanktons use dissolved organic phosphate (DOP), which majorly comes as phosphoesters, polyphosphates, and phosphonates (Bell et al., 2020). Pi is actively taken up by phytoplankton in the euphotic zone and assimilated into the organic molecules. The depletion of Pi in

surface-ocean waters, and the gradual increase of Pi with depth take place as a result of plankton settling and their remineralization (Karl et al., 2014). Whereas DOP exhibits an opposite distribution pattern i.e., the highest concentrations are near the ocean surface and decrease with increasing depth.

Nitrogen and phosphate are two major growth supporting macronutrients for algae. In nutrient-limited conditions the metabolic pathway of the organism shifts. For example, in nitrogen and phosphorus starved conditions the lipid metabolism shifts from membrane lipid synthesis, and neutral lipid storage gets stimulated. This leads to an increase in the total lipid content of green algae (Sharma et al., 2012). Most of the phytoplankton can use NH_4^+ and NO_3^- as the nitrogen source (Raven and Giordano, 2016). Several studies observed a higher photosynthesis and growth rate in macro-and microalgae for certain N-source and changes in a broad range of physiological parameters (Ale et al., 2011). For *Chlamydomonas* species, NH_4^+ is considered to be preferred N-source and negatively signals NO_3^- assimilation (Fernandez and Galvan, 2007). These preferences might be a factor for competition, as the concentration of both nutrients varies throughout the year in lakes (Kolzau et al., 2014).

1.10 Role of CO_2 and pH condition in algal growth

The pH of the growth medium can be affected by dissolved CO_2 and SOx from the flue gas. With high CO_2 concentrations, pH may drop down to 5, and it can further drop to 2.6 with higher SOx concentrations (Westerhoff et al., 2010). It has been reported that the pH change due to CO_2 does not have a major influence on algal growth, but a noticeable change in pH caused by the SOx inhibits the algal growth. With buffered medium, pH drop in the medium can be prevented as compared to SOx mediated change in the pH (Maeda et al., 1995). This indicates that the effect of SOx

on the growth is, to certain limits mainly due to change in pH than sulphate concentrations in the medium, which can be controlled by buffering pH change.

Algal cells have a tolerance limit for CO_2 beyond which it becomes detrimental for cell growth as the environmental stress caused by the high level of CO_2 concentration causes a biological reduction in the carbon sequestration potential of the algal cells. Under high CO_2 concentration, the pH of the medium reduces due to the formation of a high amount of bicarbonate buffer. Hence it can be concluded that the biomass productivity increases on increasing CO_2% (v/v) in the gas mixture up to a certain limit beyond which productivity begins to decline.

1.11 Pathway of CO_2 utilisation

In most of the microalgae, CO_2 is utilized by the Calvin-Benson cycle and RuBisCo enzyme plays a role in it. RuBisCo acts as a catalyst in carboxylation of Ribulose 1,5-bisphosphate to yield two molecules of 3-phosphoglycerate. One of these molecules moves into the central metabolic pathway while the other one is used for maintaining the calvin cycle. However, RuBisCo is less incompetent carboxylase in comparison to its oxygenase activity leading to photorespiration (Busch, 2020). The atmospheric concentration of O_2 is much higher than CO_2, this is the reason RuBisCo is more prone to its oxygenase activity, which further leads to photorespiration. To cope with this nature of RuBisco most of the microalgae have adaptive mechanisms that use energy in elevating concentrations of CO_2 in the proximity of RuBisCo. These mechanisms are known as CO_2 concentrating mechanisms (CCMs). In algae and cyanobacteria, most of the CCMs depend on the active transport of CO_2 or/and HCO_3^- across membranes separating RuBisCo from the bulk medium. In the case of cyanobacterial species, the CCM is mainly based on either HCO_3^- or CO_2 transport that takes place at either thylakoid or plasmalemma membrane (Mishra et al., 2018).

The various transporters present in the microalgae are required for delivering HCO_3^- to the cytosol, irrespective of dissolved inorganic carbon (DIC) species.

1.12 Microalgae: Resource of biofuel

In this regard, the removal of atmospheric CO_2 via microalgae has been gaining attention because the resulting biomass increase can lead to the generation of energy and other useful products (Kondili and Kaldellis, 2007). Photosynthetic CO_2 fixation is considered a feasible technology as it is energy efficient, environment friendly, and sustainable. Biofuel produced by microalgae is a feasible solution for lowering the pressure on conventional fuels. In India, petroleum consumption is about 120 million tonnes per year and only microalgae can meet out this huge demand for petroleum (Darzins et al., 2010). According to a study, almost 21% of the available agricultural land is needed for the production of biodiesel from crops such as soybean or palm which can be a substitute for petro-diesel. However, cultivation of microalgae as a biofuel resource should be preferred because of its high yield of oil per acre of cultivation and therefore, it would require even less than 2–3% of the total land used for energy crops (Bajhaiya et al., 2010). The captured CO_2 in the biomass of photosynthetic organisms can be easily utilized for the conversion of biomass energy into biofuels and other value-added products, including dimethyl ether (DME), methanol, and methane (CH_4). Using the captured CO_2 in algal biomass production in combination with wastewater is one of the promising strategies. Various methods that involve direct combustion, pyrolysis, fermentation, transesterification, anaerobic digestion (AD), and gasification, are used for converting algal biomass into biofuel. Biofuel can be defined as fuels derived from renewable raw materials. The application of algal biomass for biofuel production involves a procedure similar to biofuel production from terrestrial biomass (Daroch et al., 2013). Algal species are now being

used for the production of renewable energy (Fig.1.4) such as biodiesel, bioethanol, biogas, bio-hydrogen, etc. (Demirbas, 2011). The algal feedstocks can be used directly or converted into liquid fuel and gas mediated by various biochemical or thermochemical processes (Amin, 2009).

The growth kinetics of microalgae also affects the lipid synthesis in microalgal cells. The growth rate of the microalgal population influences the lipid accumulation rate and the nutrient concentration also determines the lipid productivity per unit of microalgal biomass. Generally, microalgae contain protein, carbohydrate, and natural lipid as organic substrates. Under environmental stress conditions, the microalgal cell division is hampered and the synthesis of the carbohydrate is switched over to lipid production for the storage of energy. Nutrient limitation can be efficiently used to increase lipid accumulation (Rodolfi et al., 2009). For example, nitrogen limitation leads to a decrease in the cellular content of the thylakoid membrane, stimulates the acyl hydrolase, and activates phospholipid hydrolysis. These metabolic changes end up increasing the intracellular content of fatty acid acyl-CoA. Whereas, nitrogen limitation activates the diacylglycerol acyl transferase, which transforms acyl-CoA to triglyceride (TAG) (Takagi et al., 2000). Therefore nitrogen deprivation increases both lipid and TAG content in the microalgae.

The use of suitable CO_2 sequestering organisms is a sustainable biological approach for managing atmospheric carbon levels (Mondal et al., 2016). The benefit of biological sequestration of CO_2 is that the organism bio converts the atmospheric CO_2 into beneficial biomolecules such as proteins, lipids, and carbohydrates. In addition, different organisms exhibit variable efficiency of CO_2 fixation, and therefore, the success of the strategy will depend upon the choice of selection of organisms, complete knowledge of their photosynthetic characteristics and stress tolerance behaviour of the organisms, and adaptation strategy. The dried biomass of

microalgae may also be subjected to the production of energy by direct combustion (Kadam, 2002), but this method of algal biomass utilization for biofuel production is least preferred. Thermochemical conversion of algal biomass to yield gas or oil-based biofuels involves several procedures such as gasification, pyrolysis, hydrogenation, and liquefaction of the algal biomass (Rittmann, 2008). However, the procedure of biochemical conversion includes anaerobic digestion and fermentation of the biomass to yield bioethanol or methane (McKendry, 2002a, b; Miao and Wu, 2004). Besides, bio-hydrogen can be produced through bio-photolysis. Nevertheless, lipids are extracted from the microalgal biomass in the form of triacylglycerol and trans-esterified to produce biodiesel of variable carbon chain length (Chisti, 2007). It has been reported that biodiesel is lesser toxic, releases lesser gaseous pollutants after combustion, and contains very little quantity of CO_2 or sulphur in comparison to petro-fuels (Rawat et al., 2013). Therefore, biodiesel is now being accepted worldwide as eco-friendly fuel among the scientific community which is an alternative to traditional fuel resources. This third-generation biofuel addresses the limitation of plant-/food-derived biofuels (Sivakumar et al., 2012).

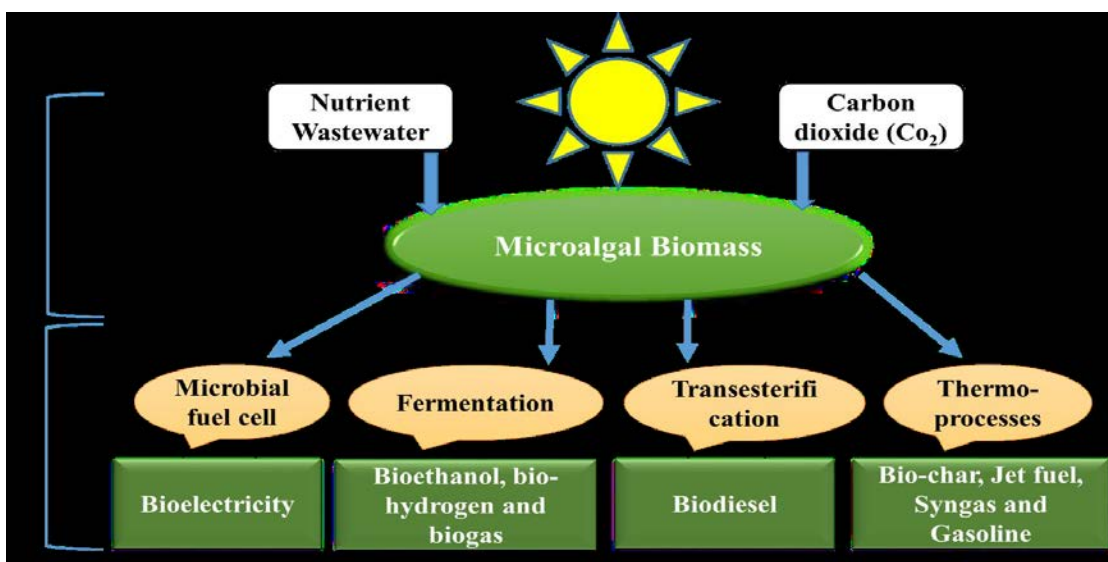

Fig.1.4 Different scenarios and conversion processes of algae for the production of biofuels. Source: Saad et al., (2019)

1.13 Carbon capture by microalgae: Promises and challenges

Presently, various physico-chemical methods of carbon capture and sequestration are jointly termed as (CCS) methodologies. CCS methods involve following 3 major steps: (a) CO_2 capture, (b) CO_2 transportation, and (c) CO_2 storage. Generally, CO_2 capture is performed at high CO_2 emission sources such as power plants and cement manufacturing sites. The capture of CO_2 from other gaseous exhaust is done via chemical absorption or physical adsorption, cryogenic distillation, and membrane separation (Figueroa et al., 2008; Pires et al., 2011, 2012). Microalgae now have become an alluring biofactory for sequestration of gaseous CO_2 and simultaneous production of biofuels, feed for aquaculture, food, and other value-added products such as bioactive products, cosmetics, nutraceuticals, pharmaceuticals, and bio-fertilizers (Ryan, 2009; Harun et al., 2010). Microalgae have a very well-defined CCM for photosynthesis by taking up carbon even from very low CO_2 concentrations (Whitton, 2012). The microalgae surpass many other feedstocks in terms of their ability to survive and flourish under extreme environmental conditions. Recent techno-economic analyses and life-cycle assessment of microalgal system-based biomass production suggested that the only way of realizing the full potential of microalgae is to use the algal biomass in an integrated biorefinery set-up, wherein every valuable component is extracted, processed, and valorized (Chew et al., 2017). However, it is still believed that successful utilization of microalgal biomass for production of bioproducts within a biorefinery setup has poor economic feasibility as it is very much expensive (Lam et al., 2017; Zhou et al., 2017). To attain economic feasibility and sustainability, both upstream processing (USP) and downstream processing (DSP) are required to be simple, efficient, and integrated. The efficiency

of USP depends upon the selection of microalgal strains, nutrient supply (CO_2, N, and P), and culture conditions such as light, temperature, pH, etc. (Vanthoor-Koopmans et al., 2013). The constraints of DSP involve harvesting, cell disruption, and extraction methods. In the DSP, harvesting alone accounts for 20–40% of the total production cost and in a multi-product biorefinery, the total increase in the cost is about 50–60% (Lam et al., 2017).

Microalgal species for biomass production can be enhanced by induced acclimation through alteration of various environmental stresses (Chen et al., 2017; Schuler et al., 2017). Aslam et al. (2017) found that a mixed diverse community of microalgae, dominated by *Desmodesmus spp.*, could adapt over few months to survive in unfiltered flue gas (100%) containing 11% CO_2. It has been reported that carbohydrate and starch accumulation in the *Chlorella* sp. AE10 could significantly improve by a two-stage process, wherein the level of CO_2, light intensity, and nitrogen concentration was drastically changed; and the cells were diluted with the onset of the 2nd stage which resulted in a 42% increase in carbohydrate accumulation (Cheng et al., 2017). Besides stress manipulation and acclimatization, desirable traits in microalgal strains can be effectively achieved through genetic and metabolic engineering.

A deep understanding of the microalgal CCM, biosynthetic pathways, and stress-mediated metabolic response of microalgae accompanied by modern genetic/metabolic engineering approaches can revolutionize the whole concept of microalgal biorefinery.

The primary aim of the present investigation was to study the carbon sequestration in the microalga *C. vulgaris*, using gaseous carbon CO_2, inorganic carbon as bicarbonate

ions, and organic carbon sources and their impact on cell biomass, photosynthesis, macromolecules including lipid. The present study has also focused on the optimization of various environmental and physiological factors such as pH, light intensity, availability of carbon sources, and nutritional conditions (nitrogen and phosphorous) on the functioning of photosynthetic machinery and accumulation of cellular macromolecules (pigments, proteins, carbohydrates, lipids, and total organic compounds) in the unicellular eukaryotic microalga *C. vulgaris*. Following objectives were proposed for the present study-

Objectives

1. Isolation and screening of microalgae with great potential for CO_2 sequestration.
2. Measurement of algal growth and biomass as a function of CO_2 and HCO_3^{-1}at different pH conditions.
3. Effect of light intensity and light quality on the photosynthetic efficiency, carbon fixation, and lipid yield.
4. To study the effect of Physico-chemical conditions on growth, biomass, and lipid production.
5. Study on the role of various physiological factors involved in regulating the carbon sequestration in algal biomass.
6. Role of nitrogen sources in the modulation of lipid production and nitrogen assimilation.

Chapter II

Review of Literature

Review of literature

Microalgae include prokaryotic or eukaryotic photosynthetic microorganisms which grow very rapidly in a wide range of habitats ranging from terrestrial to aquatic environments. Various environmental factors such as light, temperature, pH, drought, and salinity are known to affect microalgal growth and biomass production. Microalgae are tiny photosynthetic organisms and are known to possess high CO_2 fixation efficiency as compared to terrestrial plants (Cheah et al., 2015). Approximately 1.83 kg of CO_2 can be fixed by every 1 kg of microalgal biomass (Jiang et al., 2013). Generally, the CO_2 fixation ability of the microalgae depends upon the characteristics of individual microalgal species. Species of *Scenedesmus, Chlorella, Nannochloropsis*, and *Tetraselmis* have shown the capability to assimilate the CO_2 at a higher rate (Choix et al., 2018). Algae are used as a source of food, fuel (bio-oil, biodiesel, bioethanol, biohydrogen, and biogas), and other value-added products. They can also be used for wastewater treatment and mitigation of CO_2 emissions in power plants. Certain species of algae accumulate lipid up to 60% of their total biomass. Microalgal lipids and fatty acids are the membrane components, storage products, and sources of energy. More than 70,000 species of algae have been identified but all of them are not fit for human requirements. Algae can be grown under conditions that are unsuitable for conventional crop production like soybean and others. Algae has the capability of fixing CO_2 in the atmosphere, thus facilitating the reduction of increasing atmospheric CO_2 levels, which are now considered global problems. Algae contain 50% of their weight in oil. The biofuel from algae is non-toxic, contains no sulfur, and is highly biodegradable. Fifty percent of the photosynthesis process takes place on Earth by algae (Goldman et al., 1972). It removes inorganic carbon from the environment.

Various environmental parameters such as irradiance, UV-light, temperature, nutrients, salinity, temperature, and pH not only influence the accumulation of biomass but also influence the biochemical composition of microalgal cells. The ambient pH condition is the most crucial environmental condition for microalgal cultivation as it determines the solubility and availability of CO_2 and various nutrients, which ultimately impact the microalgal metabolism (Morales et al., 2018). Especially, pH of the medium is a critical factor for determining the relative concentrations of the carbonaceous species in water. Though, each microalgal species has an optimal pH range for biomass production (Su et al., 2012). Higher pH limits the availability of CO_2 and inhibits cell growth (Qiu et al., 2017). However, microalgal cultivation at high pH can also suppress undesirable biological contaminants (Azov et al., 1982). Microalgae efficiently convert inorganic CO_2 into organic biomass which contains various value-added biochemicals, such as high-density biofuels and pharmaceutical ingredients (Khan et al., 2018)

Microalga *Chlorella* is considered a protein-rich source of food (Yun et al., 2020) and an important resource for biofuel production (Yeh et al., 2011). More details about physiology, biochemistry, culture and applications of *Chlorella* have been presented in various reviews (Safi et al., 2014). Due to its abundance and commercial relevance, *Chlorella* is widely studied as an important functional food and source of nutraceuticals and food supplements (Bishop and Zubeck, 2012). The microalga *Chlorella* is already being used to fight infant malnutrition and neurosis (de Mello-Sampayo et al., 2013). The main lipid constituents *of Chlorella* include mainly oleic, palmitic, and linolenic acids, with 41%, 22%, and 9% of its total fatty acids, respectively (Lordan et al., 2011). The glycoprotein, peptide, and nucleotide contents of *Chlorella* are used for the prevention of atherosclerosis and hypercholesterolemia,

as well as tumors (Gouveia et al., 2010). Due to the presence of lutein, α-carotene, β-carotene, ascorbic acid, and α-tocopherol in *Chlorella,* it is often used as an antioxidant and dietary supplement (Yun et al., 2011). Nevertheless, the β-1,3-glucan in *Chlorella* is an important nutritional compound that is used as an active immune stimulator, free-radical scavenger, and reducer of blood cholesterol (de Morais et al., 2015).

2.1 Biological remedy of greenhouse gases and global warming-

Anthropogenic activities such as excessive use of fossil fuel, deforestation, and rapid industrialization have led to an unexpected rise in greenhouse gases (GHG). Global warming or climate change due to greenhouse gas emissions has drawn the worldwide attention of environmentalists and politicians. Carbon dioxide in the atmosphere is identified as a major, GHG causing about 52% of total global warming (Cheah et al., 2015). Flue gas emission from the cement industry, metallurgical and power industries has a major share in the global CO_2 emissions (Raeesossadati et al., 2014). To combat global warming and climate change, efforts are being made by most of the industrialized countries to reduce CO_2, particularly the flue gas CO_2 emissions (Bodansky, 2010). During the last decade, the CO_2 concentration in the air has risen from 260 to 380 ppm, but now it has reached an alarming level of 400 ppm. It is now considered that 450 ppm concentration of CO_2 could be destructive to global climate (Hansen et al., 2007). Fossil fuels account for approximately 85% of global energy demand. The effect of elevated CO_2 coupled with temperature can result in additional carbon entering the environment. The mechanism of storage and transformations of carbon requires a holistic approach to resolve the issue of global climate change. Besides, there is a need to enhance the annual production of food grains to encounter

the future requirement of food. It is also significant to maintain a sustainable environment and crop production in equilibrium. In this regard, it is mandatory to understand the dynamics of the carbon cycle under the changing climate conditions. Wetlands are now recognized as important global carbon reservoirs with large biological carbon pools corresponding to 20–25% of the world's organic carbon, which play important role in the global cycle of carbon (Wang et al., 2016). The anaerobic condition prevailing in the wetlands allows slower decomposition of organic matter, which depends upon the number of parameters including biomass, organic matter content, hydrology regime (Kayranli et al., 2010). Unfortunately, the stored carbon is susceptible to climate change, which may result in the loss of stored carbon in the form of carbon dioxide and methane. The consequence of climate change will have a profound effect on carbon mineralization and sequestration. The stored organic matter would rapidly decompose and release the organic carbon as CO_2 which would further contribute to global warming and disturb the global carbon cycle and ecosystem functioning (Zhang et al., 2007). Global carbon cycling may depend upon the factors such as temperature, pressure, depth of water column, and microorganisms. The major source of carbon loss from the soil is from the lowland rice ecosystem in the form of CH_4 and CO_2. Although there is considerable gaseous carbon emission, efforts are made to account for all the components of carbon balance. It has been suggested that about a 10% increase in methane consumption by methanotrophs may stabilize the present level of greenhouse gases in the environment. There is a necessity to adopt various strategies to stimulate gaseous carbon sink activities.

The potential of methane-oxidizing microbial communities has been provided a future scope to mitigate the methane. Microalgae, on the other hand, are known to

help in nutrient management and carbon sequestration. The combination of methanotrophic bacteria and microalgae would be a feasible approach to moderate the climate change issue especially from wetland systems and also a step forward for restoring carbon status and reducing carbon footprint under anticipatory climate change. The use of microalgal-bacterial consortia has been attempted by (Van der Ha et al., 2011) various workers to test its suitability and wastewater treatment. The co-cultivation of methanotrophic communities with algae, giving rise to "*methalgae*" would be the best option for the bio mitigation approach. Algae support minimal emission of CO_2. A continuous co-culturing of *Methylomicrobium alcaliphilum* and *Synechococcus* PC7002 (Hill et al., 2017) and co-culture of *Methylocystis parvus* and *Scenedesmus* sp. proved successful in the microbial conversion of CH_4 and CO_2 (Van der Ha et al., 2012). Methanotrophs-algae decomposes the organic matter and methane to CO_2 which in turn is consumed by the algae. The algal metabolism releases O_2, which helps to catalyze methane oxidation and assist in plant metabolism. The consortium application in the soil would be directly added to soil as fertilizer, encouraging long-term stability and soil quality. Algal bacterial co-cultures are widely used for the treatment of hazardous contaminants like phenanthrene, acetonitrile, and salicylate (Borde et al., 2003; Munoz et al., 2003).

Various biological processes for reducing CO_2 emissions have been used. The use of blue-green algae or microalgae is considered better as they can grow much faster and are productive than terrestrial plants. The CO_2-fixation efficiency of microalgae is about 10 to 50 times better and serves as an efficient tool in CO_2 mitigation strategies (Wang et al., 2008). Several algal strains have shown the potential to grow on 5–20% CO_2 (Van Den Hende et al., 2012). Recently, algae have been considered as the main

tool of bioengineering to mitigate the global environmental change in carbon dioxide and solar radiation management.

2.2 CO_2 capture by using microalgae

In view of the rising concentration of CO_2, there is a growing demand for effective (CO_2) mitigation technologies. Among all the post-combustion CO_2 capture technologies viz. physic-chemical, oceanic storage, and biological fixation, the microalgal CO_2 fixation offers several advantages such as faster growth rates and higher CO_2 fixation rates (10–50 times) than the terrestrial plants. Hence, microalgae are the most suitable candidates for the biological fixation of CO_2 (Yoo et al., 2013; Cheng et al., 2019). As a result of CO_2 fixation through photosynthesis, microalgal biomass accumulates a significant amount of lipids, proteins, carbohydrates, and other valuable compounds such as carotenoids and vitamins, which are used as active ingredients of nutraceutical, food, and feed supplements or production of biofuels (Francisco et al., 2010; Cheah et al., 2015). As compared to terrestrial plants, microalgae and cyanobacteria exhibit high CO_2- fixation efficiency which is about 10-50 times higher (Costa et al., 2000). The biological mitigation of CO_2 using microalgae offers several advantages with no additional CO_2being generated. Biological CO_2 fixation appears to be the only economical and environmentally viable technology of the future (Ho et al., 2011; Kumar et al., 2010). Plants and other photosynthetic organisms naturally capture CO_2 as part of their photosynthetic process.

Microalgae-mediated CO_2 fixation and biofuel production coupled with wastewater treatment could be a promising technology for CO_2 mitigation strategy (Wang et al., 2008; Lam et al., 2012). The biological fixation of $CO_2$is, therefore, viewed as a

strategy (Salih et al., 2011) for successful removal of CO_2 from the point source (Li et al., 2012). Thus, capturing the carbon and biologically sequestrating it in the form of organic carbon is considered safer to remove carbon dioxide from the atmosphere. The selection of microalgal species is very crucial to achieve the successful strategy of carbon sequestration. The amount of carbon dioxide in the air plays a major role in the growth of microalgae. Furthermore, aerating CO_2 into a microalgal culture medium has been used to promote photosynthetic efficiency and enhance microalgal biomass production (Jin et al., 2020). It is also worth mentioning that the cultivation of microalgae using CO_2 in flue gas as a source of carbon not only reduces the cost of microalgal biomass production but also prevents industrial carbon emissions (Douskova et al., 2009). For efficient CO_2 sequestration, the most crucial step is to search and identify hyper CO_2-tolerant strains of microalgae (Khan et al., 2009; Huang et al., 2010). Sampling for microalgal strains from aquatic ecosystems is largely influenced by environmental factors (Mutanda et al., 2011). A brackish aquatic environment appears to be an ideal area for sampling better carbon-sequestering microalgae due to dissolved CO_2 as well as the location of most of the power plants near coastal areas. Thus, the success of any microalgal cell culture depends on faster growth and adaptation to local climatic conditions (Mutanda et al., 2011). However, the growth and biomass of microalgae also depend upon other environmental factors such as light quality and intensity, water temperature, nutrient concentration, dissolved CO_2, pH, and salinity.

2.3 Role of environmental factors in algal growth

Algae are ubiquitous photosynthetic organisms and their growth is influenced by various external and internal factors. Microalgae, especially diatoms and flagellates, in

the aquaculture industry serve as feedstock for animal and fish production (Slocombe et al., 2016). Biomass productivity in microalgae is dependent on their gross photosynthetic activity, which relies on the prevailing environmental conditions. The optimization of the environmental conditions needed for microalgal growth supports the growth potential of microalgae (Chen et al., 2016). Light is an essential factor to support photoautotrophic growth driven by photosynthetic activity. Algal cells contain light-harvesting chlorophyll a and b, which are sensitive to blue and red light. Studies have revealed that algae grow better in blue and red light as they contain chlorophyll a and b which are major light-harvesting pigments. Temperature is another important factor that strongly influences the cellular chemical composition, uptake of nutrients, CO_2, and the growth rate of every species of algae. Thus, temperature, light intensity, quality of light, light-dark cycle as well as various environmental factors play important roles in algal growth, biomass accumulation, and biodiesel production.

2.3.1Temperature

The average global temperature is rising owing to gaseous imbalances produced by anthropogenic activities (Arora et al., 2018). It is estimated that by the end of the 21st century, the global average temperature of sea surface would rise by 1.4^{o}C–5.8^{o}C (Tait and Schiel, 2013). Temperature plays a critical role in the growth of algae and therefore, optimization of this factor is essential to control the growth and biomass of algae (Ras et al., 2013). Temperature strongly influences the gross photosynthetic activity of microalgae, which in turn, affects biomass productivity. Some studies have developed a model for relating the growth rate with temperature which is commonly expressed as the Arrhenius equation. According to the Arrhenius equation, every 10°C increase in temperature leads to a doubling of the growth until the temperature has

reached a point when the algal growth declines. In most cases, increasing temperature elevates the growth of microalgae up to an optimum value, and then decreases with any further increase in temperature (Ahmad et al., 2020). The temperature-induced decline in growth occurs due to heat stress resulting in the denaturation of proteins and enzymes involved in the photosynthesis process (Gatamaneni et al., 2020). The change in temperature regime also influences the absorption of nutrients and the composition of cells in microalgae (Chen et al., 2012). A temperature of 35°C or beyond is considered to be detrimental for microalgal growth (Pachiappan et al., 2015). According to a study, the optimum temperature for *C. vulgaris* was found to be between 25°C and 30°C (Chinnasamy et al., 2009). Another study on unidentified *Chlorella* sp. and *Chaetoceros calcitrans* at temperatures of 20°C, 25°C, and 30°C revealed optimum growth rate of the algal species at 25°C (0.35 – 0.04/day) and 30°C (0.27 – 0.02/day), respectively (Adenan et al., 2013). The growth rate of four species of microalgae (*Phaeodactylum tricormutum*, *Tetraselmis gracilis*, *Chaetoceros sp*., and *Minutocellus polymorphus*) was found to be better between 11°C to 36°C. (Sigaud and Aidar, 1993). But the suitable temperature range for the growth of microalgae is between 22°C to 31°C. The growth of the microalgae begins to fall rapidly at 34°C after the first few days of culturing, suggesting that higher temperatures are generally not suitable for the growth of microalgae. Kessler (1985) studied the growth rate versus optimal temperatures for 14 different strains of *Chlorella* sp. and showed that microalgal strains grew successfully between 26-36°C. Another study demonstrated that the optimal temperature for the growth of *Scenedesmus almeriensis* was 35°C, but it was capable of withstanding the temperature rise to 48°C, followed by cell death (Sanchez et al., 2008). A study reported that the three strains of *Dunaliella salina* isolated from saline soil exhibited

the highest growth at 22°C (Wu et al., 2016). It was also observed that *Dunaliella* was able to withstand a temperature range between 0°C and 45°C. A case study on *Nannochloropsis salina* exhibited an optimal temperature of 26°C with no growth detected at 35°C (Van Wagenen et al., 2012). Another study disclosed that *Nannochloropsis oculata* could grow at a temperature of 20°C, but showed a gradual decrease in the growth as the temperature was increased (Converti et al., 2009). Thus, various studies revealed that temperature tolerance in microalgae depends upon the species and its adaptation traits.

2.3.2 pH

The pH condition of the growth medium is an important underlying parameter that controls the cell metabolism and formation of biomass in microalgae. The growth of the majority of microalgal species favoured at neutral or slightly alkaline pH and they seem to have a limited optimal range of pH (Lutzu, 2012). At optimal pH, the bicarbonate present in the medium is converted into carbon dioxide by the action of the algal enzyme through carbonic anhydrase enzyme with the release of hydroxyl ions that tends to increase the pH of the surrounding medium (Gerardi, 2015).

HCO3_ _ _ _ _Carbonic anhydrase_ _ _ _ > CO2 + OH^{-}

It has been observed that the thylakoid or chloroplasts carry out their vital functions between a specific pH range and therefore, the pH of the medium determines the efficiency of photosynthesis in microalgae. The extreme pH conditions viz. high as well as low pH, reduce the rate of photosynthesis. The pH also influences the availability and absorption of certain nutrients such as iron and organic acids (Lutzu, 2012). Therefore, pH is considered a major environmental factor that is critical for

carbonate equilibrium in both oceans and in land waters. Both higher, as well as lower pH conditions, may induce the inactivation of the enzymes in the photosynthetic process. There is also a very high possibility of the growth medium getting contaminated by micro-organisms (Bakuei et al., 2015). There is a correlation between carbon dioxide concentration in the medium and pH of the medium, as there is a steady increase in the pH with gradual consumption of carbon dioxide. The optimal pH range for photosynthetic activity in the microalgae is in between pH 6 - 10, wherein the bicarbonate form is considered to be the predominant form (Rastogi et al., 2017).

Low pH natural water is often found in the volcanic regions that receive strong mineral acids such as sulfuric acid, and hence the pH of the water is often less than 4. High pH values are often found in lakes that belong to endorheic regions where sodium carbonate or sodium bicarbonate abundance is observed (Weisse and Stadler, 2006). Algae are known to survive under both alkaline and acidic pH (Ying et al., 2014). In a study on the effect of pH on *C. vulgaris*, the microalga showed reduced growth under both acidic (3.0–6.2) and alkaline (8.3–9.0) pH conditions. However, optimal growth was observed between pH 7.5- 8.0 (Sakarika and Kornaros, 2016). The optimum pH for the growth of *Spirulina platensis* was found to be in between pH 7.0- 9.0. However, growth maxima of microalga were observed at pH 8.0, suggesting that moderate alkalinity was necessary for the ideal growth (Fagiri et al., 2013). *Scenedesmus almeriensis* grew effectively at pH 8.0, followed by a decrease in the growth with a further increase in pH (Sanchez et al., 2008). The *Scenedesmus obliquus* grew well at neutral pH as well as at weakly alkaline pH, but optimum growth was observed at a pH 8.0 (Yang et al., 2018). A study on the growth of *Scenedesmus sp*. under various pH conditions ranging from 5.0 to 9.0 showed the

highest specific growth rate and biomass productivity at pH 7.0. However, acidic pH conditions (5.0 and 6.0) could lower biomass productivity (Difusa et al., 2015). The *Scenedesmus sp.* strain R-16 showed strong tolerance to varying pH from 3.0 to 12.0 and it grew well in the pH range between 4.0 and 11.0. A study on *Dunaliella salina* under different pH conditions revealed that the maximum growth occurred at pH 9.18 (Abu-Rezq et al., 2010). The growth of *D. bardawil* and *C. ellipsoidea* over a wide pH range (pH 4.0 to pH 11.0) showed that the ideal pH for the growth of these species was pH 7.5and 10.0, respectively. Nevertheless, the growth of both *D. bardawil* and *C. ellipsoidea* was retarded at a pH >10.0, as a source of inorganic carbon was not available for the algae (Khalil et al., 2010).

In addition to the effect on the growth of microalgae, a higher pH of the culture medium increases the TAG content, but this comes at the cost of a sharp decline in the growth rate (Breuer et al., 2013; Santos et al., 2012). These data suggest that the high-pH condition not only inhibits the cell cycle but effectively triggers cellular lipid accumulation. Moreover, Santos et al., (2012) demonstrated that high-pH stress not only increases the lipid content but also improves the lipid quality. The pH regulates the cell composition and distribution of inorganic carbon in the medium and organic carbon within the cell (de Morais and Costa, 2007). During carbon dioxide fixation and microalgal growth, pH variation influences the carbon dioxide dissolution, equilibrium of the dissolved inorganic carbon, and uptake of nutrients. However, these changes also depend on the input of carbon dioxide and the carbon concentrating mechanism in the microalgae. Recently, Duarte-Santos et al., (2016) have used the strategy of pH control with an injection of carbon dioxide-rich gases that allowed them to meet the carbon requirements of microalgal cultures and also optimize biomass production. Thus, the pH of the culture medium is an important

factor, affecting many physiological processes associated with microalgal growth, metabolism, and uptake of ions (Khalil et al., 2010). In general, the optimum pH for the growth of microalga is a species-specific trait.

2.4 Light-harvesting and photosynthesis

Sunlight provides the required photons for supporting the photosynthetic metabolism, but the excess photons cause photo-oxidative stress and photoinhibition of photosynthetic machinery (Caverzan et al., 2014). Algae absorb the light energy and convert it in the form of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide phosphate (NADPH), which are used to drive the growth and biomass production during the dark cycle (Fig.2.1). It is evident that light influences the cultivation of algae and optimal exposure of cells to light is essential to achieve maximum biomass productivity. Light conditions can directly regulate the growth and photosynthesis of microalgae (duration and intensity). Microalgae also need a proper light/dark cycle for efficient biomass productivity, it needs light for a photochemical phase to produce ATP and NADPH, and also a dark period for the biochemical synthesis of essential cell constituents required for growth (Khan et al., 2018). Experimental shreds of evidence have revealed that an increase in the duration of light exposure is directly proportional to an increase in the number of cells of microalgae. Khoeyi et al., (2012), used three algae placed under different light conditions (photoperiod, intensity) and they found a huge difference in the cell biomass. They observed that maximum cell biomass was achieved in the algal culture exposed to 62.5 µmol photons m^{-1} s^{-1} for 16:8 h light/dark photoperiod. Whereas the maximum percentage of total saturated fatty acids (SFA) was 33.38 % at 100 µmol photons $m^{-2}s^{-1}$ for 16:8 h light/dark photoperiod. It has been also reported that photoperiod has a

significant effect on the growth of microalgae. For example, a study on the effect of photoperiod on *Dunaliella salina* CCAP, using 19/30 h light/dark cycle, revealed that longer photoperiods lead to an increase in the cell density of microalga (Xu et al., 2016). Though algae grow well under light conditions, the cell division preferably occurs under dark conditions which have significant implications on the overall biomass productivity of microalgal cultures (Bisova and Zachleder, 2014). Carvalho et al., (2009) reported that during the light cycle, water is hydrolyzed to form oxygen and during the dark cycle carbon dioxide is incorporated in the cell through the Calvin cycle. Therefore, it is the dark cycle when microalgae can build up carbohydrates, proteins, and lipids (AlQasmi et al., 2012; Rastogi et al., 2017).

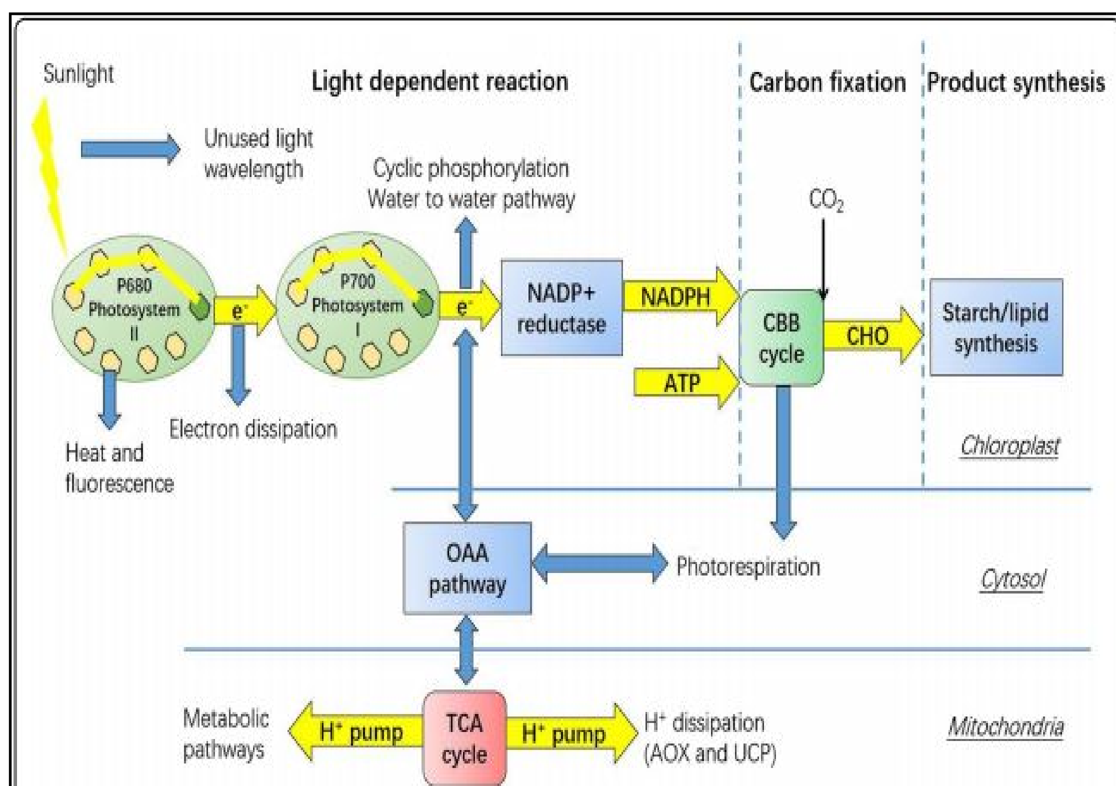

Fig. 2.1 Schematic diagram showing energy capture, delivery and dissipation in microalgae. Source: Sun et al., (2018).

Light affects the microalgal growth with any one of the three different conditions; namely light limitation, light quality, and light saturation. Under the light limiting condition, the growth of algae increases with an increase in the light intensity. Light quality relates to the spectral quality of light such as green, blue, or red light, being maximally absorbed by the cells depending upon the pigment composition. At light saturation, the photosynthetic activity is optimum and any increase in the absorption of photons will adversely influence the photosynthesis. When the light intensity is very high, irreversible photodamage occurs in the photosynthetic apparatus which is often termed as photoinhibition (Chang et al., 2017). Both the duration of exposure and intensity of light directly affect the growth and photosynthesis of microalgae. In the case of *Scenedesmus almeriensis*, it has been reported that cells exhibit greater resistance to high-intensity irradiances and show no sign of photoinhibition (Sanchez et al., 2008a). The *D. salina* CCAP 19/30 can modify their photosystems to achieve maximum photosynthesis even when they are exposed to high light intensities (Xu et al., 2016).

A study on the growth of *Odontella aurita* under two light intensities of 11,100 and 22,200 lux (150 and 300 μmol photons m^{-2} s^{-1}) revealed that the microalga was capable of growing under 11,100 lux; however, the alga grew faster at early stages under high light (22,000 lux). The results were interpreted as low cell density at early stages of microalgal culture enabled it to consume an additional amount of irradiance due to fast metabolic activities (Xia et al., 2013). Direct exposure of the microalgae to high light intensity could potentially cause damage to the cells, whereas low light condition negatively impacts the growth of microalgae (Harun et al., 2014). The growth of four microalgal strains, namely *C. vulgaris*, *Pseudokirchneriella subcapitata*, *Synechocystis salina*, and *Microcystis aeruginosa*, studied under various

light irradiances (2,664,4,440, 8,880, and 13,320 lux) with varying light/dark cycles (10/14, 14/10, and 24/0) showed that highest growth rate and biomass productivity for all the species at an irradiance of 13,320 lux with continuous illumination for 24 h (Goncalves et al., 2016). Another study using LED lights (red, natural white, warm white, and blue light) on the biomass productivity of *C. vulgaris* revealed that warm white light (380–760 nm) with 80 µmol photons. m^{-2}. s^{-1} was optimal for enhancing the biomass productivity and photosynthetic rate (Khalili et al., 2015). Another study on marine microalgae *Tetraselmis sp*. and *Nannochloropsis sp*. under blue light (420–470 nm) and red light (660 nm) of100 µmol photons. m^{-2}. s^{-1} with 24/0 light/dark cycle revealed maximum growth and biomass under the blue light (Teoa et al., 2014). Another study on red alga *Pyropiahaitanesis* under blue, red, green, and white fluorescent light of 100 µmol photons. m^{-2}. $s^{-1}$with 12/12 hour light/dark cycle showed the highest growth under fluorescent light (Wu, 2016).

The most important contribution of pulse ampliude modulated (PAM) fluorometry has been the development for analysis of photochemistry of photosynthetic apparatus and fluorescence quenching analysis (Schreiber et al., 2004), which provides detailed information on the characteristics of photosynthetic machinery and distribution of the absorbed energy: its use in photochemistry. Presently, the rapid chlorophyll fluorescence induction or relaxation kinetics by using the PAM method (Maxwell and Johnson 2000; Strasser et al., 2004; Schreiber 2004, Baker, 2008; Masojídek et al., 2011), the information on the reduction of the photosynthetic electron transport chain and the Calvin- Benson cycle can be derived. The last phase of fluorescence induction kinetics (~1 s) begins upon illumination (continuous light) of the dark-adapted microalgal culture. The signal rises rapidly from the origin (O) to a peak (P), also termed as maximum fluorescence (*F*m) along with 2 inflections i.e., J and I This

'polyphasic fluorescence rise' is also termed as 'Kautsky' curve (Kautsky and Hirsch, 1931) or the OJIP test (Strasser et al., 2004, Papageorgiou et al., 2007). It reflects the changes in the redox state of the reaction centre of PSII that reflects the primary processes of photosynthesis (Stirbet & Govindjee, 2011). The origin of the fluorescence induction curve is termed as minimum fluorescence yield (*F*0, measured after 10 to 50 μs). This signal is emitted from the Chl*a* molecules when primary acceptor QA is fully oxidized. The *F*0 or *F*m values are the most common empirical parameters. The difference between *F*m and *F*0, designated as variable fluorescence (*F*V), and the ratio *F*V/*F*m is a widely used parameter related to the maximum quantum yield of PSII photochemistry (Papageorgiou and Govindjee, 2004).

When the dark-adapted cells are subjected to saturating pulse of light, it induces a maximum value of fluorescence (*F*m) by closing the reaction centres. At this point, there is no non-photochemical quenching (NPQ) of fluorescence. The difference between *F*o and *F*m is termed variable fluorescence (*F*v) in unstressed cells. The empirical *F*v/*F*m value is a robust indicator of the maximum quantum yield of PSII chemistry (Jia et al., 2019). Any 'stress' may result in damage to PSII reaction centres of photosynthetic cells (often referred to as photoinhibition) (Long et al., 1994). The stress-induced sustained quenching of fluorescence (Demmig-Adams and Adams, 2006) results in a lowering of *F*v/*F*m. This is the reason that measurement of *F*v/*F*m, following a period of dark adaptation has been used as one of the common techniques for measuring the effect of stress. The value of *F*v/*F*m in the dark-adapted cells is commonly used to assess plant 'stress (Murchie et al., 2013), reflecting the high sensitivity of PSII to environmental stimuli, either directly or indirectly. The NPQ refers to high energy state quenching or qE that occurs due to an increase in the proton gradient across the membrane (Murchie and Horton, 2009). Two other

components of non-photochemical quenching include one state transition (qT) - refers to the migration of peripheral LHCIIs from PSII to PSI, associated with low light conditions, but absent under the high light. The second photoinhibitory quenching (qI) is small at moderate and low light but becomes significant under the high light intensity. The qI often refers to any sustained quenching (not removed by dark adaptation). Photoinhibition of PSII occurs due to photooxidation, followed by a repair cycle where damaged D1 protein is repaired by *de novo* synthesis (Yokthongwattana et al., 2001). An initial rise in *F*o is also associated with photoinhibition damage, but not with zeaxanthin retention.

2.5 CO_2 sequestration and biomass production by microalgae

The microalgal CO_2 fixation has several advantages such as they do not require farming land, can be easily harvested by using wastewater which served as a pool of all nutrients required for the growth of microalgae. Microalgae accumulate a very high content of lipids (for biodiesel), carbohydrates (for bioethanol), proteins (single-cell protein), and many other bioactive compounds which are used as antiviral and antibacterial agents. They are photosynthetic organisms, cultivated with the help of sunlight energy, minimal requirement of nutrients, and CO_2 as a carbon source. Sometimes, plastic tubes in the form of photo-bioreactors are used for higher biomass productivity than open ponds and lagoons. For cost-effective biomass production and uncontaminated outputs especially from homogeneous algal biomass, microalgae are cultivated using closed photo-bioreactors. Beer et al., (2009) performed cultivation in the closed photobioreactors to attain high yields. Bioreactors are found to be the most suitable system for CO_2 sequestration and have the flexible design of using CO_2-rich gas with a mixing facility for providing nutrients. Generally in the photobioreactors, agitation is done through

non-mechanical means like airlift, bubble column, tubular reactor, flat panel etc. There are also few bioreactors where agitation is done by mechanical means along with bubbling through CO_2-rich gas inlet. The gaseous phase CO_2 transfer inside the algal cells through the liquid phase increases the mass transfer resistance. Various bioreactors employed for cultivating microalgae are horizontal tubular reactor, vertical tubular reactor, helical tubular reactor, fermentor-type reactor, flat plate reactor, and hollow fiber membrane reactors. Among these different photobioreactors available for CO_2 sequestration, the airlift reactor system seems the most suitable reactor for CO_2 sequestration from the flue gas. Based on the geometric features of different photobioreactors and their performance in CO_2 sequestration, the following types of photobioreactors are listed.

Table.2.1 Types of algal photobioreactors and their functioning.

S.no	**Type of photo-bioreactor**	**Structure and functioning**	**Designed by**	**Advantages**
1.	Vertical tubular photobioreactor	Transparent in nature. Sparger is attached to the bottom, which converts the gas into tiny bubbles.	Loubiere et al., (2009)	Better mixing characteristics and also the high photosynthetic efficiency
2.	Flat panel photobioreactor	(a) cuboidal shape with minimal light path. (b) high surface area to volume ratio and open gas disengagement systems. (c) Agitation is provided either by bubbling air from its one side through a perforated tube or by rotating it mechanically through motor	Barbosa et al., (2005)	Provides high volumetric mass productivity (1.7 times higher) than a similar bubble column reactor.
3.	Horizontal tubular photobioreactor	A parallel set of tubes, loop shape, a shape, near-horizontal tubular shape, or horizontal	Tredici and Zittelli (1998)	Its shape gives an advantage in outdoor culture for its orientation towards

		tubular reactor.		sunlight resulting in high light conversion efficiency.
4.	Helical type photobioreactor	Consists of a coiled transparent and flexible tube of small diameter with a separate or attached degassing unit.	Morita et al., (2001)	Photobioreactor has an advantage concerning the balance between energy input and photosynthetic efficiency. Less energy requirement for its operation and less mechanical stress imposed on algal cells are the other advantages of this reactor.
5.	Stirred tank photobioreactor	The most conventional reactor where agitation is provided mechanically with the help of an impeller of different sizes and shapes.	Pohl et al.,(1988)	
6.	Hybrid type photobioreactor	This reactor has an integrated airlift system and an external tubular loop placed horizontally in a thermostatic pond of water.	Lee et al., (1995)	Its advantage includes better control over culture variables, enabling higher productivities and reducing. power consumption

Unlike the cultivation of other oil crops, the growth of microalgae is extremely fast and the biomass production rate is almost doubled every 24 h (Shokravi et al., 2020). The most effective way to curb carbon emission is by sequestrating it into solid biomass of microalgae. A very recent study was conducted by Shabani et al., (2016) on the bio-sequestration of CO_2 by *C. vulgaris* and *Spirulina platensis* in response to salinity showed that salinity has a significant effect on growth and biomass productivity. Tripathi et al., (2019) reported that carbon sequestration potential and biodiesel production in microalga *Scenedesmus* sp., isolated from a marble mining site, showed not only maximum growth of the microalgal isolate under different concentrations of gaseous CO_2, but it exhibited improvement in chlorophyll content,

biomass production, and lipid contents. The fatty acid profile of the lipid was dominated by monounsaturated fatty acids of C-16 and C-18 above 80%, which are good indices of quality biodiesel. Microalgal cells contain a high amount of protein (51–58%), carbohydrate (12–17%), and lipid (14–22%) which is useful for various purposes including biofuels (Cheah et al., 2015). *Chlorella* is one species of green microalgae that is very commonly used for carbon sequestration experiments. It is a freshwater, single-cell organism containing chlorophyll a and b and has high photosynthetic efficiency to convert CO_2 to cell biomass. *Chlorella* species can grow under ambient CO_2 concentration (0.036%) as well as at elevated CO_2 concentrations (20%). The *C. vulgaris* ARC1 could fix 18.3 mg and 38.4 mg CO_2 L/day at ambient (0.036%) and elevated CO_2 (6%) level, respectively, with 47 μmol photons $m^{-2}s^{-1}$ (Rawat et al., 2011). Highest chlorophyll concentration and biomass in *C. vulgaris* ARC1 was produced 11 μg/mL and 210 μg/mL respectively at 6% CO_2 level, which were respectively 60 and 20 times more than that of *C. vulgaris* at ambient CO_2 (0.036%) level (Chinnasamy et al., 2009). The *Chlorella* cell biomass contained 25–30% protein, 6–10% carbohydrate, and 30–40% lipid. In biological CO_2 sequestration, the *Chlorella* species and cyanobacterium *S. platensis* showed about 46% and 39% mean CO_2 fixation efficiency, respectively, at an input CO_2 concentration of 10% (v/v). Calcite deposition coupled CO_2 fixation is a commercially utilizable biomass producer, which is effective for 6% CO_2 level sequestration (Ramanan et al., 2010).

This microalga *Scenedesmus sp*. shows a high level of CO_2 tolerance and thus, making it more capable of growing within the range of 10%–20% (v/v). Keeping in view the level of CO_2 tolerance, the *Scenedesmus dimorphus* biomass at 10% (v/v) and 20% (v/v) concentration of CO_2 was 4.51 and 3.82 g/L, respectively. However,

optimum biomass of *Scenedesmus* species (5.17 g/L) was obtained at 2% (v/v) CO_2. The *Nannochloropsis sp.* showed an increase in the growth rate (58%) at 15% (v/v) CO_2 (Ma et al., 2016). Elevated CO_2 promotes the efficiency of microalgal photosynthesis leading to the proliferation of cells within a short period. However, CO_2 concentration above 5% (v/v) is considered lethal for the growth of some microalgae species.

Flue gas is made up of CO_2, water vapor, NOx, SOx, and heavy metals such as nickel, vanadium, and mercury (Packer, 2009). The use of flue gas for culturing microalgae is considered advantageous in that a carbon-free nutrient medium as the carbon requirement is met by CO_2 (Van Den Hende et al., 2012; Kumar et al., 2014). The pre-treatment cost of flue gas can be minimized by direct utilization of flue gas if the microalgae are used to remove the CO_2 (Benemann, 1993; Ono and Cuello, 2007). Some researchers have pointed out that the presence of NOx in the flue gas poses a problem to microalgal growth, while the main difficulty comes due to the presence of SOx, which tends to decrease the pH by forming sulfurous acid (Maeda et al., 1995; Packer, 2009; Kumar et al., 2011). However, some strains of microalgae show resistance to SOx, but their growth is inhibited by CO_2 when NOx is also present (Ho et al., 2011). Therefore, it has been suggested that denitrification and desulphurization of the flue gas are necessary as pre-treatment. Further, nickel concentration above 1 ppm and vanadium above 0.1 ppm decrease microalgal productivity, while mercury is phycoremediated by certain microalgal species (Packer, 2009). Thermal stability is another essential characteristic required by microalgal strains involved in CO_2 in sequestration from flue gas. It has been observed that flue gas temperature is around 120°C which can adversely affect the system (Ono and Cuello, 2007; Kumar et al., 2011). Therefore, the feasibility of sequestering CO_2 from flue gas would require the

use of thermophilic microalgal strain or the installation of a heat-exchange system. Though flue gas technology is an inexpensive and rich source of CO_2, where CO_2 is approximately 400 times more concentrated than atmospheric CO_2, the development of a successful technology has to go a long way as a tool of biomass generation (Cheah et al., 201 5). The major constraints are the presence of toxic compounds such as NOx, SOx, and CO, which are inhibitory for microalgal growth and biomass productivity (Kumar et al., 2014), mainly due to acidification of the growth medium (Van Den Hende et al., 2012). The efficiency of CO_2 fixation by microalgae from flue gas is generally less than 50% (Cheng et al., 2013). To treat flue gas by microalgae in a cost-effective manner, there is a need to develop effective strategies which will not only reduce the cost of its pre-treatment but also increase CO_2 fixation.

2.6 Salinity tolerance in microalgae

The salinity stress influences the water potential of the cells, disrupts the ion balance inside the cells, and destroys the membrane structure of organelles (Yang et al., 2012). The most important effect of salinity stress is down-regulation of photosynthesis, which limits the growth and development of photosynthetic organisms. Salinity stress adversely affects the activity of key enzymes, thereby it influences several metabolic processes (Munns, 1993). Thus, the salinity stress is injurious to growth, photosynthesis, protein synthesis, energy accumulation, and lipid metabolism (Wang et al., 2008). Microalgae are ubiquitous across most of the environments including extreme environments such as soda pans and salt lakes. Soda pans are shallow, highly saline aquatic systems due to a high concentration of sodium and carbonate ions. Whereas salt lakes represent a hypersaline environment, often changing the salt concentrations, as compared to that of seawater. Despite the extreme conditions, eukaryotic green algae along with cyanobacteria and euglenophytes

remain the major primary producers (Costelloe et al., 2005). Eukaryotic algae exhibit great plasticity and adaptability to extreme environmental conditions. Some species of microalgae are capable of growing in both fresh and saline water. Thus, the study on salt-tolerant algal species is a first good step in understanding the adaptation properties of these photosynthetic organisms against salinity stress. Unlike their freshwater counterparts, the cellular machinery of marine algae is capable of tackling the high salinity as their salinity tolerance mechanisms are engraved in the genome. In contrast, freshwater species have devised salt tolerance mechanisms by metabolic adaptation to cope with high salinity stress. The metabolic adaptation involves drastic changes in morphology and osmolyte concentrations in the short term. Intensive researches on salinity tolerance have shaped our understanding of how microalgae respond, acclimatize, and grow under such extreme conditions. Thus, a comparative account of salinity tolerance across different species can give us a complete picture of different mechanisms used by microalgae. However, a thorough understanding of these mechanisms and the propensity of the mechanisms can be successfully exploited for commercial purposes.

Since the algal cells have rigid cell walls with limited scope for change in the cell volume, they heavily rely on organic solutes for osmoregulation. These solutes, termed as compatible solutes, are typically small organic compounds with a neutral charge and low toxicity. These compatible solutes at higher concentrations are required for the efficient functioning of different enzymes and metabolic pathways. Compatible solutes get accumulated in the cytosol and counter the salinity-induced osmotic imbalance in the cells. The growth of microalgae is reduced under the salinity stress due to the accumulation of compatible solutes like proline and glycine to balance the external salt concentrations (Rhodes et al., 2002). Glycerol is a good

example of an effective compatible solute produced by most salt-sensitive algal strains. It is also a highly soluble, chemically inert, and non-toxic compound. Since it is an end-product metabolite, its accumulation does not interfere with other metabolic pathways. The glycerol accumulation in the microalgae corresponded to salt concentration i.e., higher salt concentration inducing accumulation higher glycerol content as reported in *C. reinhardtii*, *C. mexican*, *Chlamydomonas sp*. JSC4, and *C. pulsatilla*. Glycerol plays an important role in salinity tolerance by *Dunaliella*, *Scenedesmus*, and *Micrasterias* species. Some unicellular green alga like *D. salina* var. *Bardawil* responds to salinity stress by regulating carbon fluxes between the synthesis of starch and the synthesis of glycerol in the cytoplasm (Cowan, 1991). The high concentration of intracellular glycerol enables the salt-stressed *Dunaliella* cells to resume their original cell volume even under extreme salinity stress. It has been shown that glycerol accumulation in *C. pulsatilla* was derived from the degradation of starch. Application of proline is known to reduce the adverse effects of high salinity by reducing the Na^+ and Cl^-ions in *C. reinhardtii* and plants. Even some amino acids like lysine and leucine have been implicated in the growth promotion of *Chlamydomonas* under high salinity stress. Up-regulation of genes involved in the proline synthesis has been reported in *Picochlorumokla homensis* and *Picochlorum* SE3 during the salt stress. Unlike the glycerol derived from degradation of starch as in *Chlamydomonas* sp. and *D. salina*, proline is the principle osmolyte in *Picochlorum* species and starch synthesis is upregulated and starch degradation is limited. Besides proline and glycerol, trehalose is also an established osmolyte required for the stabilization of protein during the transition temperature of proteins. An increase in cellular trehalose reduces the negative effect of salinity in *Chlamydomonas*, *Chlorella*, and *Scytonema*. Few polyols such as sorbitol and mannitol also play an

important role in osmoregulation. *Platymonassuecica*-salt-tolerant algae, shows the salinity-induced gradual accumulation of mannitol. *Stichococcus chloranthus* and *Stichococcus bacillaris* exhibit accumulation of sorbitol with increasing salt concentrations.

Efficient transport of ions across the cell membrane is another important cellular strategy to counter the high salt stress. As per reports in certain plants, a high concentration of Na^+ interferes with the uptake of other cations, especially K^+ (Chakraborty et al., 2016; Shabala, 2003) Since K+ participates in maintaining the cytosolic K^+/Na^+ ratio under extreme saline conditions, therefore, active membrane transport of K^+ in halotolerant algae under the influence of high salinity confers tolerance to the cells against the salinity stress. Genes for K^+ ion transport are significantly upregulated in the salt-sensitive *C. reinhardtii* cells when subjected to salt stress (Wang et al., 2018), possibly to compensate for the impaired K^+ uptake system caused due to high concentration of Na^+ ions. The data on intracellular ions in *C. pulsatilla* revealed a remarkable capacity to increase sodium and chloride, and to a lesser extent, potassium and magnesium in response to increasing the salinity stress. The salt-tolerant strain of *Dunaliella* maintains reduced intracellular Na^+ concentration as compared to that in the outside medium (Wang et al., 2018). The Na^+/H^+ antiporter catalyzes influx of Na^+, followed by export of Na^+ through the Na^+-ATPase system. The Na^+ efflux system in *Dunaliella* is an adaptive mechanism evolved by the organisms under the hypersaline condition. Therefore, unlike marine or hypersaline algae, the freshwater salt-sensitive algae are unable to maintain ionic balance by preventing intake of Na^+ and by the accumulation of K. Besides, the Na+ and K+ transport across the membrane, the amount of two membrane proteins P150 and P60 are accentuated with increasing salt concentration in the *Dunaliella salina*

(Fisher et al., 1996; 1997). A drastic hyper-osmotic shock induces the synthesis of these proteins along with a concomitant increase in growth, suggesting their role in salt tolerance. P150 is a 150 kD transferrin-like plasma membrane protein involved in iron uptake and helps the cells to overcome any possible limitation of iron under high salinity (Fisher et al., 1997). However, this protein is not detected in other algae.

Most photosynthetic organisms exhibit a significant decrease in photosynthetic activity under high salt stress, which is attributed to a deficiency in different cations required for photosynthesis. Higher salinity stress causes a significant increase in the chlorophyll-a (Chl a) content of salinity tolerant microalgal strains (Tabeli et al., 2013). The salt-tolerant *D. salina* showed an increase in Chl a/b ratio with an increase in salt concentration up to 3 M NaCl (Mishra et al. 2008). In salt-sensitive *C. vulgaris*, the chlorophyll content was enhanced at lower concentrations of salt (0.2 M), but it was reduced at higher salt concentrations (0.3 M–0.4 M) (Hiremath et al., 2010). A slight increase in the photosynthetic activity under moderate levels of salinity stress could be due to enhanced energy requirement to produce energetically expensive molecules which protect the cells against salinity stress. Salinity-induced production of reactive oxygen species (ROS) and osmotic stress also interfere with various biochemical and physiological processes including photosynthesis (Sudhir and Murthy, 2004). Pigment analysis in *C. reinhardtii* has shown that the photosystem I light-harvesting complexes (LHCs) are damaged by oxygen radicals under high salinity, and photosystem II proteins required for photolysis of water are also impaired (Neelam et al., 2013; Subramanyam et al., 2010). Salinity stress causes a change in the turnover of D1 protein in the PS II of the Cells (Neelam et al., 2013). Transcriptome studies in *C. reinhardtii* have shown several PSI, LHC genes are down-regulated in the salinity induced impaired photosynthesis. Under the salinity

stress, the level of most of the chloroplast encoded transcripts (e.g., psaA, B, C, J, M) in photosystem I (PSI)are unchanged, while the genes like psaD, E, G, F, Hare down-regulated (Wang et al., 2018). Several transcriptomic studies have demonstrated that salinity-induced upregulation of different genes is required to resist the extreme salinity stress and loss of photosynthetic activity. In *C. reinhardtii* cells, upregulation of several genes involved in scavenging of ROS under salinity stress includes Fe superoxide dismutase (SOD), thioredoxins, glutathione transferase, and heat shock factor binding proteins. Photosynthetic pigment carotenoids are lipid-soluble antioxidants, located inside the chloroplast envelope, protect the LHC against ROS-induced damage. *Dunaliella* sp. is a good example of algal strains that produce a very high quantity of carotenoids in response to salinity stress. A study on salt-sensitive *C. reinhardtii* and *C. vulgaris* showed an increase in carotenoid production at moderate levels of salt stress (0.05 M–0.15 M).

2.7 Microalgal biofuel systems

Recently, there has been growing interest and investment in the development of microalgae as a feedstock for biofuel production. Advantages of microalgae-based biofuels are greater yield and fewer requirements for the land area as compared with terrestrial crops. Algae are estimated to produce about 2-10 fold more biomass per unit land area than terrestrial crops (Chisti 2008; Packer, 2009).

There are many reasons for greater biomass yields of algae such as algae have higher photosynthetic efficiency than land plants and efficient light capturing and minimal requirement of nutrients (Melis, 2009). Under ideal growth conditions, algae use most of their metabolic energy into cell division and allow rapid biomass accumulation. In addition, algae have carbon concentrating mechanisms and suppress photorespiration

due to high CO_2 concentration (Jansson and Northen, 2010). When the microalgae are grown under stressful conditions (e.g., low N, P, S) or the mixotrophic condition, the carbon metabolism is redirected toward the production and accumulation of high-energy storage compounds such as lipids**.** Many unicellular algae are capable of producing about 60% of neutral lipids (triacylglycerol [TAG]) per gram of dry weight, making them one of the most efficient biofuel production systems (Weyer et al., 2010).

Factors influencing microalgal biodiesel production include efficient photosynthesis, nutrient availability leading to the production of high biomass (Musa et al., 2019). In addition to offering biologically ideal biofuel production conditions, different physico-chemical conditions and engineering conditions such as dilution, surface area and volume of microalgal culture need to be optimized. Biofuel production by using lipid/carbohydrate-rich microalgae would be a highly promising solution to biofuel production technology (Sun et al., 2014). For a considerable increase in microalgae-based biofuel production, biotechnological approaches need to be considered for a cost-effective increase in the lipid/carbohydrate content under favorable environmental conditions. The primary step for efficient biofuel production is to select a suitable microalga and study its response to varying environmental conditions (Shukla et al., 2019). Hopefully, the development of microalgal-based biofuel production technology by using metabolic and genetic engineering may be economically viable as well as ecofriendly for sustainable cultivation and harvesting process.

In many cases, salt-adapted algae are not considered suitable candidates for biodiesel production as they lose their lipid accumulation capacity. A lot of commercial algae

cultivations prefer indoor and closed cultivation of salt-sensitive strains to avoid fatal contaminations. However, the use of salinity stress is a measure to harvest high-quality biomass. The *Picochlorum* SE3, isolated from a shallow mesophilic brackish-water lagoon, could tolerate a hypersaline environment and thus, making it a suitable candidate for large-scale open-pond cultivation of biomass. Previously, an increase in the fatty acids with increasing salinity has been reported in *Navicula oculata* (Mohy El-Din, 2015). Many reports have also suggested that excess unsaturated lipids in the cells under the salinity stress might be the cellular defence of the cells against salinity (Ritter and Yopp, 1993).

Microalgae are considered as a potential feedstock for bioethanol production as they have cellulosic cell walls and starch as the main carbohydrate reserve. Both starch and some of the cell wall polysaccharides are converted into fermentable sugars, which are subsequently transformed into bioethanol through microbial fermentation. To increase the economic feasibility of using algal carbohydrates for biofuels production, biomass productivity is the first step required for cost-effective biofuel technology. Enzymatic saccharification, involving cellulases, amylases, and glucoamylases, is extensively used for the hydrolysis of microalgal sugars. The microalgal cellulose is mainly located in the inner cell wall where lignin and the hemicellulose content are very low. Therefore, lignin degradation enzymes such a laccase and lignin peroxidase, and xylanase is not required in the enzymatic saccharification process. Besides acidic or alkaline pretreatment of microalgal biomass, or steam explosion is not needed, which makes the saccharification of microalgae easier and cheaper than other lingo cellulosic materials. To hydrolyze microalgae-based cellulose, endo-1,4-d-glucanase attacks the amorphous cellulose and cleaves it into smaller components, and then the exo-1,4-d-glucanasehydrolyzes them into simple sugars, such as cellobiose and

cellodextrin. Finally, the cello-oligosaccharides are degraded to glucose by glucosidase enzymes. In the case of hydrolysis of starch inside the microalgal cells, endo-amylase first attacks the internal β-1,4-glycosidic bond of starch to produce dextrin, and then glucoamylase further cleaves the dextrin into glucose and oligosaccharides such as maltose. Enzymatic hydrolysis has certain advantages over hydrolysis by acid or alkali, including mild conditions with low requirement of equipment and higher glucose yields.

Therefore, there is a great potential of using microalgae for low-cost and environmentally friendly wastewater treatment as compared to other more commonly used treatment processes (Mehrabadi et al., 2015). The major problem with most wastewaters is that they have very high concentrations of nutrients, particularly total N and total P concentration as well as toxic metals, which require costly chemical-based treatments to remove them during wastewater treatment (Gasperi et al., 2008). Total N and P concentrations can be found in the range of 10–100 mg L^{-1} in municipal wastewater and >1000 mg L^{-1} in agricultural effluent (de la Noue et al., 1992). The ability of microalgae to effectively grow in nutrient-rich wastewater makes it an extremely attractive, sustainable, and low-cost nutrient source for the production of biomass and treatment of wastewater (de-Bashan and Bashan, 2010; Hoffmann, 1998; Mallick, 2002). Thus, efforts are required to integrate wastewater treatment with biomass and biofuel production to minimize the cost of biofuel technology (Oswald and Golueke, 1960).

Nitrogen is an essential nutrient for the growth of microalgae as it is involved in the synthesis of proteins, amino acids, chloroplast, enzymes, and coenzymes etc. (Bellido-Pedraza et al., 2020). A variety of inorganic nitrogen sources such as nitrate,

nitrite, ammonia, and urea are assimilated by microalgae, and the nature of nitrogen sources influence the biochemical composition of cells. It has been extensively reported that nitrogen-deprivation in many microalgal strains synthesize the higher lipid content of carbohydrates as an energy reserve (Ikaran et al., 2015). Illman et al., (2000) reported that about 55% carbohydrate content was obtained in the *C. vulgaris* cells grown on a low-nitrogen-containing medium. D'Souza and Kelly, (2000) also reported that *Tetraselmis suecica* under nitrogen starvation with CO_2 feeding showed a dramatic increase in the cellular carbohydrate content from 10 to 57%. Several other examples demonstrated that the nitrogen limiting condition is the most effective way of triggering the accumulation of carbohydrates and lipids in microalgae (Pancha et al., 2014). However, some investigators indicated that there was a competition between lipid and carbohydrate synthesis under stress conditions (e.g., nitrogen-deficient) because the metabolic pathways for synthesis and degradation of energy-rich compounds like lipids and carbohydrates are closely interlinked (Yang et al., 2018). Precisely, starch biosynthesis of microalgae is on the contrary to lipid synthesis where the degradation product of starch acetyl-CoAis the precursor of fatty acid synthesis (Li et al., 2015). Thus, decreasing the starch degradation by genetic modification would be necessary to block the synthesis of lipids. The most difficult part is that the accumulation of microalgal carbohydrates occurs only when the microalgal cells are subjected to stresses, particularly nutrient limitation, which also results in poor cell growth and lower biomass productivity. Therefore, efforts are being made to enhance the carbohydrate content without compromising cell growth which is crucial for biomass production. Now various cultivation strategies are employed such as irradiance, nitrogen depletion, temperature variation, pH change, and CO_2 sequestration to enhance lipid and biomass production. Earlier studies have

revealed that an increase in the light intensity in the range of 30–400 μmol m^{-2} s^{-1} could increase the accumulation of carbohydrates (Rayati et al., 2020). In another study, it has been reported that a significant increase in starch content from 8.5% (dry weight basis) to 40% was recorded with an increase in the light intensity from 215 to 330 μmol m^{-2} s^{-1}. However, in some cases, no obvious positive correlation was observed between the light intensity and carbohydrate accumulation. This indicated that the accumulation of carbohydrates in microalgae is not only dependent on the light intensity but also on other environmental factors.

Chapter III

Materials and Methods

Materials and method

3.1 Study Material

Pure culture of green microalga *Chlorella vulgaris* was obtained from an algal biology lab, CSIR-NBRI Lucknow, India.

3.2 Sterilization

All the glassware and the growth medium used in the study for culturing *C. vulgaris* were steam sterilized in an autoclave operated at a steam pressure of 15 lbs/inch^2 with a temperature of 121°C. Cotton plugs wrapped with aluminum foil were used to tightly cover the mouth of the Erlenmeyer’s flasks containing growth medium. The glassware and media were placed in the autoclave for 15 min under prescribed standard conditions.

3.3 Classification of the organism under study

For the microscopic study of the microalga, *C. vulgaris* multifunctional digital microscope (Model No. US3, U1407007) was used.

Division	Chlorophyta
Class	Trebouxiophyceae
Order	Chlorellales
Family	Chlorellaceae
Genus	*Chlorella*
Species	*vulgaris*

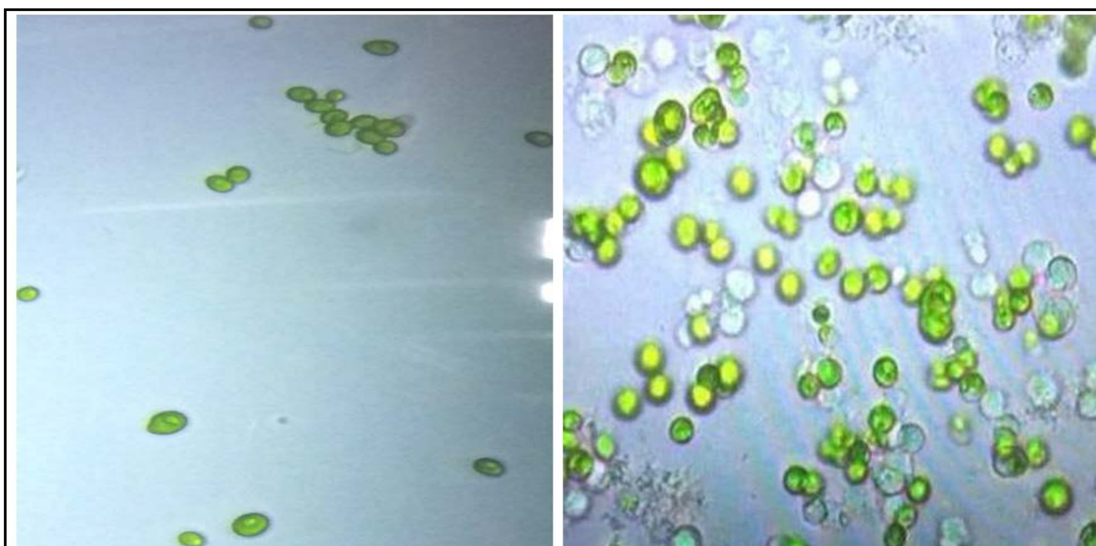

Fig: 3.1 Microscopic images of *C.vulgaris.*

3.4 Culture Condition

The axenic culture of *C. vulgaris* was routinely grown in Erlenmeyer's flasks (1000 mL) containing 500 mL of sterile BG-11 growth medium. The culture flasks inoculated with microalga were maintained in a culture room with a temperature of 25±2°C. The culture flasks were illuminated by using cool white fluorescent tubes (20Wm^{-2}) with 16/8 hour's light/ dark cycle. The cultures flasks were shaken thrice daily to deflect settling and sticking of the cells to the bottom of the flask.

Fig: 3.2 Microalgae *C.vulgaris* cultivated under varying bicarbonate concentrations.

3.5 Nutrient Medium

C. vulgaris was grown in BG-11 medium as described by Stainer et al., (1971). The composition of the nutrient medium is as follows:

Macro-elements	Concentration (g L^{-1})
$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
Na_2CO_3	0.02 g
Trace metal mix A5	1.0 ml
Distilled water	1.0 L

Trace metal mix A5	Concentration (g L^{-1})
H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$SO_4 \cdot 7H_2O$	0.22 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L

For preparing BG-11 medium, an adequate amount of macro-elements and micronutrients were mixed and autoclaved. The pH of the medium was adjusted to 7.2

3.6 Determination of growth

The microalga *C. vulgaris* was grown in an Erlenmeyer flask (500 mL) containing BG-11 medium (250 mL). The flask was inoculated with an inoculum density of 0.1 OD. The flasks were kept under favourable conditions of growth such as temperature (±30˚C) and light (20 W/m^2). The turbidity of microalgal cell suspension was monitored periodically at 680 nm up to 20 days by using a double beam UV-visible spectrophotometer (Shimadzu, Japan 1601). The specific growth rate (μ, d-1) was calculated using the following formula as suggested by Yu et al., (2017):

Specific growth rate μ, (d-1) = (ln X2 – ln X1)/ (t2-t1)

Where, X1= Initial density of population at t1 time;

X2= Final density of population at t2 time

3.7 Estimation of Protein

Protein content in the cell suspension was estimated by the method of Lowry et al., (1951) modified by Herbert et al., (1971).

Reagents:

I. 1N – NaOH

II. (i) 5% (w/v) solution of Na_2CO_3 (Sodium carbonate)

(ii) 0.5% (w/v) solution of $CuSO_4$ (Copper sulphate)

(iii) 1% (w/v) solution of Na-K tartarate

For making the reagent II, 50 ml of solution

(i) Mixed with 1 ml of solution (ii) and (iii)

III. 1 N Folin Phenol (Ciocalteau reagent)

Procedure:

To 0.5 ml of the cell suspension, 0.5 ml of 1 N - NaOH was added and the mixture was placed in a boiling water bath at 100°C for 10 min. After sufficient cooling of the mixture, 2.5 ml of reagent II was added to it. This mixture was thoroughly shaken and was incubated at room temperature for another 10 minutes. Thereafter, 0.5 ml of Folin-Phenol reagent (1N) was added to the mixture. After 15 min. of the incubation at room temperature, the color intensity of the mixture was read at 650 nm by using a double beam UV-visible spectrophotometer (UV – 1601, Shimadzu, Japan) against an appropriate blank. The concentration of protein in the unknown solution was estimated from a calibration curve prepared by using Bovine Serum Albumin (BSA) as a known standard.

3.8 Estimation of carbohydrate

The total carbohydrate content in the cell suspension was measured by the phenolsulfuric method as described by Dubois et al., (1956).

Reagents

I. 5% (w/v) of phenol solution

II. Sulphuric acid (96%, v/v, reagent grade)

To 1.0 ml of the cell suspension, 0.5 mL of phenol reagent was added and the mixture was shaken well. Thereafter 2.5 mL of 96% (v/v) sulphuric acid was added to each tube, using sidewalls of the test tube. The reaction mixture was shaken well and left for 20 min for developing a green-colored complex. The color intensity of the clear reaction mixture was measured at a wavelength of 490 nm in a UV-visible spectrophotometer (UV–1601, Shimadzu, Japan) against an appropriate blank. The known concentrations of glucose solution were used for the preparation of the

standard curve. The total carbohydrate content of the sample solution was calculated by using the standard graph and expressed in terms of µg carbohydrate/mL-1.

3.9 Estimation of total organic carbon (TOC)

The (TOC) of the cell suspension was measured by the Walkley-Black method as described by Grobler, (1979).

Reagents

I. 49.04 g of $K_2Cr_2O_7$ dissolved in 1.0 L distilled water (DW) (dichromate solution)

II. $Fe(NH_4)2(SO_4)2.6H_2O$ (1961.1 g) dissolved in 800 mL DW containing 200 mL sulphuric acid (H_2SO_4) and made up to 1L. This solution was standardized against the dichromate solution.

Procedure

100 mL microalgal culture was concentrated and the pellet was evaporated to dryness at 80˚C overnight. The dry biomass sample was taken in a boiling tube with 10 mL potassium dichromate solution followed by 20 mL concentrated sulphuric acid with gently swirling and mixing. The reaction mixture was left to cool down at room temperature for 30 min before making the final volume up to 50 mL in a volumetric flask. The turbidity of the reaction mixture could be cleared by either leaving the mixture to stand overnight or by centrifugation at 2000 rpm for 20 min. The concentration of the chromium- III ions chromophore was determined by measuring the color intensity of the green color at a wavelength of 649 nm in a UV-visible spectrophotometer (UV – 1601, Shimadzu, Japan) against an appropriate blank. The standard prepared curve was with a known concentration of the chromium-III ion. The TOC of the sample was calculated by using a standard curve and expressed in terms of mg $L^{-1.}$

3.10 Lipid extraction by gravimetric method

A modified Bligh and Dyer, (1959) protocol were used for extraction and estimation of lipid content in *C. vulgaris*. The microalgal biomass was harvested after 20 days of cultivation. 500 mL of cell suspension was centrifuged for 5 min at 5000 rpm. The supernatant was removed and the pellet was washed thrice with DW. The cell residue was allowed to dry overnight in a hot air oven at 70 ℃ and then dried biomass was grounded by using a mortar and pestle. The powdered dried biomass was weighed and then washed twice with phosphate buffer saline (PBS) of pH 7.0±2. Subsequently, 0.5 mL PBS was added to the dried biomass along with 1 mL glass beads. The obtained suspension was vortexed for 10 min (stop vortex every one min. and maintained cooling by keeping it in the cold water). Further, 0.4% (v/v) phosphoric acid and sulfuric acid were added in the ratio of 2:0.75. The mixture was incubated at 50°C for 5 min. Thereafter, the solution was centrifuged at 2000 rpm for 5 min and the precipitate was decarded. The chloroform: methanol (2:1 v/v) solution was added to the residual biomass. The mixture was left for 24 h at room temperature after vortexing for 5 min. After 24 h, 1mL distilled water and 2 mL chloroform: methanol (2:1 v/v) solution were added and again the mixture was vortexed for 1 min and then centrifuged at 4000 rpm for 5 min. The bottom organic phase layer was withdrawn to another flacon tube (50 mL) and washed with 5% NaCl solution (1:1 v/v) to remove the impurities. The organic phase (lipid) was allowed to evaporate at room temperature and the lipid residue was weighed and recorded. The lipid content was calculated gravimetrically and expressed as percent (%) lipid.

Lipid content (%) = weight of lipid/weight of algal biomass ×100

3.11 Estimation of proline

Proline content in microalgae was estimated according to the method of Bates et al., (1973).

Reagents

Chemical	Amount
Toluene	Pure
Sulfosalicyclic acid	3 % (w/v)
Glacial acetic acid (GAA)	3 % (v/v)
Phosphoric acid	6 M
Acid ninhydrin	1.25 g ninhydrin warmed in 30 ml glacial acetic acid (100 %) and 20 ml 6 M phosphoric acid

Procedure

To measure the proline content 3 ml of microalgal cell suspension was crushed in 3% aqueous sulfosalicyclic acid, centrifuged at 5000 rpm for 20 minutes. 2 ml of ninhydrin and 2 ml glacial acetic acid (3 % v/v) were added to a test tube containing 2 ml of filtrate. The obtained mixture was heated in a water bath for 1 h at 95°C. The samples were allowed to cool using an ice bath. The reaction mixture was further mixed with 4 ml toluene (pure), mixed, and stirred for 15-20 min. The chromophore containing toluene was extracted from the aqueous phase and the absorbance was taken at 520 nm, using toluene as blank. The proline content in each sample was calculated using the standard curve.

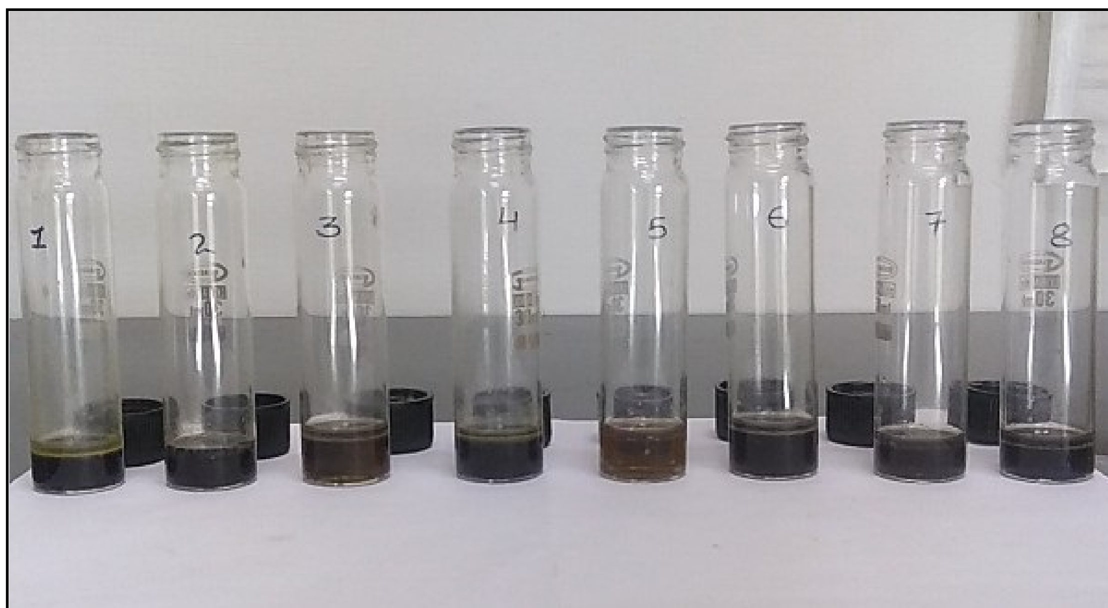

Fig: 3.3 Lipid extraction by gravimetric method.

3.12 Measurement of photosynthetic pigments

For the extraction of photosynthetic pigments, an aliquot of cells suspension (4.0 mL) was withdrawn at regular intervals and was subjected to repeated freezing and thawing to break the cells. The broken cell pellet was suspended in equal volume (4.0 mL) of acetone (80%, v/v) and was shaken well. The suspension was kept at 4°C for 24 h. After thawing of the broken, the cells suspension was centrifuged for 5 min at 4000 rpm to collect the supernatant. The collected supernatant was used to take absorbance at different required wavelengths in a double beam UV-visible spectrophotometer (UV – 1601, Shimadzu, Japan) by using a light path of 1 cm. Total chlorophyll (chlorophyll a, b) contents were calculated by using the following equations (Lichtenthanler et al., 2001)

Chl*a* (µg/ ml) = 5.19 ($A_{649\ nm}$ –$A_{750\ nm}$) + 13.36 ($A_{649\ nm}$ –$A_{750\ nm}$)

Chl*b* (µg/ ml) = 27.43 ($A_{649\ nm}$ –A_{750nm})–8.12 ($A_{665\ nm}$ –$A_{750\ nm}$)

3.13 Estimation of physiological parameters (photosynthetic performance) of the algae

The physiological studies of microalgae were conducted by measuring chlorophyll fluorescence induction kinetics in microalgae cells. The photosynthetic parameters were measured by pulse modulated Photon Systems Instruments (Aqua Pen-C portable fluorometer, Czech Republic). The cells were dark-adapted for 10 to 15 min at room temperature before the measurement of chlorophyll fluorescence induction kinetics (OJIP curve) at fixed excitation (450 nm) and emission wavelengths (650 nm). Chlorophyll-a fluorescence induction (OJIP) curve was used for calculation of photosynthetic parameters such as performance index (Pi ABS), maximum quantum yield (Fv/Fm), photochemical quenching (PQ), Non-photochemical quenching (NPQ), Net closing rate of the reaction centre (Mo), Trapping flux (TRo/RC), effective antenna size expressed as absorbance per reaction centre (ABS/RC). The microalga *C. vulgaris* cells under different experimental conditions were examined as described by Strasser et al., (2000) and Lichtenthaler et al., (2005). Measurement of Non-photochemical quenching (NPQ) was measured by using the protocol described by Schreiber (2004). A measuring light (0.09 $\mu mol.m^{-2}.s^{-1}$) was used to acquire the minimal level of fluorescence Fo. A short saturating flash of light (100 $\mu mol.m^{-2}.s^{-1}$) was then applied to reduce the plastoquinone pool, measured as maximum fluorescence, Fm. After a short dark relaxation, the sample is exposed to actinic irradiance (20 $\mu mol.m^{-2}.s^{-1}$) for tens to hundreds of seconds to elicit a transient of the Kautsky effect. Moreover, a sequence of saturating flashes is applied on top of the actinic light to probe the non-photochemical quenching NPQ and effective quantum yield of photosynthesis. Relative fluorescence decrease ratio (RFD) has been often used in eco-physiological studies as a measure of the photosynthetic activity of higher

plants, for estimation of photosynthetic CO_2 assimilation rates and chlorophyll content in algae. Following equations were used for calculation of various photosynthetic parameters, 1-3 is described by Strasser et al., (2000; 2004). The equations 4, 5, 6 are given by Gashi et al., (2013); Schreiber et al., (1986) and Lichtenthaler et al., (2005), respectively.

$$1 - Absorption of energy per active reaction centre \left(\frac{ABS}{RC}\right) = M_O \times \left(\frac{1}{Vj}\right) \times \left(\frac{1}{\Psi P_O}\right)$$

$$2 - Initial slope of induction curve\ (M_O) = 4 * (F2ms - F_O)/(Fm - F_O)$$

$$3 - Performance Index on absorption basis PIabs = \left(\frac{RC}{ABS}\right) = \frac{\varphi P_O}{(1-\varphi P_O)})\Psi o/(1 - \Psi_O)$$

4- $Relative fluorescence decrease ratio RFD = \frac{Fp-Fs}{Fs}$,

5- $Energy dependent quenching\ (q_{E}) = \frac{(Fv)m-(Fv)s}{(Fv)m}$

$$6 - Non - photochemical quenching\ (NPQ) =_N F/F'_{M,}\ Where\ ({}_N F = F_M - F'_M)$$

Fo- Initial fluorescence in the dark-adapted cells, Fm- Maximum fluorescence in the dark-adapted cells, Fm'- Maximum fluorescence in the light-adapted cells, Fv- Variable fluorescence, F2ms- fluorescence after 2 milliseconds, Vj- Relative variable fluorescence at point J in the OJIP curve, Mo- initial slope of the induction curve, φP_O*- Quantum yield of QA reduction,* Ψo *– the probability that a trapped exciton is used for electron transport beyond QA. Fp- initial fluorescence increase caused by the actinic light exposure and Fs-steady-state fluorescence after the actinic light exposure.*

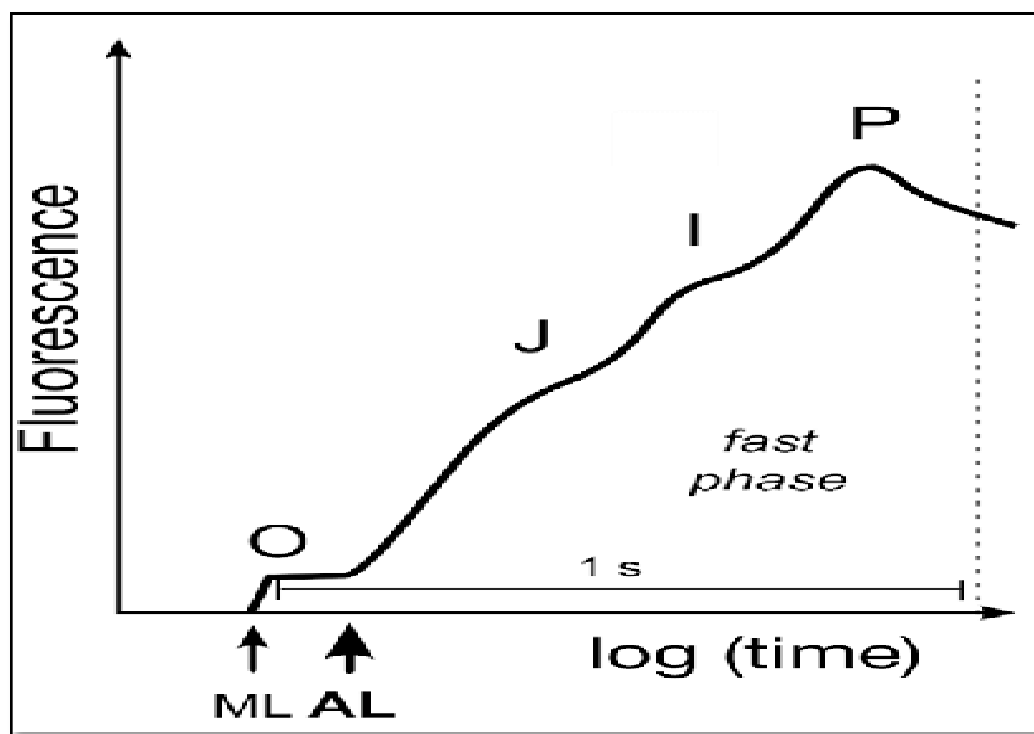

Fig.3.4. Schematic illustration of chlorophyll -a fluorescence induction curve known as OJIP.

3.14 Fourier transform infrared (FTIR) spectroscopy

FT-IR spectroscopy was used to detect the changes in the macromolecular composition of *C. vulgaris* under various stress conditions or nutrient treatments. A measured amount of biomass was mixed with KBr (1:100). Then the mixture was grounded into fine particles and was compressed into a translucent sample disk by manual hydraulic pressure. The spectra for both control and treated cells were measured within the range of 500-4000 cm-1 using a KBr window by FTIR pectrophotometer (Nicolet 6700, Thermoscientific USA). The background obtained from the scan of pure KBr was automatically subtracted from the sample spectra and the obtained FTIR spectra were used for further analysis. The biochemical constituents were assessed based on the characteristic IR absorbance of different macromolecules (Duygu et al., 2012; Laurens et al., 2011) and monitored by the

analysis of total lipid, lipid/carbohydrate (L/C ratio), and lipid/protein (L/P ratio), amideI/amide II ratio, unsaturation/saturation ratio (Dean et al., 2010; Mahapatra et al., 2013).

3.15 Scanning electron microscopy coupled energy dispersive X-ray spectrometer study

Sample preparation

The *C. vulgaris* cells were fixed in 2.5 % of glutaraldehyde at 4°C for 4 h. Thereafter, the cells were washed with phosphate buffer and subsequently dehydrated by ethanol (10 %, 30 %, 50 %, 70 %, 95 %, and 100 %) for 5 min at each concentration (Chandra et al., 2017). The final dehydration was carried in 100 % ethanol for 30 min. The samples after dehydration were mounted on an aluminium stub using carbon tape. SEM coupled with an energy dispersive X-ray spectrometer (EDS) detector was used for the X-ray spectra. X-ray plotting was done using EDS in conjunction with Scanning Electron Microscopy (SEM) (JEOL, Japan; model JSM-6490LV).

3.16 Chemicals

All the chemicals used in the present course of study are of analytical reagent (AR) grade and were products of SD-Fine chemicals (India), Loba Chemicals Ltd. (India), Sigma chemicals (USA), or Merck (Germany).

3.17 Statistical analysis

All experiment was performed in triplicate and demonstrated as mean ± SD with error bars. To confirm the variability of data and validity of results, all data were subjected

to one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT, $P<0.05$) to see the significant level (Gomez and Gomez, 1984).

3.19 Equipments

i. Spectrophotometric readings were taken by a Double beam UV- visible spectrophotometer (Shimadzu, 1601, Japan).

ii. The pH of the solution was measured by using a pH meter of Toshniwal Inst. Mfg. Pvt. Ltd., Ajmer.

iii. The microscopic study was done by using a Multifunctional digital microscope (model, US3, U1407007).

iv. Fluorescence spectra of cells were taken by Spectro-fluorometer (Cary Eclipse 100, India).

v. Infra-red spectra of the samples were recorded on Fourier Transform Spectrophotometer (Nicolet 6700, Thermo Scientific, USA).

vi. Chlorophyll a fluorescence transient was measured by using Pulse Amplitude Modulated (PAM) (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic).

Chapter IV

Antagonistic effect of bicarbonate on salinity induced changes in microalga Chlorella vulgaris

Antagonistic effect of bicarbonate on salinity induced changes in microalga *Chlorella vulgaris*

4.1 Introduction

Microalgae are a promising resource for biodiesel production as they exhibit higher growth rates, short generation time, and higher lipid content, which confers them an edge and makes them useful as green resources for biofuel production (Chisti 2008, Davis et al., 2011; Srinivasan, 2018). However, cell biomass and biochemical composition of the microalgal cells are generally controlled by various environmental and nutritional conditions (White et al., 2015; Pal et al., 2011). It has been reported that lipid production in microalgal cells is usually enhanced under unfavourable growth conditions (Benavente-Valdes et al., 2016; Xin et al., 2011), particularly under high light stress, UV stress, salinity stress and nutritional stress etc. (Paliwal et al., 2017; Pancha et al., 2015). Salinity stress is one of the most common environmental stresses which cause varying degrees of damage to plants and algae, depending upon the interactive effect of other growth conditions (Haghjou et al., 2014). The salinity stress has been reported to induce an increase in the lipid content in a host of microalgal strains, including *Chlamydomona s*sp. JSC4 (Ho et al., 2014). Stress-based strategies in cultured microalgae are widely recognized as an environment-friendly approach to induce overproduction of lipids – a source of biodiesel (Behera et al., 2015). However, a stress-induced increase in the lipid content of microalgae is simultaneously accompanied by a significant decline in the growth and biomass of microalgae.

Physiological impact of microalgae on salinity stress is generally observed in terms of salt-induced changes in the anti-oxidative defence system, damage to proteins and

DNA, ion toxicity, reduced photosynthesis, and decrease in the membrane permeability of the cell (Parida and Das, 2005). One of the important effects of hyper-saline conditions in plants and algae is to create an ionic and osmotic imbalance in the cells which impairs the membrane functions in cell organelles (Yang et al., 2015). Though the microalgal cells easily adapt to such stress conditions by bringing about changes in their physiology and biochemical processes (Asulabh et al., 2012). Some unicellular halophilic microalga like *Dunaliella salina* are well known to respond to salinity stress by regulating carbon fluxes between starch production in the chloroplast and synthesis of glycerol in the cytoplasm (Shetty et al., 2019). It has been demonstrated that the percentage of highly unsaturated fatty acid is enhanced with increasing salt concentration, while the overall quantity of cell constituents declines due to unfavourable stress conditions (Kirrolia et al., 2011). Most of the studies concerning the effects of salinity stress on lipid production in microalgae have been mainly confined to seawater microalgae. But little effort has been made to know about the effect of salinity stress on the overall synthesis of biochemical constituents including lipids in the freshwater microalgae (Yang et al., 2011).

Previous studies have shown that the biomass, cell composition of microalgae respond to the addition of bicarbonate (Peng et al., 2017). Earlier it has been suggested that the presence of bicarbonate not only influenced the carbon sequestration as an external source of carbon, but it also interacted with other metabolic systems including nutrient uptake (Bruggemann et al., 2011). There is still no clear understanding of how bicarbonate affects the nutritional status and other metabolic functions in the microalgae. To achieve the desired level of commercial production of biofuel, a concerted effort would be required to optimize the salinity stress-induced overproduction of lipid and changes in the nutritional status of microalgal cells to

overcome the stress-induced limitations on biomass production. The present study is an effort to study the role of sodium bicarbonate in the regulation of salinity stress-induced alterations in the growth, nutrient status of the cells, photosynthetic performance, and level of cell constituents including lipid in the microalga *C. vulgaris*.

4.2 Materials and Methods

4.2.1 Experimental setup

For routine growth, the microalga *C.vulgaris* was grown in a 250 ml Erlenmeyer flask containing 100 ml of BG-11 medium (pH 7.5). The cell suspension was incubated in a culture room (25°C) fitted with cool fluorescent light (20 Wm^{-2}) with an alternate light/dark cycle of 16/8 hours. In the present study, fresh monoculture of *C. vulgaris* was first grown in presence of sodium bicarbonate (0, 5, 10, 15, 20, 25, 30 mM) concentrations, to obtain the suitable bicarbonate dose for higher biomass production. Next, the combined effect of optimum concentration of sodium bicarbonate and NaCl was studied. Effect of NaCl (0-400 mM) on the cell constituents of microalga growth was recorded in terms of chlorophyll, protein, carbohydrates, lipid, total organic carbon, and proline content. To see the effect of sodium bicarbonate (0-30 mM) or NaCl (0-400mM) or NaCl + HCO_3^-(200 mM + 20 mM) as and when required, the growth medium was supplemented with these salts before the cells were suspended.

4.2.2 Determination of growth rate and biomass productivity

Turbidity of the cell suspension was monitored at 680 nm in a double beam UV-visible spectrophotometer (Shimadzu, Japan 1601). The specific growth rate (μ d^{-1}) was calculated as described in chapter- III of materials and methods.

For determination of the dry weight of algal biomass, 200 ml cell suspension of microalga was filtered through pre-weighed dried filter paper and the cell residues were washed with distilled water to remove the impurities. Thereafter, the filter paper was oven-dried at 60^{0}C for 20 hours to obtain the dry weight of algal biomass.

$$Biomass\ productivity - \frac{B2 - B1}{T}$$

(*where B2 and B1 represent the dry weight biomass densities at the time T (days), at the end and start of the experiment, respectively)*

4.2.3 Estimation of macromolecules

Total carbohydrate content was quantified by the phenol-sulphuric acid method as described by (Dubois et al., 1956). Proline content was estimated according to the method described by Bates et al., (1973). The protein content of the cell suspension was estimated by following the method of Lowry et al., (1951), using bovine serum albumin (BSA) as standard. The total lipid in the algal biomass was extracted by mixing chloroform–methanol (4:2, v/v) with the algal sample as described by (Bligh and Dyer, 1959). These methods are described in detail in chapter III of materials and methods. (Page no. 66-71)

4.2.4 Measurement of Chlorophyll Fluorescence Induction Kinetics

Chlorophyll fluorescence induction kinetics (OJIP) Non-photochemical quenching (NPQ) in dark-adapted cell cultures was measured by PAM fluorometer (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic).

4.2.5 Analysis by scanning electron microscopy (SEM-EDS)

Scanning Electron Microscope coupled with Energy dispersion X-ray spectroscopy was used to study the morphological alterations and mineral status of the different elements in the algal cells. A scanning electron microscope—EVO LS 15 (Oberkochen, Germany), operating at 20 kV was used in the study.

4.2.6 Statistical analysis

All the experiments were carried out in triplicates and the results were expressed as mean values and standard deviation (SD). To compare the validity and variability between groups and among each other, a one-way analysis of variance (ANOVA) was done followed by Duncan's multiple range test (DMRT, $P \leq 0.05$). The analysis was performed using SPSS version 21.0 (IBM Co., Armonk, NY, USA).

4.3 Results

4.3.1 Effect of sodium bicarbonate addition on specific growth rate and biomass productivity of *C. vulgaris*

Microalgae generally grow photoautotrophically by utilizing CO_2 as an inorganic source of carbon. As per the previous reports bicarbonate significantly increases lipid and biomass content (White et al., 2013). Bicarbonate addition is preferred over CO_2 for large-scale cultivation of microalgae as the solubility of CO_2 is very low in the water. In the present study addition of sodium bicarbonate significantly affected the specific growth rate and biomass productivity of *C. vulgaris* (Fig. 4.1). In comparison to control (without bicarbonate) the specific growth rate increased gradually with the increasing concentrations of $NaHCO_3$, the highest growth rate (0.66 μ/d) was

observed at 20 mM bicarbonate concentration. Whereas, at 25 mM bicarbonate addition decline in the specific growth was recorded. A similar pattern of rising and fall was found in biomass productivity of *C. vulgaris* on the addition of bicarbonate (0-25 mM). Control showed the lowest biomass productivity (0.275 g/d/L) and the highest biomass productivity (0.85 g/d/L) was found on 20 mM bicarbonate supplementation. From the above-mentioned results, it can be inferred that bicarbonate addition to the growth medium increases the cell division and metabolic process in *C. vulgaris*.

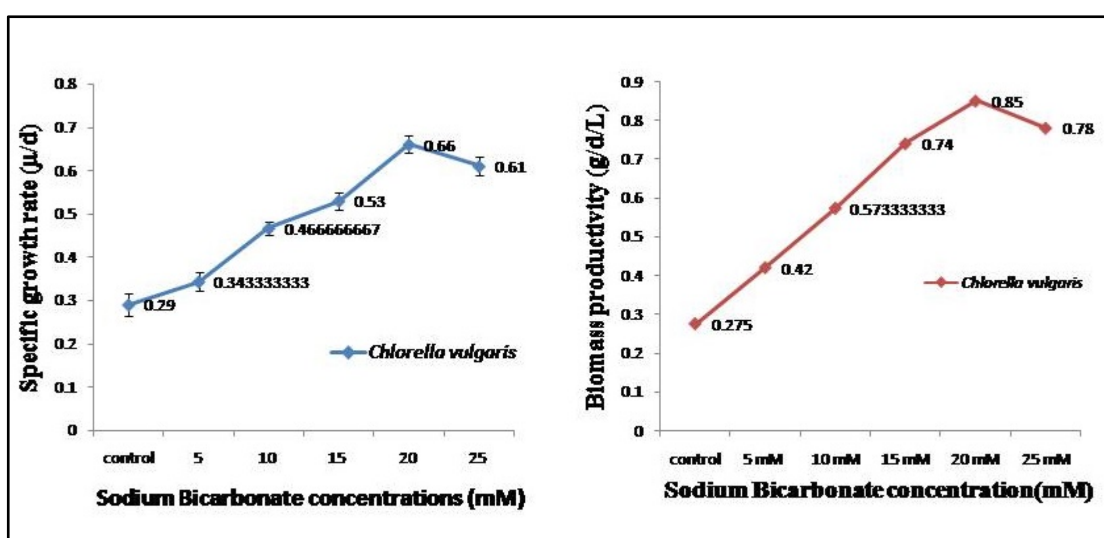

Fig. 4.1 Effect of sodium bicarbonate treatments (0-25 mM) on (a) Specific growth rate and (b) Biomass productivity of *C. vulgaris.* Data are the mean of three replicates ± SD.

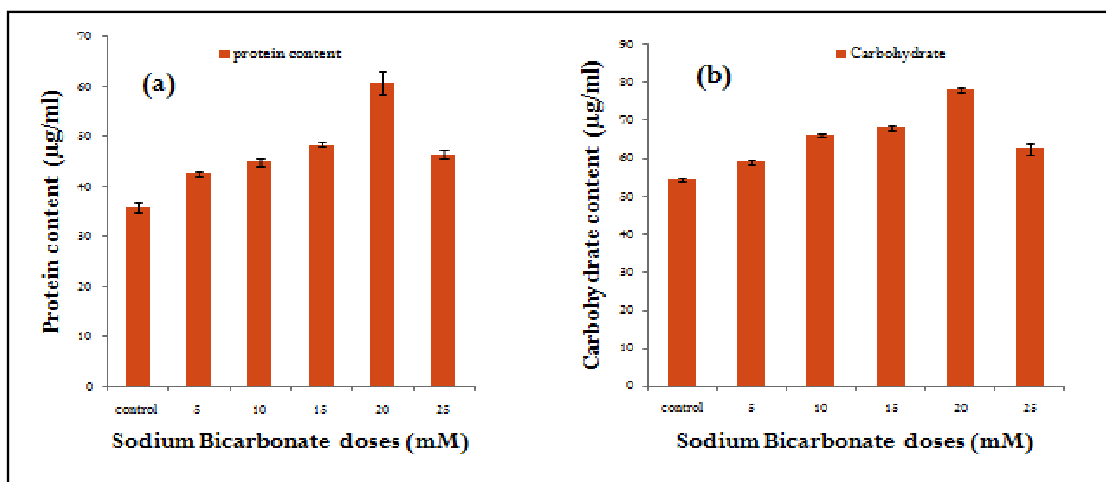

Fig. 4.2 Effect of sodium bicarbonate treatments (0-25 mM) on (a) protein content and (b) carbohydrate content of *C. vulgaris.* Data are the mean of three replicates ± SD.

4.3.2 Effect of sodium bicarbonate addition on protein content and carbohydrate content of *C. vulgaris*

The addition of sodium bicarbonate to the growth medium (BG-11) resulted in increased protein content in *C. vulgaris* in a dose-dependent manner (Fig. 4.2). The highest protein content was found in *C. vulgaris* cells grown with 20 mM bicarbonate, and the lowest protein was found in cultures grown without additional bicarbonate (control). In the case of carbohydrate content, no significant difference was observed on the addition of 5 mM and 10 mM bicarbonate, the maximum carbohydrate content was recorded under 20 mM bicarbonate supply. A slight decline in carbohydrate content was noticed at 25 mM bicarbonate addition.

According to Peng et al., (2014) in presence of high inorganic carbon, the activity of ribulose-1, 5-bisphosphate carboxylase/oxygenase (RuBisCO) plays an important role in the conversion of 3-phosphoglycerate, gets stimulated. This key enzyme acts as a substrate for the biosynthesis of carbohydrates and fatty acids in microalgae. Fan et

al., (2014) mentioned in their study that the availability of carbon and supply to the algal system is a major metabolic regulator which regulates the synthesis of lipid and carbohydrates.

4.3.3 Effect of NaCl on the specific growth rate, protein and chlorophyll content in *C. vulgaris* cells

Effect of different concentrations of NaCl (0-400 mM) on the specific growth rate of microalga *C. vulgaris* was studied in the absence and presence of maximum growth supporting concentration of $NaHCO_3$ i.e.20 mM. Results (Fig. 4.3.a) exhibited a concentration-dependent decline in the specific growth rate of microalga under bicarbonate supplemented as well as deprived conditions. A maximum decrease in the specific growth rate was observed at 400 mM concentration of NaCl. The 50% growth inhibitory (I-50) concentration of NaCl was found to be at 100 mM, which increased to 160 mM in the presence of bicarbonate. Similarly, the effect of NaCl concentrations was observed on the protein (Fig. 4.3.b) and chlorophyll (Fig. 4.4 a) contents in the microalga *C. vulgaris*. However, the addition of bicarbonate (20 mM) to the growth medium could significantly improve the protein as well as chlorophyll content throughout the concentration range of NaCl (0-400 mM). These results suggested that the addition of bicarbonate to the growth medium, to a great extent, was able to mitigate the toxic behaviour of NaCl. Besides, the NaCl toxicity in the microalga was not attributable to Na^+ ions, perhaps NaCl toxicity was due to Cl^- ions.

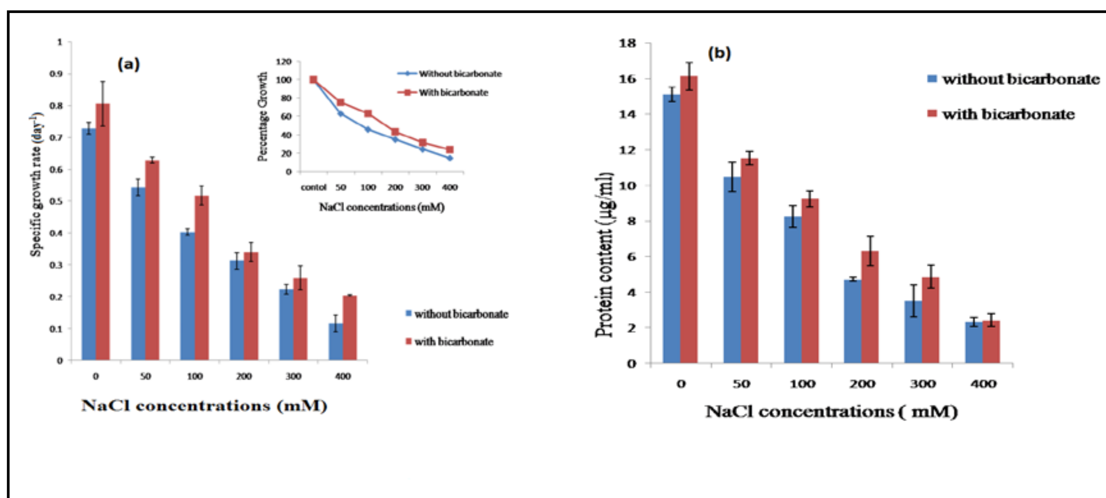

Fig. 4.3. **(a)-** Optical density of *C. vulgaris* in response to various salt concentrations (0-400 mM) in the presence and absence of sodium bicarbonate (20 mM). The inset derived from a specific growth rate depicts the percent growth in response to NaCl concentrations. **(4.3.b)-** Protein content of *C. vulgaris* in response to various salt concentrations (0-400 mM) in the presence and absence of sodium bicarbonate (20 mM). Data are the mean of three replicates ± SD.

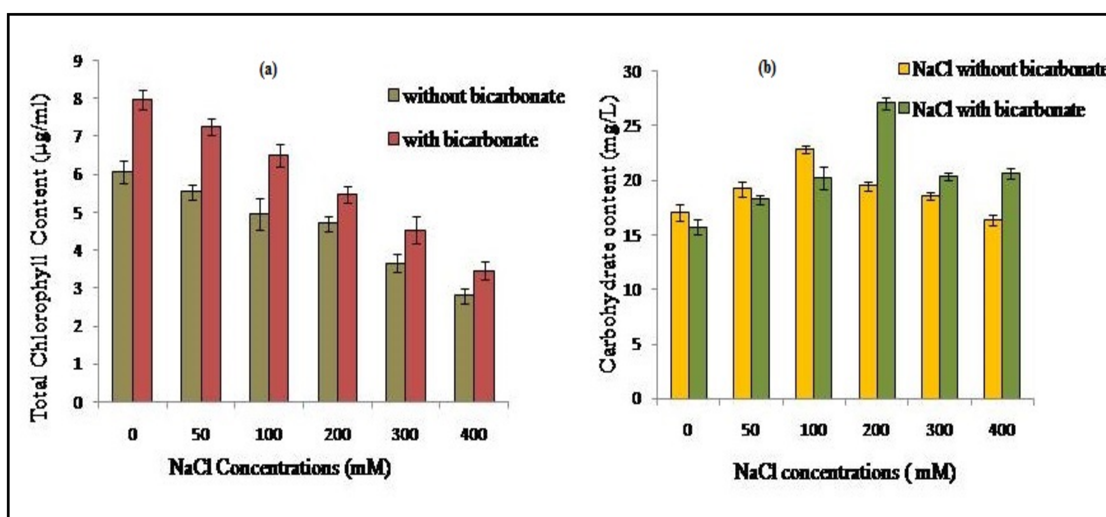

Fig: 4.4 **(a)** Effect of NaCl concentrations (0-400 mM) on the chlorophyll content in *C.vulgaris* cells in the absence and presence of $NaHCO_3$ (20 mM). **(b)** Carbohydrate content of *C.vulgaris* in response to various salt concentrations (0-400 mM) in the presence and absence of sodium bicarbonate (20 mM). Data are the mean of three replicates ± SD.

4.3.4 Effect of salinity on carbohydrate content of microalga

Results (Fig.4.4 b) on the effect of NaCl concentrations (0-400 mM) on the total carbohydrate content in the microalga *C. vulgaris*, in the presence and absence $NaHCO_3$ (20 mM) exhibited a concentration-dependent initial increase in total carbohydrate content up to 100 mM, followed by a concentration-dependent gradual decline in the total carbohydrate level from 200 mM to 400 mM NaCl. The addition of bicarbonate to the growth medium resulted in significant improvement in the carbohydrate content up to a concentration of 400 mM NaCl. The bicarbonate-dependent unusual increase in the carbohydrate content of microalga up to 400 mM NaCl was in sharp contrast to NaCl alone (without bicarbonate). NaCl alone induced an increase in the carbohydrate up to 100 mM concentration of NaCl. Thus, it was inferred that NaCl induced an increase in the synthesis of carbohydrate, particularly in the presence of bicarbonate, might be due to improved functioning of photosynthesis.

4.3.5 Effect of salinity on TOC

Results on the effect of different concentrations of NaCl (0-400 mM) on the (TOC) content in the microalga *C. vulgaris* (Fig. 4.5.a), in the presence and absence of $NaHCO_3$ (20 mM), initially showed a concentration-dependent increase in the TOC content up to 100 mMNaCl as compared to control (without NaCl), followed by a declining pattern in the TOC level with increasing concentration of NaCl from 200 to 400 mM. However, the addition of $NaHCO_3$ (20 mM) to the growth medium resulted in a significantly higher level of TOC content throughout the concentration range of NaCl (0-400 mM) when compared with corresponding values in the presence of NaCl alone (without bicarbonate). Unlike the pattern of NaCl-dependent specific growth rate and protein content, the TOC content initially increased at lower concentrations

of NaCl (50 and 100 mM). The TOC content in the $NaHCO_3$ supplemented cells at 100 mM NaCl concentration was about 35% higher than the corresponding value of TOC recorded in the absence of bicarbonate. These results suggested that an initial increase in the level of TOC at a lower concentration of NaCl (up to 100 mM) might be due to the osmotic requirement of the microalga, which significantly improved in the presence of bicarbonate.

4.3.6 Effect of salinity on the proline content in the microalga-

Results (Fig.4.5.b) depict the effect of varying concentrations of NaCl (0-400 mM) on the proline content of microalga *C. vulgaris* under $NaHCO_3$ (20 mM) added and deprived conditions. Results exhibited a concentration-dependent gradual increase in the proline content throughout the concentration range of NaCl. The maximum level of NaCl induced increase in the proline content was observed at 400 mM concentration of NaCl. The addition of $NaHCO_3$ (20 mM) to the growth medium resulted in a significant increase in the level of proline in *C. vulgaris* cells throughout the NaCl concentration range (0-400 mM). However, it was observed that level of proline content was significantly enhanced in the presence of bicarbonate. Thus, it was inferred that the addition of bicarbonate was perhaps helping the microalgal cells in terms of salinity tolerance due to an increase in the proline synthesis.

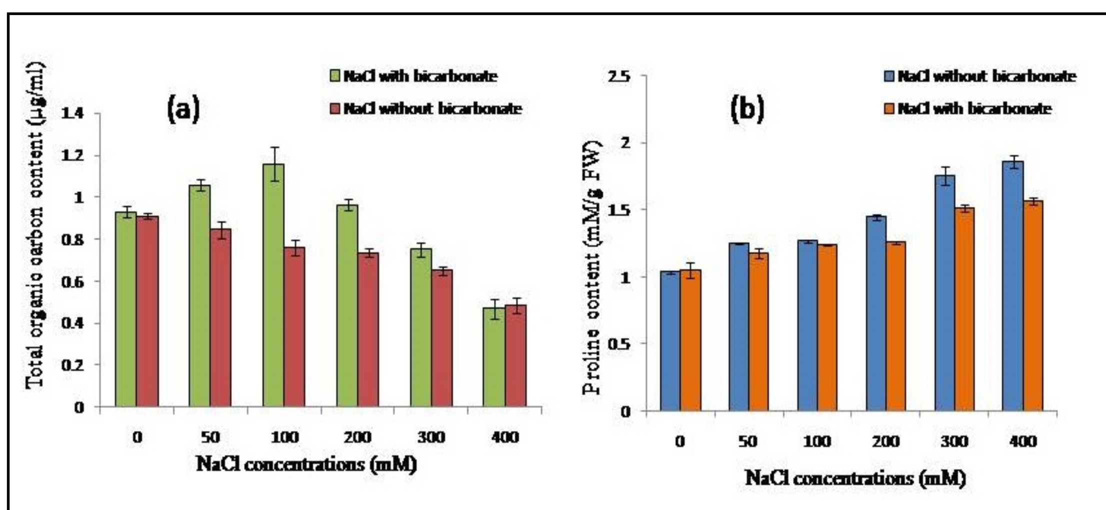

Fig. 4.5 (a) Total organic carbon (TOC) content of *C.vulgaris* in response to various salt concentrations (0-400 mM) in presence and absence of sodium bicarbonate (20 mM) **(b)** Proline content of *C.vulgaris* in response to various salt concentrations (0-400 mM) in presence and absence of sodium bicarbonate (20 mM). Data are the mean of three replicates ± SD.

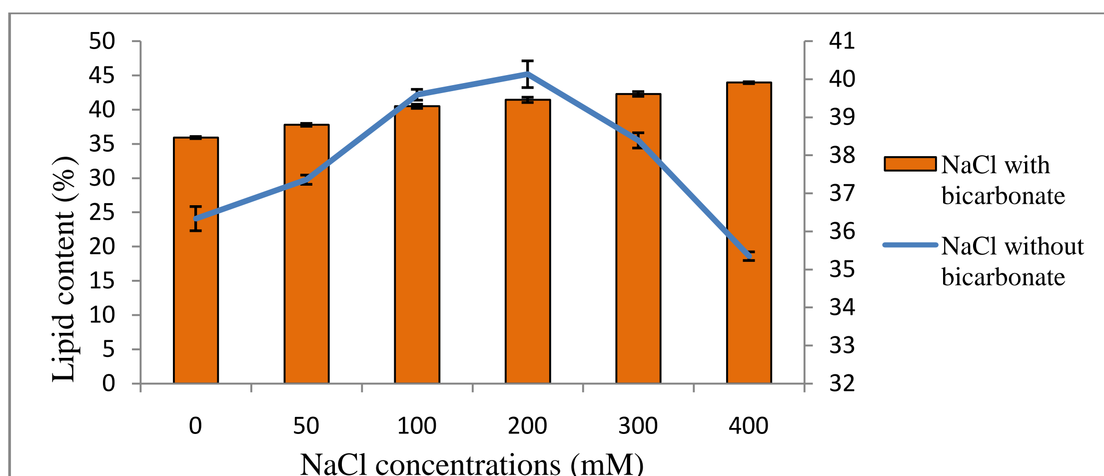

Fig.4.6 Lipid content of *C. vulgaris* in response to various NaCl concentrations (0-400 mM) in presence [primary axis] and absence of sodium bicarbonate (20 mM) [secondary axis].Data are the mean of three replicates ± SD.

4.3.7 Effect of salinity on lipid content of microalga

The lipid content of *C. vulgaris* was measured in response to a range of NaCl concentrations (0-400mM) under $NaHCO_3$ (20 mM) supplemented and deprived

conditions. As evident from the results (Fig.4.6), the total cellular lipid content in the *C. vulgaris* cells initially increased with increasing NaCl concentrations (50, 100, 200 mM), and subsequently, the cellular lipid content registered a gradual decline with increasing NaCl concentrations (300-400 mM). Further results showed that the addition of bicarbonate to the growth medium exhibited significant improvement in the overall lipid content of the microalga throughout the concentration range of NaCl (0-400 mM) when compared with the corresponding values obtained in the absence of bicarbonate. The present results suggested that lipid accumulation in the microalga was better at lower concentrations of NaCl and the addition of bicarbonate significantly improved the lipid content.

4.3.8 Analysis of the cell morphology and nutrient status by SEM-EDS

The SEM analysis (Fig.5.7 & 5.8) of cell samples treated with NaCl (100), $NaHCO_3$ (20 mM), NaCl (100mM) + $NaHCO_3$ (20mM) was compared with untreated cells (without NaCl and bicarbonate). The results showed that both NaCl and HCO_3 exerted their effect on morphology and cell surface appearance. In the absence of NaCl and bicarbonate (untreated control), the microalgal cells were small spherical shaped (3-6µm) with a smooth surface. There was no cell clumping tendency. However, the cells treated with 100 mM NaCl showed rounded cells (4-7µm) with fuzzy appearance and no clumping. But the cells treated with $NaHCO_3$ exhibited larger cells (4-7µm) with profuse cell clumping tendency. When the cells were given a combined treatment of NaCl (100 mM) + $NaHCO_3$ (20 mM), the cell size was enlarged (4-5 µm) and exhibited a fuzzy appearance with a greater tendency to form clumps.

The EDS analysis of phosphate, sodium and chloride elements in the NaCl treated *C. vulgaris* cells showed substantial increase in the respective values of weight % and atomic % of Na (3.12 % & 1.84 %), Cl (2.96 % & 1.14 %) and P (1.49 % & 0.65 %) when compared with the weight% and atomic% of elements in the untreated control (Na-2.5 % & 1.19 %, Cl-1.29 % & 0.49 %, P -0.73 % & 0.32 %, respectively). However, bicarbonate supplemented cells exhibited reduced weight% and atomic% for these elements (Na- 1.15 % & 0.66 %, Cl- 0.90 % & 0.33 % and P- 0.67 % & 0.29 %) as compared to untreated control (without NaCl). However, a combined treatment of cells with 100 mM NaCl+ 20 mM $NaHCO_3$ resulted into drastic decrease in the weight% and atomic % of these elements (Na- 0.96 % & 0.55 % ,Cl- 0.75 % & 0.28 % and P- 0.43 % & 0.19 %) as compared to NaCl treated cells as well as untreated control. These results demonstrated that presence of HCO_3^- was able to prevent the NaCl induced larger accumulation of elements (Na, Cl and P) and thereby, it might be preventing the NaCl toxicity.

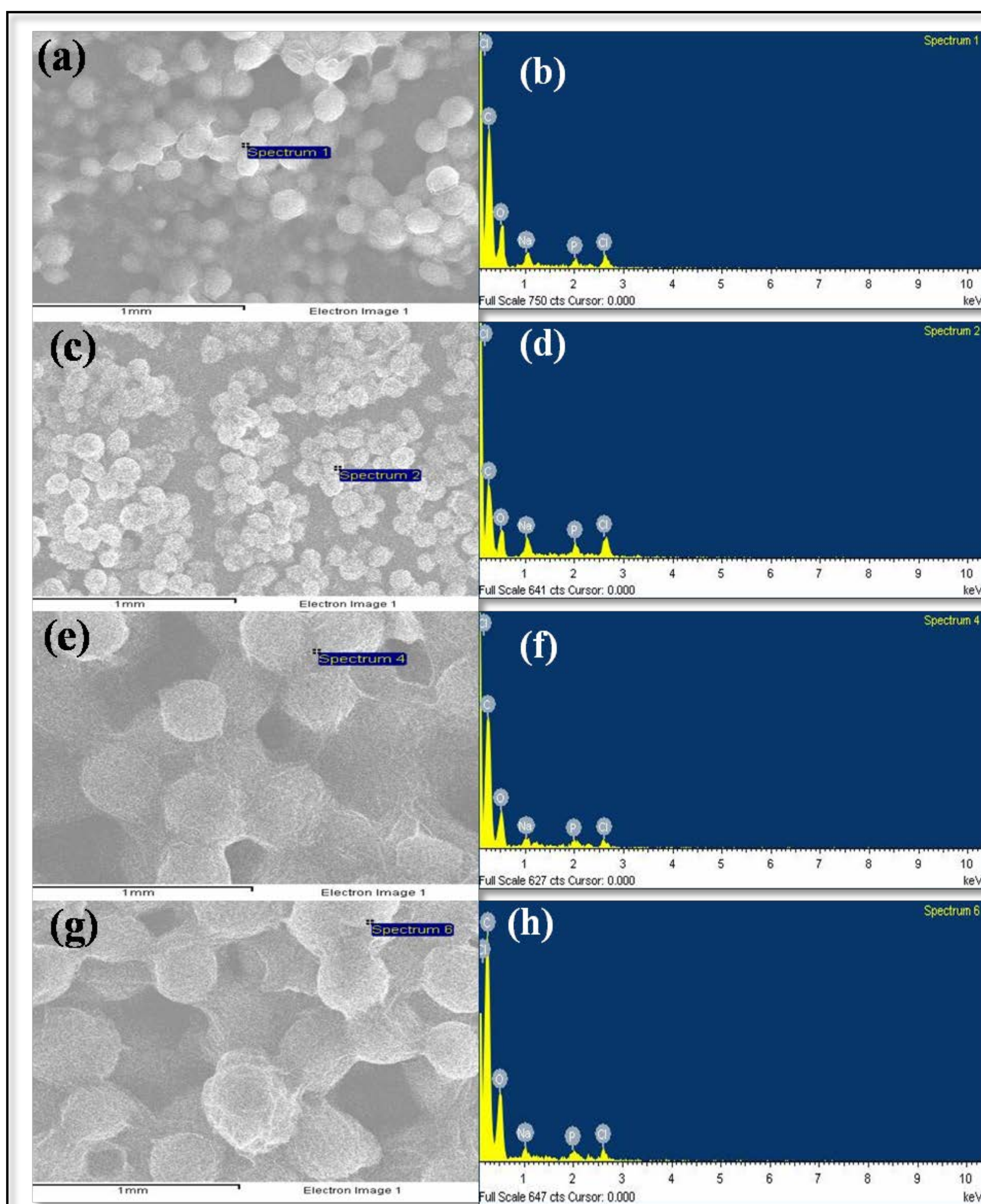

Fig.4.7 SEM-EDS results of *C. vulgaris* cells **(a, b)** Control cells (without NaCl or $NaHCO_3$), **(c, d)** Cells treated with NaCl (200 mM) alone, **(e, f)** Cells supplemented with $NaHCO_3$ (20 mM) and **(g,h)** Cells given combined treatment of NaCl (200 mM) + $NaHCO_3$ (20 mM).

Element	Weight%	Atomic%
C K	64.06	71.35
O K	31.87	26.65
Na K	2.05	1.19
P K	0.73	0.32
Cl K	1.29	0.49
Totals	100.00	

(a)

Element	Weight%	Atomic%
C K	63.63	71.93
O K	28.80	24.44
Na K	3.12	1.84
P K	1.49	0.65
Cl K	2.96	1.14
Totals	100.00	

(b)

Element	Weight%	Atomic%
C K	68.11	74.70
O K	29.17	24.02
Na K	1.15	0.66
P K	0.67	0.29
Cl K	0.90	0.33
Totals	100.00	

(c)

Element	Weight%	Atomic%
C K	63.57	70.45
O K	34.29	28.53
Na K	0.96	0.55
P K	0.43	0.19
Cl K	0.75	0.28
Totals	100.00	

(d)

Fig. 4.8(a) *C. vulgaris* Control cells (without NaCl or $NaHCO_3$), **(b)** Cells treated with NaCl (200 mM) alone **(c)** Cells supplemented with $NaHCO_3$ (20 mM) and **(d)** Cells given combined treatment of NaCl (200 mM) + $NaHCO_3$ (20 mM).

4.3.9 Photochemistry of PS II

Chlorophyll fluorescence induction (OJIP) curve of *C. vulgaris* cells was recorded in response to NaCl stress (0-400 mM), under bicarbonate supplemented and deprived conditions (Table: 4.1). The results showed a concentration-dependent decrease in the overall photosynthetic performance (PIabs) and photosynthetic yield (Fv/Fm). There was little effect of exogenously added bicarbonate on the toxic effect of NaCl. The slope of rising curve Mo, proportional to the rate of QA reduction and ABS/RC, an indicator of absorption of photoenergy per reaction centre exhibited an increasing

trend with a rise in the NaCl concentrations (0-400 mM). However, there was very little effect of bicarbonate on the NaCl-induced increase in Mo values, while ABS/RC values were further significantly increased after the addition of bicarbonate. An increase in Mo value suggested that transfer of an electron from RC to QA was stimulated in the presence of NaCl, perhaps due to ionic support of Na^+ and Cl^- ions on the charge separation property of PS II. The salinity-induced increase in the ABS/RC values, irrespective of bicarbonate concentration, could be interpreted in terms of the reduced number of active PS II reaction centres. The overall photosynthetic performance (PIabs) is considered to be a good photosynthetic parameter for monitoring and evaluating the effect of abiotic stresses (Van Heerden et al., 2007). Our observation on PIabs revealed an overall decline in the photosynthetic performance (PIabs) with an increasing concentration of NaCl (0-400 mM). Similar to PIabs, the relative fluorescence decrease (RFD) (Fig.4.9) helps in fast analysis of overall photosynthetic activity under stress conditions (Rohacek, 2002). The RFD value in NaCl treated *C. vulgaris* cells increased with an increase in the concentration of NaCl up to 200 mM. A further increase in the NaCl concentrations from 300 to 400 mM, irrespective of bicarbonate concentration, caused a gradual decline in the RFD value. However, the addition of bicarbonate at lower concentrations of NaCl (0-200 mM) exhibited a general increase in the RFD values. The non-photochemical quenching (NPQ) value depicts the dissipation of excess excitation energy of PS II reaction centre in the form of heat. In the case of increasing inactivation of PS II reaction centres, the value of NPQ is increased. Our results on NPQ showed an initial increase in the value with increasing concentrations of NaCl up to 200 mM NaCl, followed by a declining pattern with the rise in the NaCl concentrations (300-500 mM). The addition of bicarbonate (20 mM) to the algal cell culture resulted in further

reduction in the NPQ values throughout the concentration range of NaCl (0-400 mM). The photosynthetic parameter qE, an important component of NPQ, exhibits a decrease in qE value due to the build-up proton gradient (ΔpH) across the thylakoid membrane (Szabo et al., 2005). The present results on qE showed NaCl induced increase in the qE value throughout the concentration range of NaCl (0-400 mM). The addition of bicarbonate could slightly increase the qE values when compared with their corresponding values in the presence of NaCl alone. These results together suggested that NaCl-induced built-up of proton gradient might be responsible for the excessive reduction of plastoquinone (PQ) pool. This interpretation is corroborated by an increase in NPQ and qE values. However, the addition of bicarbonate further accentuated the value of NPQ and qE.

Table: 4.1. Salinity (0-400 mM) dependent changes in the photosynthetic parameters (Fv/Fm, PIabs, NPQ, and qE) in *C. vulgaris* cells in the absence and presence of 20 mM sodium bicarbonate. All the values are in means ± SD. ANOVA significant at the level of ($p \leq 0.05$) according to the one-way ANOVA test. Identical superscripts denote no significant difference between means in the column according to DMRT ($p \leq 0.05$).

	Without bicarbonate				**With bicarbonate**			
NaCl (mM)	*Fv/Fm*	*PIabs*	*NPQ*	*qE*	*Fv/Fm*	*PIabs*	*NPQ*	*qE*
Control	0.786± 0.004[d]	27.31 ± 0.27[f]	0.36 ± 0.02[b]	0.466 ± 0.025[a]	0.779 ± 0.005[b]	33.24± 0.07[f]	0.53 ± 0.036[c]	0.62 ± 0.025[a]
50	0.740 ± 0.30[c]	23.35 ± 0.43[e]	0.41± 0.02[c]	0.545± 0.022[b]	0.679 ± 0.25[b]	29.59± 0.32[e]	0.586± 0.025[d]	0.69 ± 0.02[b]
100	0.756± 0.005[c]	16.4 ± 0.40[d]	0.45 ± 0.15[d]	0.638 ± 0.019[c]	0.826± 0.005[b]	24.62 ± 0.09[d]	0.50 ± 0.025[c]	0.698 ± 0.02[b]
200	0.736± 0.009[c]	12.3 ± 0.19[c]	0.53 ± 0.025[e]	0.722 ± 0.035[d]	0.823 ± 0.001[b]	18.27± 0.097[c]	0.44± 0.032[b]	0.712 ± 0.005[c]
300	0.680 ± 0.005[b]	9.19 ±0.38[b]	0.43 ± 0.025[c]	0.735± 0.002[d]	0.756 ± 0.005[b]	12.21± 0.20[b]	0.45± 0.02[b]	0.728± 0.004[c]
400	0.407 ± 0.011[a]	2.31 ± 0.18[a]	0.17 ± 0.02[a]	0.751 ± 0.004[d]	0.77 ± 0.004[a]	7.89 ± 0.30[a]	0.25 ± 0.025[a]	0.74± 0.015[c]

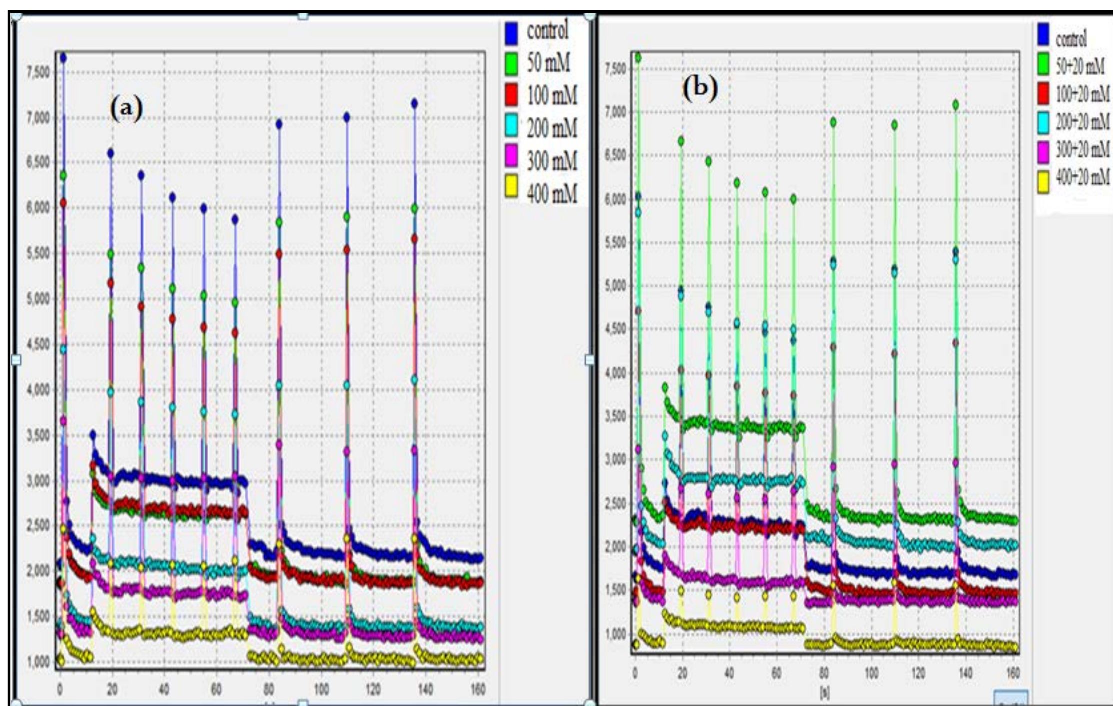

Fig.4.9. Non- photochemical quenching in C. *vulgaris* cells treated with different doses for NaCl in **(a)** absence and **(b)** presence of 20 mM sodium bicarbonate.

4.4 Discussion

Various stress conditions have been used as an eco-friendly strategy to achieve higher lipid and biofuel production (Sharma et al., 2012; Fan et al., 2014). Recently, salinity stress has also been used to induce an increase in the lipid synthesis in microalgae (Pandit et al., 2017; Liang et al., 2013). It has been observed that salinity stress influences the activity of some of the metabolic processes including photosynthesis (Romaneko, 2017), and results in reduced growth and biomass. It would be highly beneficial for bioenergy technology if we can manipulate the nutritional conditions and biomass production. The present study is an attempt to study the changes in the physiological response of *C. vulgaris* cells against the salinity stress in the presence of sodium bicarbonate as a nutritional supplement. Earlier workers have also reported that the addition of sodium bicarbonate stimulates the growth and biomass of various

microalgae (White et al., 2015). However, there is no general agreement on the exact role of bicarbonate in the regulation of the cell constituents and photochemistry of PS II, particularly under the salinity stress (Lu and Vonshak, 2002).

The present findings showed that overall growth and protein content in the *C. vulgaris* gradually declined with an increasing concentration of NaCl (0-400 mM). Our study was in resonance with Peng et al., (2014), which reported higher biomass in diatom *Phaeodactylum tricornutum* when grown under bicarbonate supply. However, total carbohydrate and TOC content increased up to 100 mM concentrations of NaCl. On the contrary, lipid content in the *C. vulgaris* cells increased with rising concentrations of NaCl up to 200 mM, while the proline content increased throughout the concentration of NaCl (0-400 mM). An initial increase in the level of carbohydrate, TOC, and proline in the presence of NaCl might be due to the osmotic requirement of the microalga (Fatma et al., 2007; Shetty et al., 2019). They suggested that proline and specific sugars are important osmolytes under salinity stress and the genes responsible for the synthesis of proline and carbohydrate are upregulated under the stress condition (Hayat et al., 2012). Further results suggested that an optimum concentration of NaCl was necessary for the synthesis of carbohydrates and lipids (Chokshi et al., 2015). A higher concentration of NaCl (200-400 mM) was stressful and injurious to the cell metabolism as indicated by a rising level of proline. Addition of bicarbonate in the presence of NaCl, not only enhanced the NaCl tolerance of microalga by about 70% as evident from the increase in I-50 concentration of NaCl in the presence of $NaHCO_3$, but it also significantly improved the synthesis of all the cell constituents. Our findings align with the earlier suggestions that salinity tolerance depends upon the tolerance limit of individual strains and nutritional conditions (Ruangsomboon, 2012; Alvensleben et al., 2016). In the present investigation, it has

been shown that cell morphology and accumulation of Cl by the *C. vulgaris* under the salinity stress were altered with the addition of sodium bicarbonate. These results suggested that NaCl induced cell toxicity in the microalga *C. vulgaris* was modulated by sodium bicarbonate by preventing the accumulation of excess Na and Cl elements within the cells. It has been suggested that NaCl toxicity in photoautotrophs is due to the accumulation of Cl ions, but an optimum level of Cl^- ions play important role in the primary photochemistry of PSII and photoautotrophic growth of alga (Demetriou et al., 2007).

Various reports available on salt-induced inhibition of photosynthetic activity suggested that reduced supply of carbon dioxide due to a partial closure of stomata might be the limiting factor for salt-induced inhibition of photosynthesis (Zhang et al., 2010). Therefore, the addition of bicarbonate was expected to ameliorate the salinity stress-induced inhibition of photosynthetic carbon assimilation (Salbitani et al., 2020). To assess the exact role of bicarbonate in the regulation of salinity stress-induced impairment of photosynthetic performance in microalga *C. vulgaris*, the chlorophyll fluorescence induction kinetics (OJIP) was used. Present results showed that overall photosynthetic performance (PIabs) and photosynthetic yield (Fv/Fm) (Strasser, 2004) decreased with increasing salinity. There was a little or negligible effect of bicarbonate on the NaCl-induced changes in the primary photochemistry of PS II as evident from the results on Fv/Fm and PIabs. The measurement of PIabs reflects the functionality of both the photosystems and gives us quantitative information on the current state of photosynthetic performance under stress conditions (Strasser et al., 2000; 2004). Van Heerden et al., (2007) had also observed a very good positive correlation between CO_2 assimilation capacity and PIabs values under water stress. Therefore, it was inferred that the overall photosynthetic performance of *C. vulgaris*

cells was not influenced by the presence of $NaHCO_3$. However, Mo- slope of the induction curve, proportional to the rate of QA reduction, and ABS/RC, an indicator of absorption of energy per reaction centre (Kalaji, 2012), exhibited an increasing trend with rising concentrations of NaCl (0-400 mM). The NaCl-induced increase in Mo values indicated a faster rate of electron transfer from PSII reaction centre to primary acceptor QA, perhaps due to NaCl dependent ionic support for charge separation in the photosystem II (Kana and Govindjee, 2016). But NaCl induced increase in the ABS/RC could be due to a decline in the number of active PS II reaction centres (Lu and Vonshak, 2002; Zhang et al*.,* 2010). Earlier reports also suggested that the decrease in PSII activity under the extreme salt stress condition was due to inhibition of repair of PSII and reduced turnover of D1 protein (Allakhverdiev et al*.*, 2002).

The non-photochemical quenching (NPQ) value depicts the dissipation of excess excitation energy of PS II reaction centre in the form of heat (Ware et al*.,* 2015) and RFD value is an important parameter of fast analysis of photosynthetic activity under stress conditions. Both RFD and NPQ values in *C. vulgaris* cells increased up to 200 mM concentration of NaCl and showed a decrease in their values at higher concentrations of NaCl. However, the addition of bicarbonate exhibited a general increase in the RFD values and a decrease in NPQ value throughout the concentration range of NaCl. A decrease in the RFD and increase in the NPQ values have been correlated with the down regulation of PSII under the salinity stress (Neelam et al., 2013). However, a decline in the NPQ value at higher concentrations of NaCl could be due to salinity-induced inactivation of the light-harvesting complex of PSII reaction centre (Stroch et al*.,* 2004; Szabo et al*.*, 2005). Amongst the three major components of NPQ, the qE is triggered by the proton gradient (ΔpH) across the

membrane during photosynthetic electron transport (Hill et al., 2005). The results on qE showed an increasing trend with a rising concentration of NaCl (0-400 mM). The addition of bicarbonate could further increase the qE values when compared with the corresponding values of qE at each concentration of NaCl. A positive increase in qE due to bicarbonate could be due to its ionic role at the level of charge separation in the PS II reaction centre as suggested by Klimov and Baranov, (2001). A contrasting result on NPQ and qE could be interpreted in terms of the cumulative effect of several other factors regulating the NPQ (Logan et al., 2007).

In the present study, it can be concluded that the addition of sodium bicarbonate to the growth medium (BG-11) significantly enhanced biomass production, and 20 mM is the best-suited dose of bicarbonate for the growth and synthesis of biochemical compounds in *C. vulgaris*. The microalga *C. vulgaris*, particularly at a lower level of salinity, undergoes various metabolic adjustments by altering the synthesis of different cell constituents (protein, carbohydrate, lipid, and proline). The antagonistic role of bicarbonate against the salinity stress was observed in terms of improved metabolic functions and reduced accumulation of Na and Cl in the cells. At higher salinity, the overall photosynthetic performance of *C. vulgaris* cells was severely impaired, which could not be relieved by the addition of $NaHCO_3$. A corollary of the results on the antagonistic role of $NaHCO_3$ against the salinity stress suggested that the effect of bicarbonate, during the salinity stress, was more pronounced in terms of its ameliorating effect on intracellular Na and Cl accumulation, which probably mediates the improved synthesis of cell constituents and charge separation in the PS II reaction centre as evident from the increase in NPQ and qE values.

Chapter V

Effect of pH Conditions on Photosynthetic Efficiency Andmacromolecules Of Microalgae Chlorella vulgaris

Effect of pH conditions on photosynthetic efficiency and macromolecules of microalgae *Chlorella vulgaris*

5.1 Introduction

Microalgae are considered to be a very promising resource for biofuel production as they produce many bioactive compounds. The algal biomass is a potentially useful resource for many bioactive products used in the pharmaceutical and cosmetic industries (Bhattacharjee, 2016). The major advantage associated with the microalgae, as compared to plants, is their metabolic plasticity which opens the possibility of modifying their biochemical pathways and cellular composition by manipulating the culture conditions (Aslan and Kapdan, 2006). In microalgal biotechnology, the production of algal biomass is limited due to various nutritional and environmental conditions such as salinity (Bartley et al., 2014), temperature (Van Wagenen et al., 2012), and pH (Moheimani and Borowitzka, 2011). It has also been demonstrated that environmental conditions such as pH, light, and temperature have been used to manipulate the cultivation and biomass production in algae (Richmond and Becker 1986). Various studies have demonstrated that variability in pH conditions of natural habitats is an important factor which drastically influences the growth, photosynthesis as well as solubility and availability of nutrients (Morris et al., 1974). Notably, the pH of the surrounding medium is a crucial factor in determining the relative proportion of carbonaceous species in water (Azov, 1982). Mostly the algal growth occurs around neutral pH; any change in the optimum pH condition may limit algal growth and photosynthetic activity (Goldman et al., 1982). Reports are available on the pH-dependent growth of green alga *Ulva lactuca* (Christensen, 1990) and red alga *Gracilaria secundata* (Lignell and Pederson, 1989), which revealed that the growth

rate of algae under alkaline pH conditions are strongly influenced by the CO_2/ HCO_3- ratio. It has been suggested that highly alkaline pH condition limits the availability of dissolved CO_2 as it is converted into insoluble carbonate salts, and algal affinity to free CO_2 is also lowered due to a drastic decrease in the CO_2 partial pressure (Hofmann and Bischof, 2014). It has also been reported that alkaline pH can reduce the membrane fluidity of cells and increase the accumulation of triglycerides (Yang et al., 2014). However, extremely alkaline pH conditions may favour a decrease in the membrane-associated unsaturated polar lipids due to inhibition of the cell cycle or prolongation of the cell cycle (Juneja et al., 2013).

The selection of the appropriate carbon supply might have a great impact on algal production. The inorganic carbon source plays an important role in intensive microalgal cultures. In natural conditions, carbon supply will either be in the form of bicarbonate salts or CO_2 – enriched air. In a previous study by Goldman et al., (1981) it was observed that pH tolerance limits of the microalgae are governed either by chemical influence on the growth medium or by metabolic effects on the cells. Although pH is a major parameter that determines algal growth, it has been difficult to frame a general rule for optimum pH, as it greatly depends upon the selection of the species and the prevailing growth conditions. Even if we discuss a particular strain or species such as *C. vulgaris*, the literature is somewhat confusing. However, it is safe to say that at pH-controlled cultivation conditions, the microalgae's performance is improved both in terms of growth and macromolecule formation.

Photosynthetic electron transport is highly dependent on a range of internal feedback processes (AEC and NPQ) to ensure the best efficiency, simultaneously preventing potential damage caused by excess excitation energy. NPQ which is a light stimulated

process is closely related to pigments, however, the control mechanisms are species-specific and show broad variability. The study of chemical inhibitors of the electron transport chain is used as a mechanistic way to isolate specific components of the electron transport chain, which improves the understanding of the photosynthetic pathways.

Earlier investigators have reported that external pH conditions directly influence the intracellular pH of the algal cells, leading to several alterations in the metabolic functions including photosynthesis (Kosourov et al., 2003). It has been reported that the growth and physiology of algae are influenced by the carbon speciation and abundance of inorganic carbon, albeit to a different extent (Zhang et al., 2012). However, the presence of excess bicarbonate in the aqueous solutions also raises the alkalinity of the medium (Qi et al., 2019). Therefore, it is critical to have an understanding of the effect of both pH and carbonate alkalinity on the dynamics of photosynthetic activity and cell constituents. Thus, the present investigation was aimed to study the effect of varying pH conditions on algal growth and cell constituents in the *C. vulgaris*. In addition, we have also attempted to see the interactive effect of bicarbonate and pH conditions on the photochemistry of PSII and cell constituents like protein, carbohydrate, total organic carbon and lipid.

5.2 Materials and methods

5.2.1Experimental design

Pure algal inoculums of *C. vulgaris* were used in 500 ml Erlenmeyer flasks containing 200 mL BG-11 media. The flasks were kept in growth chamber for acclimatization for 24 hours. During the experiment, cells of *C.vulgaris* were grown in BG-11 medium adjusted to different pH conditions (pH 6.5-10.5). pH condition of the BG-11 medium

with or without sodium bicarbonate was adjusted by using 0.1 N HCl or 0.1 M NaOH solution every 8 hours under sterile conditions. In this pH range (6.5 to 10.5), the pH-dependent speciation of dissolved inorganic carbon is predominantly in the form of bicarbonate (Mosley et al., 2010). All the experiments were performed in three sets under controlled laboratory conditions. The algal cells were harvested during the stationary phase.

5.2.2 Determination of growth rate and protein content

The growth of microalgal cell suspension was monitored in terms of absorbance at 680 nm, using a double beam UV VIS spectrophotometer (model UV 1601, Shimadzu, Japan). The specific growth rate (μ d-1) was calculated as described in the literature (Yu et al., 2017). The protein content of the cell suspension was estimated by following the method of Lowry et al., (1951), using bovine serum albumin (BSA) as standard as described in the chapter –III of materials and methods.

5.2.3 Measurement of cell constituents

The cells of *C. vulgaris* growing under different pH (6.5-10.5) conditions were routinely harvested by centrifugation (5000 rpm, 5 min). The washed pellet was used for the measurement of carbohydrate, lipid, and TOC content. The lipid content was measured by using sulfo-phosphovanillin (SPV) method as described by (Chabrol et al., 1973). The absorbance of the resulting chromophore was measured at 530 nm. Total carbohydrate content was quantified by using the phenol-sulphuric acid method as described by Dubois et al., (1956). Quantification was performed using glucose for a standard curve. (TOC) was estimated by the Walkely- Black method as described by

(Grobler et al., 1979). As described in chapter –III of materials and methods. (Page no. 66-71).

5.2.3 Fourier-transform infrared spectroscopy (FTIR) spectroscopy

The sample preparation for FTIR study was done as described in chapter- III of materials and methods. The cell constituents with characteristic IR absorbance were used for semi-quantitative assessment of different macromolecules such as total lipid (1740 cm-1), lipid/carbohydrate (1740/1040 cm-1), and lipid/protein (1740/1650 cm-1) ratio (Dean et al., 2010).

5.2.4 Measurement of chlorophyll fluorescence induction kinetics and non- photochemical quenching

Chlorophyll fluorescence induction kinetics was measured by using Pulse Amplitude Modulated (PAM) (Aquapen- C, AP 110-C, Photon Systems Instrument, Czech Republic) fluorimeter. Described in detail in chapter-III of materials and methods.

5.2.5 Inhibitor study on the induction of chlorophyll fluorescence

To elucidate the mechanism of specific biochemical processes, the use of energy inhibitors, uncouplers, artificial electron acceptors, and donors is the most common technique. The cell suspension of *C. vulgaris* was allowed to get adapted to different pH conditions (pH 6.5-10.5) for 04 hours under both HCO_3- supplemented and deprived conditions. In the present study metabolic inhibitors (obtained from Sigma, USA) like rotenone (0.1 mM), 2,4- dinitrophenol (0.1 mM), Carbonyl cyanide p-chlorophenylcarbazide (0.05 mM), Phenazinemetho-sulphate (0.05 mM) and 0.02 mM of N,N- Dimethyl-4,4-bipyridinium dichloride (Methyl- viologen, MV2+) were added to the cell suspension 30 minutes before the dark incubation of microalgal cell

suspension. The dark incubated cells were used for the measurement of chlorophyll fluorescence induction kinetics by PAM fluorimeter in the absence and presence of these inhibitors.

5.2. 6 Statistical analysis

The experiment was carried out in triplicate and presented as mean±SD.

5.3 Results

5.3.1 Effect of varying pH conditions on protein, carbohydrate, TOC, and lipid

Results on the effect of varying pH conditions (pH 6.5-10.5) on the protein and carbohydrate, content of microalga *C. vulgaris* (Fig.5.1b) showed pH-dependent initial increase in the protein and carbohydrate content of cells (pH 6.5 to 8.5). Maximum protein and carbohydrate content in the microalga was recorded between pH 7.5-8.5. The subsequent increase in the pH from 8.5 to 10.5 exhibited a pH-dependent decline in both the cell constituents. Extremely alkaline pH conditions (9.5-10.5) were found to be inhibitory for protein and carbohydrate synthesis. TOC and lipid content in the *C. vulagris* cells (Fig.5.1a) grown under varying pH conditions (pH 6.5 to 10.5) showed a pH-dependent gradual increase in both the TOC and lipid content with increasing pH. The lowest level of both TOC and lipid contents was recorded at pH 6.5. There was about a two-fold increase in the TOC and lipid content of microalgal cells at pH 10.5 as compared to their respective values at pH 6.5. Earlier reports have also shown that an increase in the alkalinity of medium from pH 8.5 to 9.5 results in a reduction in the growth, membrane transport of nutrients, and other metabolic functions of algae (Smith and Raven, 1979). However, an increase in the TOC and lipid content of microalga with increasing alkalinity of the

medium could be because of changes in the intracellular allocation of carbon as suggested earlier (Norici et al., 2011).

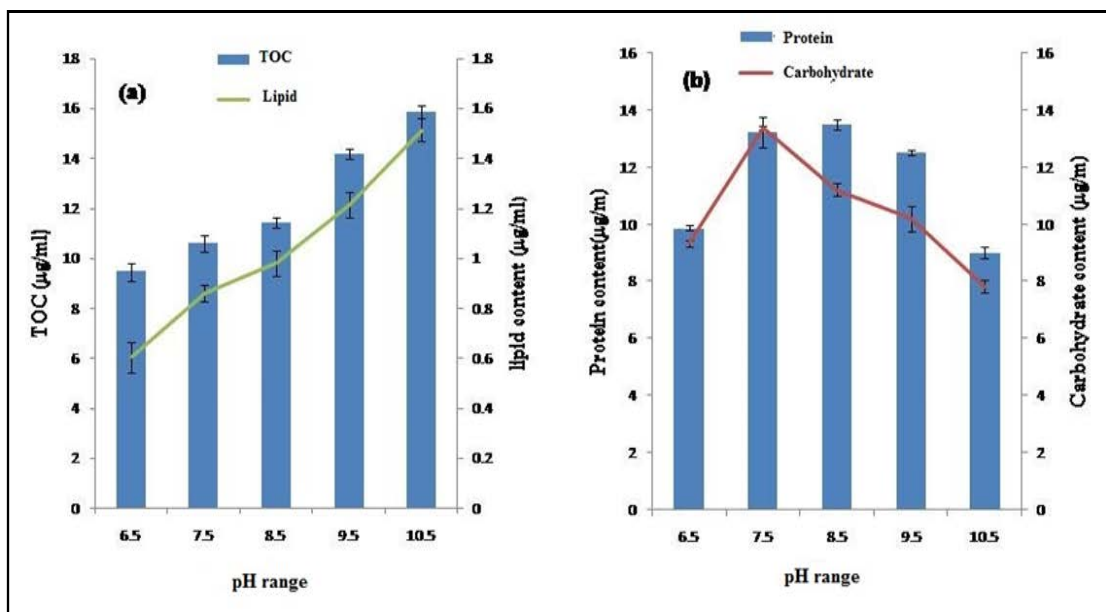

Fig: 5.1(a) TOC (primary axis) and lipid content (secondary axis) of *C. vulgaris* cells grown under different pH conditions (6.5-10.5). **(b)** Protein content (primary axis) and carbohydrate content (secondary axis) of *C. vulgaris* cells grown under different pH conditions (6.5-10.5). Data are the mean of three replicates ± SD.

5.3.2 Effect of pH conditions on the FTIR spectra of cell biomass-

The FTIR spectra of cell biomass (Fig.5.2) grown under different pH conditions (pH 6.5, 8.5, and 10.5) exhibited large variations in the characteristic signature bands of various macromolecules (950-1800 cm-1) (Giordano et al., 2001). The IR spectral region between 3000 cm^{-1} and 2500 cm^{-1}, specifically assigned to -CH_2 stretching of fatty acids and lipids, showed pH-dependent (pH 6.5-10.5) increase in the absorption peaks as well as a shift in the IR absorption peaks at wavenumber 2923 to 2850 cm-1. The IR absorbance at wavenumbers at 1540 cm^{-1} and 1650 cm^{-1}, assigned to N–H stretching of Amide I and C-O bond of Amide II groups of proteins, respectively (Giordano et al., 2001) showed an increase with increasing pH up to pH 8.5. The absorption band at wave number 1740 cm^{-1}, assigned to stretching of C-O bond of

esters of fatty acids (Dean et al., 2010), indicated an overall increase in the total lipid content with increasing pH from 6.5 to 10.5 Emergence of a new peak in the alkaline pH range (pH 9.5-10.5) at wave number 1052 cm^{-1}, associated with the carbohydrates (Pistorius et al., 2009), could be the result of an accumulation of carbohydrates due to their reduced utilization. As indicated in (Table.5.1), an increase in the lipid/ carbohydrate (1740/1040 cm-1) and the lipid/protein (1740/1650 cm-1) ratio revealed a relatively higher amount of lipid content with increasing pH (6.5-10.5). In the present study, pH- dependent increase in the lipid of microalgal cells with increasing pH (6.5-10.5) suggested that enhanced alkalinity of the medium drives preferential storage of carbohydrate and lipid as evident from a general increase in characteristic IR absorption between the wavenumbers 1200-1000 cm^{-1} and 1740 cm^{-1}. So far the actual mechanism of lipid accumulation due to pH stress is not yet fully understood.

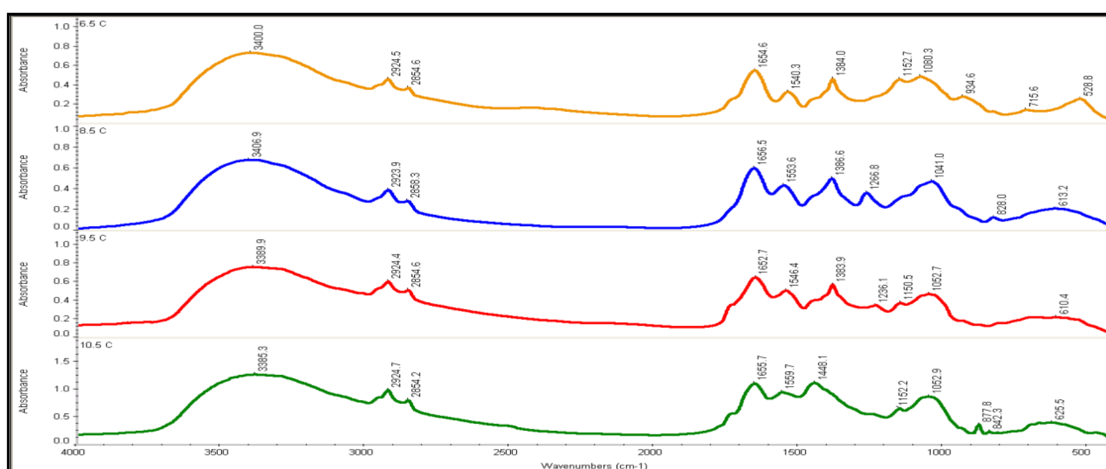

Fig: 5.2 FTIR spectra of *C. vulgaris* cells grown under different pH conditions (pH 6.5, 8.5, 9.5 and 10.5).

Table: 5.1 Total lipid, Lipid/Carbohydrate and Lipid/ Protein (Amide II) ratio derived from FTIR spectra of *C. vulgaris* cells grown under different pH conditions (pH 6.5, 8.5, 9.5, and 10.5)

pH	Total lipid ($1740\ cm^{-1}$)	Lipid/carbohydrate ($1740/1040\ cm^{-1}$)	Lipid/protein ($1740/1650\ cm^{-1}$)
6.5	0.10	0.25	0.20
8.5	0.14	0.24	0.21
9.5	0.21	0.44	0.28
10.5	0.40	0.571	0.36

5.3.3 Effect of pH conditions on the photochemistry of photosystem II

Chlorophyll fluorescence induction kinetic (OJIP) in the dark-adapted *C. vulgaris* cells grown under varying pH conditions (6.5-10.5) showed changes in various photosynthetic parameters viz. (Fv/Fm), Mo, ABS/RC, ETo/RC. Initially, photosynthetic parameters like quantum yield (Fv/Fm), the slope of the induction curve (Mo), the absorbance of energy per reaction centre (ABS/RC), flux of electron transfer per reaction centre (ETo/RC) in *C. vulgaris* cells (Table 5.2) showed pH-dependent initial increase from pH 6.5 to pH 8.5, followed by a declining pattern between pH 9.5 to 10.5. These results suggested that photosynthetic performance in C.*vulgaris* was dependent on the external pH condition of the medium. Further, all the photosynthetic parameters like Fv/Fm, Mo, ETo/RC were slightly reduced due to the addition of bicarbonate under all the pH conditions (6.5-10.5). However, the value of Fo in the OIJIP curve was increased throughout the pH range (6.5-10.5) due to the addition of HCO_3, suggesting the inhibition of PS II reaction centre. Generally, an increase in the Fo value indicates an increase in several inactive PSII reaction centres due to a lesser number of oxidized QA (Masojıdek et al., 2004). Further, an enhanced value of ABS/RC in *C. vulgaris* cells under bicarbonate supplemented conditions also

indicated a reduced number of active PSII reaction centres as reported earlier (Xiang et al., 2018). These results together suggested that charge separation in the PS II reaction centre is slowed down at alkaline pH beyond pH 8.5, which is further accentuated due to the addition of bicarbonate. So far, there is no general agreement on the reasons behind the adverse effect of bicarbonate on the photosynthetic process. The present findings (Fig.5.3b) on relative fluorescence decline (Rfd) value, an indicator of overall photosynthetic carbon assimilation (Lichtenthaler and Rinderle, 1988), showed a marginal decline in response to rising pH condition (pH 7.5 to 10.5). The highest Rfd was recorded at pH 7.5. The addition of bicarbonate under different pH conditions showed little increase in the Rfd values with an optima at pH 9.5, perhaps due to the ability of bicarbonate to overcomeCO_2 deficiency at alkaline pH. The non-photochemical quenching (NPQ) of chlorophyll fluorescence provides valuable information to interpret the effect of environmental stresses on the photochemistry of the PSII reaction centre (Perkins et al., 2006). The present study showed that the NPQ value (Fig.5.3a) in *C. vulgaris* cells increased with increasing pH (6.5-10.5). However, NPQ value was further enhanced due to the addition of HCO_3-, particularly between pH 8.5-10.5, when compared with their corresponding values obtained in the absence of bicarbonate. Amongst the three components of NPQ, the qE component is found to have a major influence on the chlorophyll fluorescence signal (Logan et al., 2007). The qE is triggered by the build-up of ΔpH across the membrane during photosynthesis, which ultimately quenches the fluorescence emission (Krause and Weis, 1984). The present results on pH-dependent (6.5-10.5) qE values indicated an increase in the trans-thylakoid proton gradient with rising pH, which was further stimulated by the presence of bicarbonate. A corollary of these results on NPQ and qE values demonstrated a pH-dependent increase in the

proton gradient (high energy state) of the membrane, which was further enhanced due to the presence of HCO_3^-. The proton gradient and charge separation in the PSII reaction centre is tightly coupled, leading to the inactivation of the PSII reaction centre of *C. vulgaris* cells (Li et al., 2018). However, at present, it is difficult to predict the exact mechanism of bicarbonate-dependent rise in the proton gradient (Hohner et al., 2016).

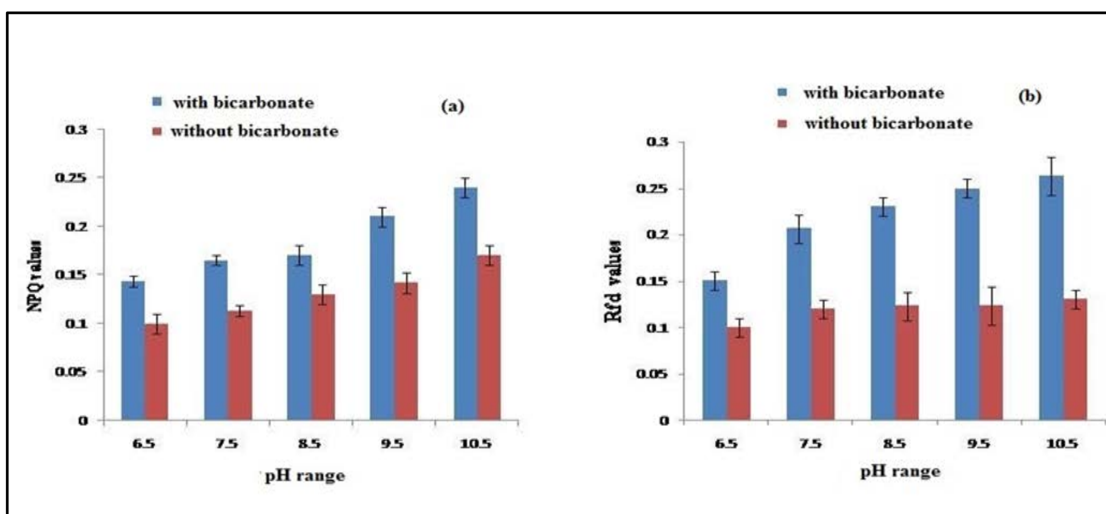

Fig:5.3 (a) Non- photochemical quenching values of *C. vulgaris* cells grown under different pH conditions (6.5-10.5)with bicarbonate (20 mM) and without bicarbonate. **(b)** Relative fluorescence decrease ratio (Rfd) values of *C. vulgaris* cells grown under different pH conditions (6.5-10.5) with bicarbonate (20 mM) and without bicarbonate. Data are the mean of three replicates ± SD.

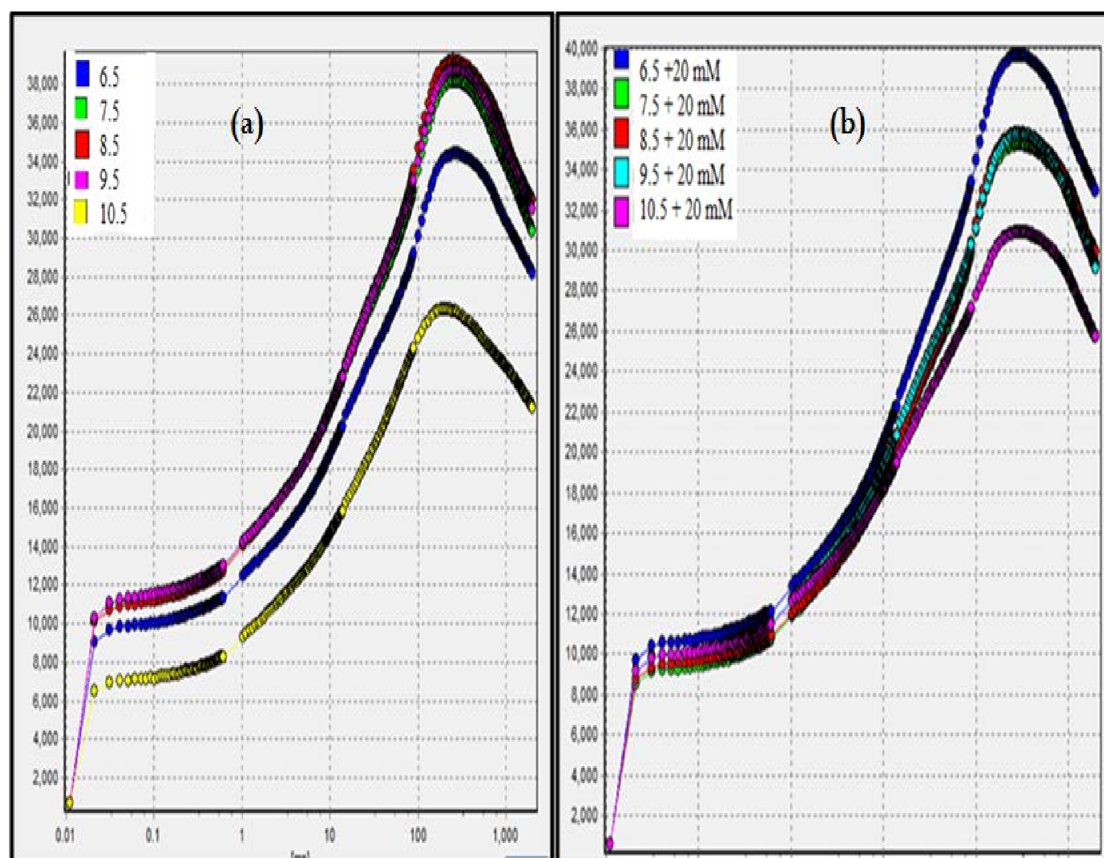

Fig.5.4 OJIP curve of *C. vulgaris* (a) In different pH conditions without bicarbonate (b) In different pH conditions with bicarbonate supplementation (20 mM).

Table: 5.2 Photosynthetic parameters Fv/Fm, Mo, ABS/RC, ETo/RC, and qE values derived from the chlorophyll fluorescence induction kinetics of *C. vulagris* cells grown without or with bicarbonate (20 mM) under different pH conditions (pH 6.5-10.5).

	Without Bicar bonate					With Bicar bonte (20 mM)				
pH	Fv/ Fm	Mo	ABS/ RC	ETo / RC	qE	Fv / Fm	Mo	ABS/ RC	ETo/ RC	qE
6.5	0.688	0.261	1.224	0.582	0.583	0.652	0.255	1.246	0.65	0.660
7.5	0.691	0.261	1.251	0.602	0.622	0.672	0.244	1.268	0.634	0.696
8.5	0.698	0.263	1.253	0.625	0.769	0.687	0.266	1.276	0.619	0.710
9.5	0.697	0.261	1.201	0.616	0.521	0.657	0.265	1.245	0.611	0.653
10.5	0.682	0.258	1.124	0.610	0.477	0.662	0.261	1.152	0.589	0.569

5.3.4 Effect of Metabolic Inhibitors on the photochemistry of PS II

Two ambient pH conditions i.e., acidic (pH 6.5) and alkaline (pH 8.5), were selected to study the effect of inhibitors on the bioenergetics of photosynthetic electron transport system in *C. vulgaris* (Table 5.3). All the photosynthetic parameters were derived from the OJIP curve of dark-adapted *C. vulgaris* cells in the presence and absence of inhibitors. The Fv/Fm, Mo, and ABS/RC derived from OJIP curve are considered to be sensitive parameters for the assessment of the health of photosynthetic machinery (Zivcak et al., 2008). Value of Fv/Fm, Mo, and ABS/RC were higher at slightly alkaline pH (8.5) than at acidic pH (6.5). The addition of protonophore, CCCP, to the cell suspension under both acidic (pH 6.5) and alkaline pH (pH 8.5) resulted in a sharp increase in the values of Fv/Fm, Mo, and ABS/RC. However, the effect of CCCP at alkaline pH (8.5) was less pronounced on these photosynthetic parameters. The results with the protonophore CCCP (Lu et al., 2020) suggested that the photochemistry of PSII reaction centre was tightly regulated by the proton gradient of thylakoid membrane, particularly under the acidic pH 6.5. The overall photosynthetic performance (PIabs and ETo/RC values) was significantly improved in the presence of protonophore throughout the pH range, indicating an important role of the proton gradient of the membrane in the regulation of transport. The addition of phenazine methosulphate (PMS), an electron donor to PS I and an inhibitor of a proton gradient across the membrane (Slooten and Branders, 1979), significantly reduced the values of Fv/Fm, ETo/RC, PIabs under both the acidic and alkaline pH conditions. It has been reported that the addition of electron donor PMS stimulates the cyclic electron flow and results in blocking of the electron flow from PS II to PSI. The resulting reduction of electron carriers of PS II leads to an increase

in the chlorophyll fluorescence as evident from higher ABS/RC values and reduced values of ETo/RC and PIabs. However, the inhibitory effect of PMS under alkaline pH conditions (pH 8.5) was less pronounced than that observed under acidic conditions. The addition of rotenone, an inhibitor of PQ reduction (Mus et al., 2005), showed a significant reduction in the photosynthetic (ETo/RC, and PIabs) parameters under both the acidic and alkaline pH conditions. However, the value of Mo and ABS/RC were enhanced by rotenone at acidic pH. The corresponding photosynthetic parameters at alkaline pH (8.5) exhibited a little effect of rotenone. The 2,4-Dinitrophenol (DNP)- a known inhibitor of oxidative phosphorylation and ATP synthesis (Edwards and Bovell, 1996), exhibited a more pronounced inhibitory effect on the Fv/Fm, Mo, ETo/RC, and PIab sunder acidic pH (6.5) condition when compared with its corresponding effect under alkaline condition. Since DNP is an uncoupler of ATP synthesis, it might be inhibiting the metabolic consumption of proton gradient, leading to over reduction of electron transfer components. Thus, an overall decrease in the electron transport with rising pH might be the main cause of a decline in the photosynthetic performance and an increase in the chlorophyll fluorescence emission. It has been suggested that the MV oxidizes the primary acceptor (ferrodoxin) of PSI and stimulates the flux of electrons from the PSII reaction centre (David et al., 2011). The addition of MV under both acidic and alkaline pH (6.5 & 8.5) conditions exhibited a significant increase in Mo and ABS/RC values as compared to control (without MV). However, a decline in the ETo/RC and PIabs values indicated a decrease in the overall photosynthetic performance of the *C. vulagris* under both pH conditions. These results suggested that MV induced stimulated rate of energy transfer between PS II and PS I might be responsible for the

reduced buildup of the proton gradient and faster electron transport (Falkowski and Raven, 2007).

Table: 5.3 Effect of different energy uncouplers, electron donor/ acceptor, and inhibitors on the photosynthetic parameters derived from chlorophyll fluorescence induction kinetics (OJIP) in *C. vulgaris* cells grown under both acidic (pH 6.5) and alkaline (pH 8.5) conditions.

			pH 6.5					pH 8.5		
Treatment	Fv/Fm	Mo	ABS /RC	PIabs	ETo /RC	Fv/ Fm	Mo	ABS /RC	PIabs	ETo /RC
Control	1.616	0.235	2.42	0.747	0.747	1.627	0.222	2.114	0.792	0.593
CCCP	1.140	0.756	9.749	0.009	0.009	1.228	0.313	3.490	0.069	0.334
DNP	1.512	0.253	2.594	0.487	0.487	1.584	0.187	1.812	0.887	0.490
Rotenone	1.319	0.319	3.317	0.146	0.146	1.611	0.262	0.705	2.861	0.205
PMS	0.981	0.800	49.9	0.001	0. 002	1.538	0.157	1.946	0.920	0.524
Methyl viologen	1.132	0.422	6.897	0.017	0.382	1.424	0.388	3.365	0.20	0.614

5.4 Discussion

It is a general notion that the optimum pH varies among different algal species. Earlier workers have also reported that change in the external pH condition influences the intracellular pH and associated metabolic functions, which influences the accumulation of several macromolecules including amino acids (Taraldsvik et al., 2000, Juneja et al., 2013). Massyuk and Yurchenko, (1962) found in their study that *D. salina* withstands a wide range of pH values between 5.5 and 10. On the other hand, McLachlan, (1964) reported that the optimum pH for growth of *D. tertiolecta* was nearly 6. Similarly, Zhang et al., (1997) mentioned that *Chlorococcum* species can grow to tolerate a pH ranges between 5.5-9, but the optimum pH was about 8. Liu

and Lee, (2000) observed that pH affected both the composition and quality of carotenoids in *Chlorococcum sp.*

But few researchers have reported that extreme alkaline pH induces lipid accumulation by reducing the cell cycle (Vadlamani et al., 2017) as a stressor, which might have resulted in a diversion of intracellular carbon pool from other metabolic pathways towards storage of high energy compounds such as lipid (Gupta et al., 2019). According to Karatay et al., (2011), three algal species grown in BG 11 medium exhibited maximum lipid production within the pH range of 7-9. As per the study of Rodolfi et al., (2009) better growth of *C.vulgaris* at pH 6.5 and 7.0, and accumulation of lipid at pH 7 and 8.5, was observed. So, pH 7.0 was suggested to be the optimal pH for growth and lipid accumulation in *C.vulgaris*. A decline in the content of both protein and carbohydrate was observed in shifting the pH value towards the alkaline or acidic side. In a study conducted on *Chlorella zofingiensis* maximum astaxanthin accumulation was observed at pH 5-6.5, whereas no carotenoids were seen at pH 7-8 (Liu and Lee, 2000).

At alkaline pH autospore release is inhibited as a result of increased cell wall flexibility of mother cells, thus the time for cell cycle completion gets increased which leads to higher lipid accumulation. Mello and Chemburkar (2018) in their research found that the microalgal culture exhibited maximum production of biomass and lipid at a pH of 7 but it was not much affected by an increase in the pH of the medium. For the large-scale production of microalgal biofuel, pH and temperature are the limiting factors.

The present findings on microalga *C. vulgaris* showed pH-dependent increase in the level of protein and carbohydrate content between pH 7.5-8.5. The (TOC) and lipid

content exhibited a gradual increase with increasing pH (6.5 to 10.5). These observations were supported by the data on FTIR analysis of cell constituents. The photosynthetic parameters like Fv/Fm, Mo, ABS/RC, and ETo/RC showed optimum values at pH 8.5. These parameters were negatively influenced by the addition of HCO_3- throughout the pH range (6.5-10.5). The other parameters like ABS/RC, NPQ, and qE values significantly increased with rising pH; but the addition of HCO_3- could further stimulate the ABS/RC, NPQ, and qE values. It has been reported that an increase in pH of the medium affects the algal photosynthesis by altering the availability of free CO_2 in the outdoor microalgal cultures (Singh and Singh, 2014). Earlier workers have also demonstrated that the capacity of green microalgae to utilize the bicarbonate declines with increasing pH conditions (Axelsonm et al., 1995). Use of energy uncouplers like DNP, CCCP, and electron acceptors/donors (methyl viologen and phenazine methosulphate) exhibited reversal of pH-induced increase in the Fv/Fm, Mo, ETo/RC, and PIabs. It was inferred that both higher pH as well as the addition of HCO_3- negatively influenced the photosynthetic performance of *C. vulgaris* cells, which was mediated by the proton gradient across the membrane. Regulation of photosynthetic electron transport by higher pH as well as the addition of HCO_3 could be reversed by the protonophores (CCCP), DNP, and electron acceptor MV, suggesting that effect of rising pH on the photosynthetic electron transport and charge separation in PS II reaction centre was due to build-up of a proton gradient across the membrane.

Chapter VI
Effect of excess CO_2 and high light intensity on microalga Chlorella vulgaris

Effect of excess CO_2 and high light intensity on microalga *Chlorella vulgaris*

6.1 Introduction

Global concern on climate change and the unceasing rise of CO_2 concentrations has put forward the strategy of microalgal-based CO_2 sequestration to reduce the atmospheric level of carbon dioxide. Microalgae have recently gained enormous attention from scientists' world over due to their application as a valuable feedstock for biodiesel. The biomass production of algae per unit of land is much higher than the terrestrial plants (Sayre, 2010) Thus, the selection of a suitable algal species can be beneficial for both mitigation of CO_2 as well as biofuel production due to its high photosynthetic conversion efficiency, rapid biomass production and ability to thrive under the adverse conditions. In outdoor mass cultures, optimization of light conditions and carbon supply, usually either in the form of CO_2-enriched air or as bicarbonate salts, would be essential parameters to enhance biomass productivity (Show et al., 2017). By using the stored carbon, microalgae can provide neutral storage lipids such as triacylglycerides (TAGs) and polar structural lipids such as phospholipids, glycolipids, and sterols (Hu et al., 2008). The CO_2 gas can freely diffuse in and out of the cell, exchange between extracellular and intracellular CO_2 is somewhat more rapid at alkaline pH and constant CO_2 pressure. There is reverse conversion ($CO_2 \rightarrow$ (H_2CO_3) $\rightarrow HCO_3^-$) of trapped CO_2 within the cells in the form of HCO_3^-.

Lipids and carbohydrates are the preferred storage products under various stress conditions because they are highly reduced forms of carbon, used by the cells during

adverse conditions for their survival (He et al., 2015). Numerous factors such as nitrogen deprivation, high salinity, and carbon source concentration have been used to enhance the lipid production in microalgae, especially neutral lipids/triglycerides (TAGs) -a precursor of biodiesel production (Sun et al., 2014). Light intensity as an environmental factor not only influences the algal photosynthesis but also alters the cell composition and causes energy imbalance in the metabolic pathway to trigger the TAG accumulation (Klok et al., 2013; He et al., 2015). Several studies suggested that photosynthetic carbon flow changes from carbohydrates to lipids under stress conditions (Pancha et al., 2014). The energy demand for the biosynthesis of lipids is much higher than that required for the carbohydrates. Therefore, lipids serve as effective energy and carbon storage compounds providing a larger sink for excess energy, especially under the high light intensity (Li et al., 2011). However, the exact mechanism of light intensity directing microalgae metabolism toward an increase in lipid/TAG production under high light stress is not yet fully explored. Most of the recent studies on high light intensity induced accumulation of lipid have shown that high intensity supported lipid accumulation is simultaneously accompanied by low biomass productivity (Breuer et al., 2013). Earlier findings on lipid accumulation under high light stress showed that photosynthetic carbon partitioning, photochemistry of photosystems, and levels of ROS are good indicators of stress-induced lipid synthesis (Gwak et al., 2014). There is still no consensus on how an abundance of carbon dioxide interacts with high light intensity at the level of compositional alteration and photosynthetic performance in phototrophs (Hymus et al., 2001). The objective of this article is to provide useful knowledge and information concerning biochemistry, bioprocess engineering, and commercial applications to assist in the viable technology development for biofuel generation, particularly when

both light intensity and CO_2 are present in excess. The study aims to investigate the effect of light intensity on growth, physiology, and synthesis of cell constituents in the oleaginous microalga *C. vulgaris* in the presence of excess CO_2 (more than CO_2 in air). In the present study, the combined role of CO_2 and light intensity in the regulation of photochemistry of PS II has been elucidated by studying the photosynthetic performance parameters of microalga under varying light intensities.

6.2 Materials and methods

6.2.1 Experimental design

The culture of microalga *C. vulgaris* was grown routinely in a photobioreactor with provision for gas inlet and outlet and the same set up was placed in a culture room at 25 ± 2^0 C. control conditions (i.e 40 LPM atmospheric air), in presence of 5% CO_2 (3 LPM pure CO_2+ 40 LPM atmospheric air) and presence of 10% CO_2 (5 LPM pure CO_2 + 50 LPM atmospheric air). The design of the algal photobioreactors is very important to achieve high biomass productivity. In the present experiment, we opted for a closed algal photobioreactor after an intensive review of literature, as it has less risk of contamination by other microorganisms. The mixing of algal culture was ensured by bubbling the ambient air along with CO_2. The light was provided to culture by using LED (Philips, India), providing different light intensities (50-300 µmol photons/$m^{2/}$s) on the surface of the vessel, as and when required. The cells were grown in BG-11 medium with continuous bubbling by using different concentrations of CO_2 viz. ambient air (0.04%), 5%, and 10% CO_2. Among the three selected treatments, 5% CO_2 was found to be the best growth supporting concentration of CO_2. The pH of the medium was adjusted every day by using a 1.0 M solution of NaOH. The growth of microalga was determined by measuring the turbidity of the

cell suspension at 680 nm in a double beam UV-visible spectrophotometer (Shimadzu, UV 1601, Japan). Other biochemical parameters like protein, carbohydrate, TOC and chlorophyll pigments were also analyzed in all sets of experiments.

6.2.2 Determination of cell constituents

The protein content of the cell suspension was estimated by following the method of Lowery et al., (1951), modified by Herbert et al., (1971), using bovine serum albumin (BSA) as standard. The total carbohydrate content was determined using the phenol sulfuric acid method as described by Dubois et al., (1956). Lipid analysis was performed gravimetrically based on a method given by Bligh and Dyer, (1959). (Page no.66-71).

6.2.3 Pigment extraction and quantification

The cells pellet was extracted by using acetone (80%, v/v), and the extract was measured by using a double beam UV-visible spectrophotometer (UV-A 1601 Shimadzu, Japan) as described by Lichtenthanler et al., (2001).

6.2.4 FTIR analysis of biochemical constituents

The cell biomass of *C. vulgaris* was grown under low (50 µmol photons/$m^{2/}$s) and high light (300 µmol photons/$m^{2/}$s) conditions with a continuous supply of air and 5% CO_2 were harvested and air-dried. Samples for FTIR analysis were prepared as described in chapter- III of materials and methods. (Page no. 74).

6.2.5 Measurement of chlorophyll fluorescence induction kinetics

Chlorophyll fluorescence induction kinetics and non-photochemical quenching (NPQ) in dark-adapted cells was measured by Pulse Amplitude Modulated (PAM) fluorimeter (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic). As described by Strasser et al., (2000) and Lichtenthaler et al., (2005). Measurement of Non-photochemical quenching (NPQ) was measured by using the protocol described by Schreiber, (2004).

6.2.6 Statistical Analysis

All the experiments were carried out in triplicates and the results were expressed as mean values and standard deviation (SD) was applied using MS excel.

6.3. Results

6.3.1 Measurement of macromolecules of *C.vulgaris* treated with varying CO_2 doses.

To investigate the effect of varying concentrations of CO_2 on *C. vulgaris* was subjected to different concentrations of CO_2 (ambient air, 5% CO_2, and 10% CO_2) for 7 days. The highest specific growth rate (Fig: 6.1 a) was found at 5% CO_2 concentration, at 10 % CO_2 supply a decline in specific growth rate was observed. A similar trend was noticed in the case of chlorophyll content in the *C. vulgaris*, in comparison to control highest chlorophyll content (Fig: 6.1 b) was observed under 5% CO_2 dose, at 10 % CO_2 supply a decline was noticed, whereas the least amount of

chlorophyll content was found in cultures grown in ambient air (control). The lipid content (Fig: 6.1a) improved upon increasing the CO_2 supply upto 5 %, whereas 10 % CO_2 was found to inhibit the lipid synthesis in the microalga. A sharp increase in the protein content (Fig: 6.1 b) was observed on under 5 % CO_2 in comparison to control, at 10 % CO_2 supply a reduction in protein content was recorded.

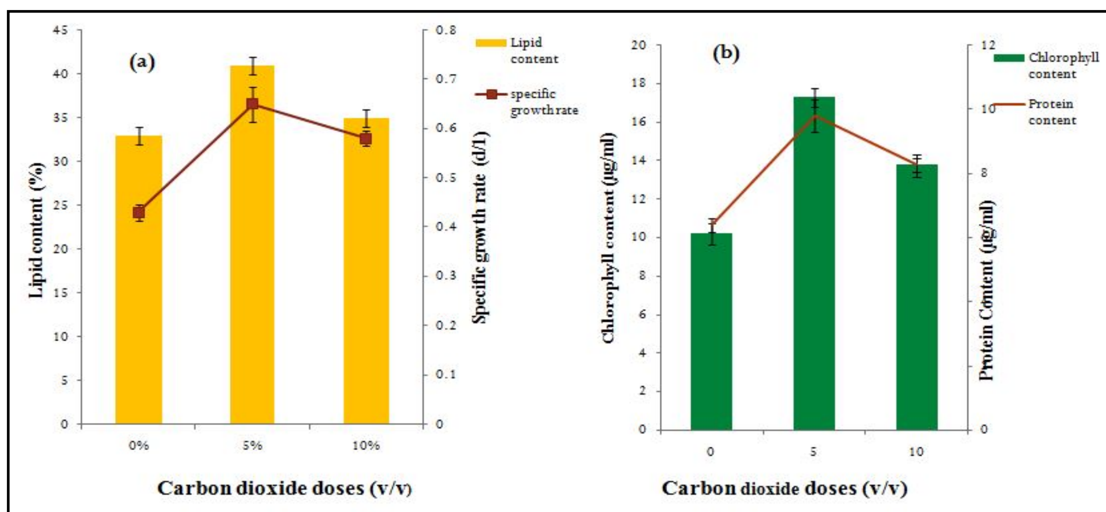

Fig.6.1(a) Effect of different CO_2 doses on lipid content (primary axis), specific growth rate (secondary axis). **(b)** Chlorophyll content (primary axis), protein content (secondary axis) in *C. vulgaris***.** Data are the mean of three replicates ± SD.

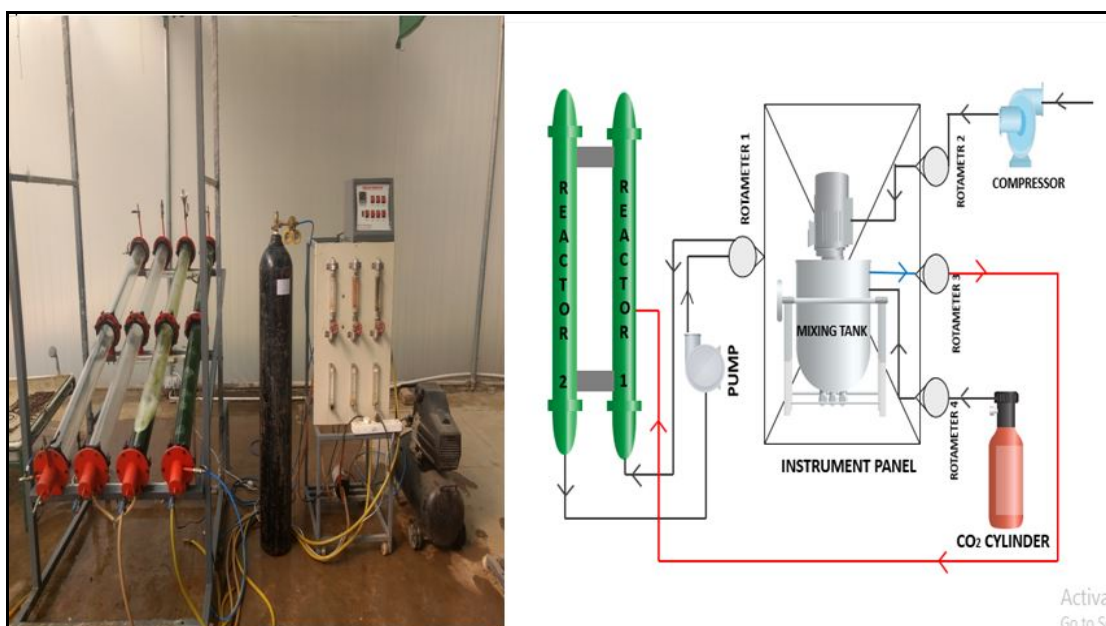

Fig.6.2 The algal photobioreactor **(a)** real image **(b)** Schematic diagram.

6.3.2 Effect of light intensity and CO_2 on chlorophyll and protein content

Microalga *C. vulgaris* was grown under different light intensities (i.e, 50, 100, 150, 200, 300 µmol photons/m$^{2/}$s) with a continuous supply of either ambient air or 5% CO_2 (v/v) in a closed photobioreactor The results (Fig.6.1 a) showed light intensity-dependent increase in the chlorophyll content in *C. vulgaris* cells up to 150 µmol photons/m$^{2/}$s, under the supply of ambient air as well as 5 % CO_2. Further increase in the light intensity (200 and 300 µmol photons/m$^{2/}$s) resulted in a gradual decline in the chlorophyll content. The results further revealed that an increase in chlorophyll content of microalga up to growth saturating light condition (i.e. 150 µmol photons/m$^{2/}$s) was higher in the presence of 5 % CO_2 as compared with the chlorophyll content in the air grown culture. However, at higher light intensities beyond 150 µmol photons/m$^{2/}$s, the decline in chlorophyll content was higher in the air-grown culture as compared to CO_2-grown cells. The protein content (Fig.6.3.b) in the *C. vulgaris* cells, measured in response to varying light intensities (i.e, 50, 100, 150, 200, 300 µmol photons/m$^{2/}$s) under the supply of ambient air as well as 5 % CO_2, showed a similar pattern in the case of chlorophyll content. The impact of high light (HL) caused a dramatic reduction in the protein content under the supply of both ambient air and 5 % CO_2. However, reduction in the protein content under the HL condition was higher in the air-grown cells than the 5 % CO_2.

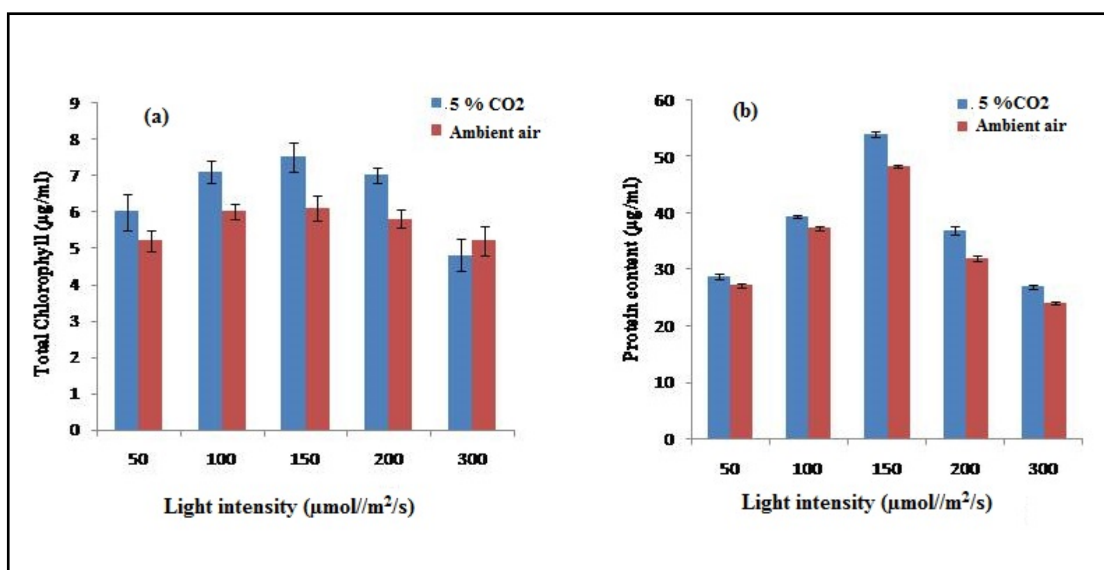

Fig.6.3. Effect of varying light intensities (50-300 µmol/m^2/s) on the chlorophyll **(a)** and protein content **(b)** in *C. vulgaris* cells grown with ambient air or 5% CO_2. Data are the mean of three replicates ± SD.

6.3.3 Effect of light intensity and CO_2 on carbohydrate and lipid content

The results on total carbohydrate (Fig. 2a) in the *C. vulgaris* cells in response to varying light intensities and CO_2 doses (ambient air 0.04% or 5% CO_2) exhibited an initial increase in the carbohydrate content up to 100 µmol photons/m$^{2/}$s of light intensity. A further increase in the light intensity from 150 to 300 µmol photons/m$^{2/}$s resulted in a gradual decline in the carbohydrate content under the supply of both ambient air as well as 5% CO_2. However, it was noticed that carbohydrate content in *C. vulgaris* cells was higher in the presence of 5% CO_2 as compared to ambient air-grown cells throughout the range of light intensity (50-300 µmol photons/m$^{2/}$s). Thus, it was presumed that carbohydrate synthesis in the microalga growing under various light intensities was supported by the excess carbon dioxide (5% CO_2) as suggested earlier (Van Den Hende et al., 2012).

Unlike other macromolecules, the lipid content (Fig.6.4.b) in *C. vulgaris* cells, grown under varying light intensities (50-300 µmol photons/$m^{2/}$s), exhibited light intensity-dependent gradual increase with maximum lipid content at 300 µmol photons/$m^{2/}$s of light intensity. Further, it was observed that up to growth supporting light intensity (50-150 µmol photons/$m^{2/}$s), the supply of 5% CO_2 was more favourable to lipid accumulation than the ambient air. However, the advantage of excess CO_2 supply diminished under the higher light intensity (150-300 µmol photons/$m^{2/}$s).

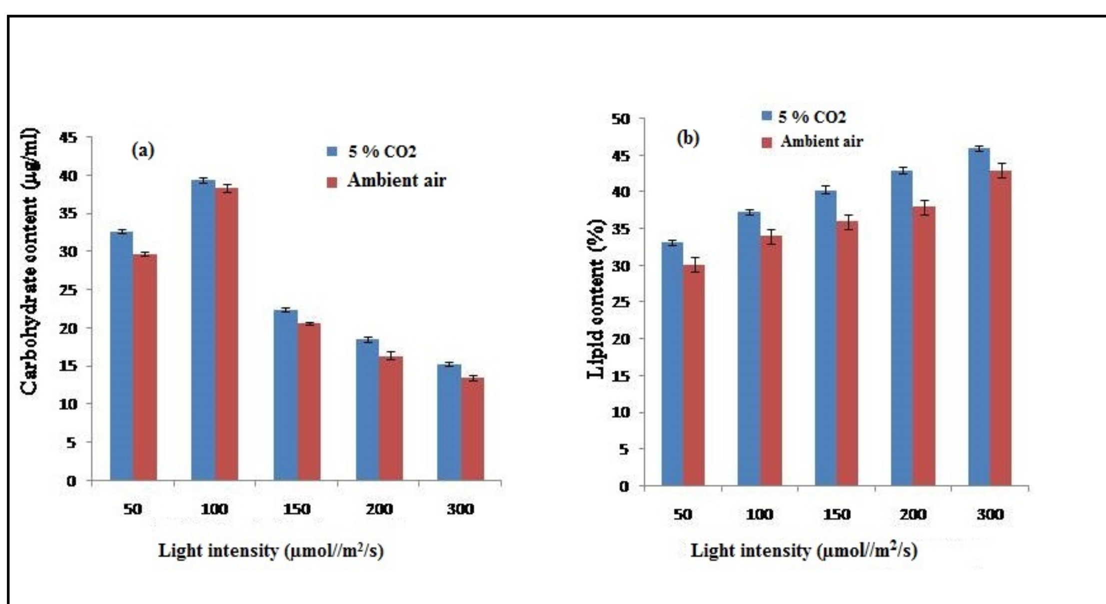

Fig.6.4 Carbohydrate **(a)** and lipid **(b)** contents in *C. vulgaris* cells grown under different light intensities (50-300 µmol/m^2/s) supplied with the ambient air or 5 % CO_2. Data are the mean of three replicates ± SD.

6.3.4 FTIR analysis of LL and HL grown cells

Biomass of *C. vulgaris* cells, harvested on 5th day of their growth under the low light (LL) at 50 µmol photons/$m^{2/}$s and high light (HL) at 300 µmol photons/$m^{2/}$s, under the continuous supply of ambient air or 5 % CO_2, were used for FTIR analysis (Table 6.1). The total lipid (1740 cm^{-1}), lipid/carbohydrate (1740/1040 cm^{-1}), and lipid/protein (1740/1650 cm^{-1}) ratio were calculated from the FTIR spectra to assess

the relative content of different macromolecules including lipid (Dean et al., 2010; Mayers et al., 2013). These results showed that the total lipid and lipid/ protein ratio in the *C. vulgaris* cells grown under LL conditions with 5 % CO_2 was higher than that grown with ambient air. The results revealed that the total lipid content in the cells grown under HL condition in the presence of elevated CO_2 levels was relatively higher than that grown under LL condition. Similarly, lipid/protein ratio in the *C. vulgaris* cells under the supply of 5 % CO_2 was found to be higher than that with ambient air. The overall results indicated high light intensity favoured the increase in the synthesis of lipids as compared to proteins. Further results showed that lipid/ carbohydrate ratio under LL condition was higher in the presence of elevated CO_2, while the same was about 80-90 % higher under the HL condition. Effect of enhanced CO_2 level increased the ratio of lipid/ carbohydrate under LL condition and vice versa under the HL condition. The results suggested carbohydrate accumulation at elevated CO_2 levels under the HL condition.

Table 6.1 The lipid (1740 cm^{-1}), lipid/ carbohydrate (1740/1040 cm^{-1}), and lipid/protein (1740/1650 cm^{-1}) ratio were derived from the FTIR spectra (4000-500 cm^{-1}) of cell biomass of *C. vulgaris* grown under low light and high light conditions with the supply of ambient air and 5 % CO_2.

	Low light			High Light		
CO_2	**Total lipid (1740 cm^{-1})**	**Lipid/ carbohydrate (1740/ 1040 cm^{-1})**	**Lipid/protein (1740/ 1650cm^{-1})**	**Total lipid (1740cm^{-1})**	**Lipid/ carbohydrate (1740/ 1040 cm^{-1})**	**Lipid/ protein (1740/ 1650 cm^{-1})**
Ambient Air	1.1	0.85	0.44	1.75	1.53	0.45
5 % CO_2	1.4	0.93	0.52	2.6	1.4	0.57

6.3.5 Effect of excess CO_2 and light intensity on photosynthetic parameters

Fast chlorophyll fluorescence induction kinetics, using PAM fluorimeter, was measured in *C. vulgaris* cells grown under high light (300 µmol photons/m$^{2/}$s) and low light (50-300 µmol photons/m$^{2/}$s) conditions with a continuous supply of air and 5% CO_2 (Fig. 6.5). The photosynthetic parameters were derived from the OJIP curve of the fluorescence induction kinetics of dark-adapted (for 10 minutes) cells (Table 6.2). The dark-adapted value of Fv/Fm is commonly used to assess the effect of 'stress' (Murchie et al., 2013), reflecting the high sensitivity of PSII to environmental stimuli. Quantum yield (Fv/Fm) of PSII in *C. vulgaris* cells grown under low light (LL) condition was higher in the presence of 5 % CO_2 (0.724) than the ambient air (0.720). However, Fv/Fm value in the high light intensity grown cells showed a decline in the quantum yield (0.664); the decline in the photosynthetic yield was more prominent in the presence of 5 % CO_2 (0.613). The other parameter Fv/F_0 is considered to be an indicator of the water-splitting site of PS II, which is widely used to assess the effect of stress conditions (Kalaji et al., 2012). The present findings on Fv/Fo in response to light intensity and CO_2 showed a pattern similar to the photosynthetic yield. These results together suggested that an improvement in the photosynthetic performance in the presence of excess CO_2 under the low light condition, but excess CO_2 showed a synergistic effect on the high light-induced inhibition of photosynthetic activity under the HL stress. There is a probability that the elevated CO_2 concentration increased the probability of utilization of photosynthetic reducing equivalents under the LL condition.

The photosynthetic parameter Vj is a measure of the fraction of primary electron acceptor QA of PSII in its reduced state, and Mo value is proportional to the rate of QA reduction under the conditions when QB and the pool of plastoquinone are mainly in their oxidized state (Saleh et al., 2014). In the present investigation, Mo and Vj values in the low light-grown cultures showed a decline in the presence of excess CO_2 supply as compared to their corresponding values in the air-grown culture.

The total flow of photons, absorbed by the pigments of the PSII antenna and normalized against the RC number (ABS/RC) and trapping of energy per reaction centre (RC) of PSII, denoted as TR_0/RC (Wang et al., 2012). These values showed a significant increase in the presence of excess CO_2 under both the LL and HL conditions. These results indicated improved energy transfer through PS II at elevated CO_2 levels. However, the increase in both the ABS/RC and TRo/ RC values under the HL condition was substantially higher than the LL condition, which could be perhaps due to an increase in the inactive reaction centre (Lawlor and Tezara, 2009). Further, PIabs is an indicator of plant vitality, representing three main functional steps of photosynthetic activity (Stirbet et al., 2018). The values of PIabs revealed that the presence of excess CO_2 under the LL condition enhanced the value of PIabs (20.380) as compared to air-grown cells (15.198), indicating a positive impact of CO_2 supply on the photosynthetic activity. On the contrary, high light condition induced decline in the PIabs (10.267) was further accentuated by an excess supply of CO_2 (4.857). Non-photochemical quenching (NPQ) of excited chlorophylls (1Chl*) denotes the dissipation of excess excitation energy trapped by the photosynthetic apparatus (Chukhutsina et al., 2014). The present results showed no effect of excess CO_2 supply on NPQ value in the LL grown cells, while excess CO_2

supply in the HL grown cells resulted in a significant decline in the NPQ (0.23) than the air grown cell under HL condition (0.23). A higher NPQ is an indicator of the protection efficiency of the cells against the high light-induced photoinhibition (Ruban, 2016). The present results indicated that excess CO_2 stimulated the photoinhibition potential of high light intensity, perhaps due to a decline in the NPQ. A simultaneous decrease in the NPQ and Rfd values under the HL condition and excess CO_2, with and no apparent change in photochemical quenching (Qp) component (data not shown) suggested that both CO_2 and HL stress acted synergistically to increase the photoinhibition of photosynthetic apparatus. The photochemistry of algae is greatly influenced by the varying light regimes (LL and HL) which ultimately provide energy and reducing power for CO_2 assimilation. The present findings demonstrated that, unlike LL condition, the presence of excess CO_2 under HL condition substantially accentuates the photo-inhibitory effect of high light intensity and inactivation of the enzymes involved in the CO_2 fixation (Franklin et al., 2003). The accumulation of lipid in *C. vulgaris* cells was also enhanced, perhaps due to the combined effect of these two environmental stress which was evident from the synergistic effect of both HL stress as well as excess CO_2 on the inhibition of photochemistry of photosystem II (PS II).

Table 6.2 Photosynthetic parameters were derived from the fast Chlorophyll fluorescence induction kinetics (OJIP curve) of a dark-adapted culture of *C. vulgaris* grown under low light (LL 50 µmol/m^2/s) and high light (HL 300 µmol/m^2/s) conditions in the presence of air and 5% CO_2. Data are the mean of three replicates ± SD.

	Low light treatment		**High light treatment**	
Photosynthetic parameters	**Ambient Air**	**5% CO_2**	**Ambient Air**	**5% CO_2**
Fv/Fm	0.720 ± 0.03	0.724 ± 0.02	0.664 ± 0.02	0.613 ± 0.01
Fv/Fo	2.576 ± 0.19	2.648 ± 0.08	1.975 ±0.05	1.585 ± 0.03
Mo	0.104 ± 0.03	0.084 ± 0.03	0.110 ±0.02	0.161 ±0.05
Vj	0.148 ± 0.05	0.105 ±0.01	0.140 ±0.025	0.196 ± 0.05
ABS/RC	0.973 ± 0.047	1.340 ±0.05	1.186 ± 0.11	1.340 ± 0.02
TRo/RC	0.701 ± 0.017	0.792 ±0.02	0.787 ± 0.03	0.822 ± 0.01
PIabs	15.198 ± 0.037	20.380 ±0.23	10.267 ±0.27	4.857 ±0.04
NPQ	0.30 ± 0.02	0.30 ±0.032	0.27 ±0.02	0.23 ±0.01
Rfd	0.39 ± 0.035	0.44 ± 0.05	0.32 ± 0.04	0.28 ± 0.03

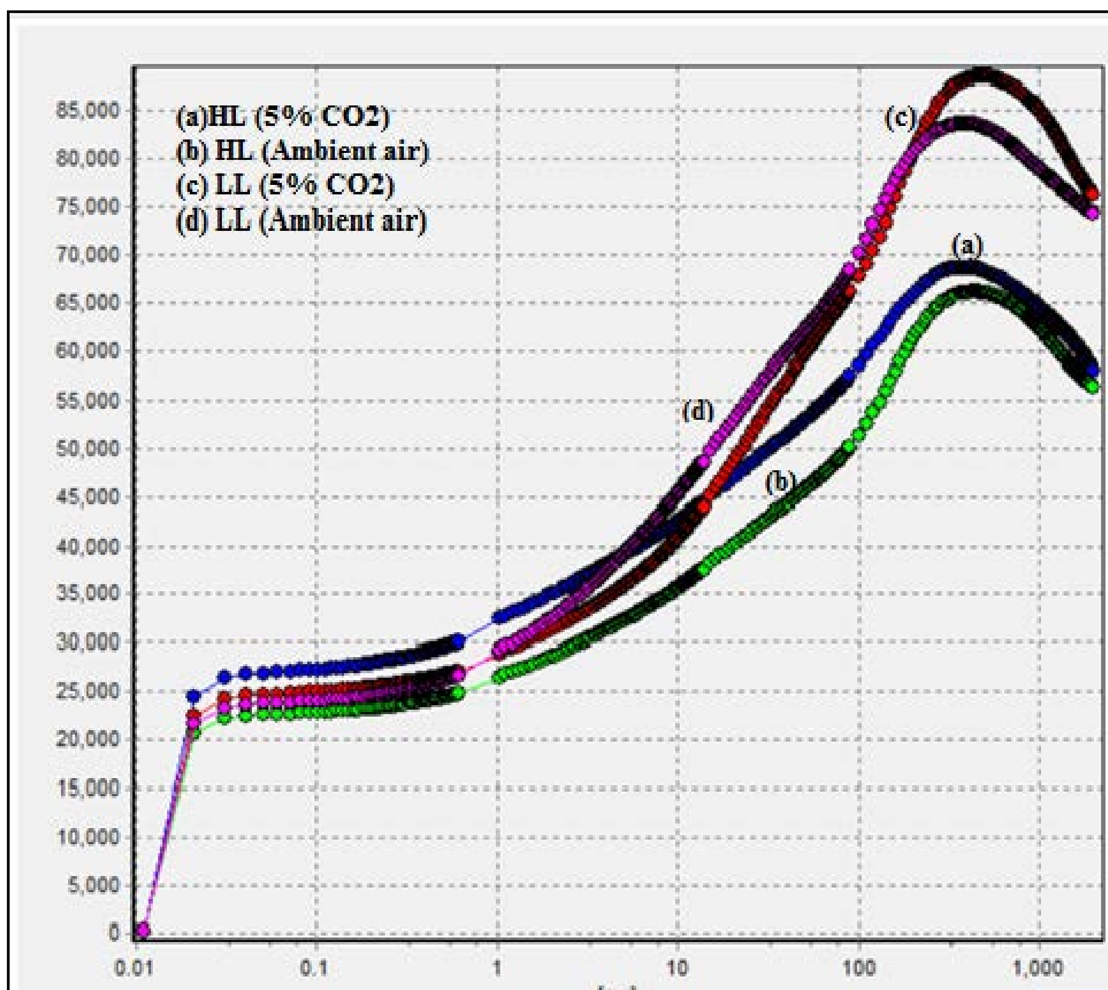

Fig.6.5 Chlorophyll fluorescence induction curve (OJIP) of *C. vulgaris* cells grown under low light (LL, 50 µmol/m^2/s) and high light (HL, 300 µmol/m^2/s) conditions in presence of air and CO_2 (5%). The symbol (a-d) refers to different treatments given to the cells.

6.4 Discussion

In the present study microalga *C. vulgaris* was grown under different CO_2 concentrations, out of which 5% CO_2 dose was found to be the optimum for obtaining maximum growth and lipid production. In a similar study, Kumar et al., (2014) observed 5% CO_2 as the most optimum concentration to sequester CO_2 from flue gas in the case of microalga *Chlorella sorokiniana.* When microalga *C. vulgaris* was subjected to varying light intensities with either continuous supply of ambient air or

5% CO_2 showed a threshold limit of light tolerance at 150 µmol photons/m$^{2/}$s. The cell constituents like chlorophyll, protein, and carbohydrate in the cells at low light intensities were enhanced by excess CO_2 (5%) as compared to air-grown cell culture. Earlier it has been demonstrated that low light intensity (LL) favors the higher protein content (Liu et al., 2012). Under the high light condition, the decline in protein content might be due to the paucity of reducing equivalents required for nitrogen assimilation (Foyer, 2018; Juneja, 2013). Earlier it has been suggested that the addition of CO_2 essentially improves the autotrophic growth of microalgal cells including biomass productivity and protein content (Chena et al., 2013). However, it is still unclear how the elevated CO_2 influences the protein and chlorophyll content, particularly under the HL conditions (150-300 µmol photons/m^2/s). Cheng et al., (2015) also reported that cell wall carbohydrate content in *C. vulgaris, C. sorokiniana,* and *C. variabilis* increased significantly in CO_2 enriched conditions. Previous studies demonstrate that an increase in the light intensity in the range of 30–400 µmol m^{-2} s^{-1} could slightly increase the accumulation of carbohydrates (Carvalho et al., 2009). Hymus et al., (2001) suggested that elevated CO_2 levels under the high light stress led to increased photo-inhibition and competitive inhibition of photorespiration which might have resulted in reduced consumption of carbohydrates. However, there was no obvious positive correlation between the light intensity and intracellular carbohydrate accumulation. This suggested that the accumulation of carbohydrates in microalgae does not solely depend on the light intensity, but it is also influenced by various other environmental factors such as nutrients and CO_2. Xia and Gao, (2005) reported that an increase in CO_2 concentration during the cultivation of *C. pyrenoidosa* and *C. reinhardtii* resulted in an enhanced level of carbohydrate content.

However, lipid content showed a light intensity-dependent gradual increase, which further increased in the presence of excess CO_2 and reached its maxima at 300 µmol photons/$m^{2/}$s. Similar to these results, He et al., (2015) have reported a higher accumulation of lipid content in *Chlorella sp*. (33.03%) under the high light intensity. Earlier workers have reported that high light intensity induced photoinhibition was primarily responsible for the decline in the biomass, whereas the lipid synthesis was enhanced as it consumes more excess energy (Ho et al., 2014). Cheng et al., (2014) also reported a decrease in the total lipid accumulation in *C. vulgaris* when the cells were grown at 2 % CO_2 concentration. Perhaps, the strain specificity or tolerance of microalgae to elevated CO_2 concentrations could be the reason for variation in lipid content of different strains of microalgae. Earlier reports suggested that lipid accumulation is directly proportional to the decline in intracellular protein content of cells (Wang et al., 2012) and intensity of stress conditions. Perhaps, stimulated accumulation of lipid under HL + 5 % CO_2 is the effect of combined stress on the photoinhibition as suggested by Franklin et al., (2003).

The high light-induced increase in reactive oxygen species could be the reason for an overall decline in the chlorophyll content of *C. vulgaris* cells (Hu et al., 2008; He et al., 2015). The photosynthetic parameters like Fv/Fm, Fv/Fo, Mo, Vj, ABS/RC, and TRo/RC showed that excess CO_2 supported an improved functioning of these parameters under low light (LL). However, under the high light (HL) condition, the photosynthetic parameters indicated that the primary photochemistry of PSII was negatively influenced by the excess CO_2 and HL.

Earlier it has been reported that a decrease in the level of reduced QA results from the rapid rate of QA oxidation where excess CO_2 could be the sink for electrons (Strasser

et al., 2004). But the excess supply of CO_2 under HL condition exhibited an increase in the Mo and Vj values, which indicated that the ratio of reduced (QA) to oxidized QA^- was increased as the electron is not transferred to QB under the high light condition. Previous findings showed that the total non-cyclic electron flow through PSII was enhanced by elevated CO_2 under the high light condition (Habash et al., 1995). Therefore, it could be inferred that the fraction of reduced QA was enhanced in response to both the high light stress as well as excess CO_2 concentration which contributed to an increase in the photoinhibition.

However, there is evidence that the growth of cells at elevated *p* CO_2 decreases the antioxidant activity (Polle et al., 1997). The Relative fluorescence decrease (Rfd) ratio represents both the photochemical and non-photochemical quenching components (Lichtenthaler et al., 2005).

The synergistic effect of CO_2 and high light intensity on the photochemistry of PSII was corroborated by the results on an overall decline in the photosynthetic performance index (PIabs), non-photochemical quenching (NPQ), and relative fluorescence decrease (Rfd) ratio in the presence of excess CO_2 and HL condition.

Chapter VII

Role of phosphate and organic carbon in the regulationmixotrophic growth of Chlorella vulgaris

Role of phosphate and organic carbon in the regulation of mixotrophic growth of *Chlorella vulgaris*

7.1 Introduction

Microalgae are one of the largest photosynthetic microorganisms on the earth and are known to produce several commercially important metabolites including the precursor of biodiesel through the process of photosynthesis. The mixotrophic nature of microalgae provides the inherent capability to grow under a variety of complex environments including wastewater, saline water, industrial effluents, ponds, river, ditches, etc (Andrade and Costa, 2007). The advantage associated with mixotrophic cultivation of microalgae is an ideal approach to get high cell density and biomass yield (Kumar et al., 2014). The growth and biomass production of the microalgae is governed by several parameters such as nutrient concentration (Aslan and Kapdan, 2006; Rodolfi et al., 2009), availability of CO_2 (Anjos et al., 2013), light conditions (Moreno-Garcia et al., 2017) and pH of the culture medium etc.

The concentrations of nitrogen and phosphorus present in the wastewater are considered to be a fundamental factor and have a direct influence on algal growth kinetics, biochemical constituents, and high energy storage compounds i.e., lipid. The level of lipid in microalgae determines the feasibility of using them as feedstock for biofuel production. There are various strategies employed by researchers to induce lipid production in microalgae (Aratboni et al., 2019). So far nutrient starvation is being recognized as one of the most successful strategies to improve lipid productivity. Nitrogen, phosphorus, and/or sulfur starvation has been widely recognized as the main lipid inducer in green microalgae (Zhu et al., 2016).

Phosphorus is an essential nutrient for the growth of microalgae as it plays a significant role in cellular metabolic processes related to energy transfer, signal transduction, photosynthesis, and respiration (Liang et al., 2013). Recent studies have shown that the phosphorous deprived conditions result in higher lipid content in microalgae *Phaeodactylum tricornutum, Chaetoceros* sp.*, Isochrysis galbana* and *Pavlova lutheri* Liang et al., (2013) also reported the effect of phosphorus on lipid accumulation in *Chlorella* sp. and demonstrated an increase in the lipid accumulation by microalga with decreasing phosphorus concentrations.

C. vulgaris is a fast-growing unicellular green microalga widely distributed in diverse habitat from freshwater to marine to waste environments (Ahmad et al., 2020). All these characteristics make it an ideal microalga for large-scale cultivation and commercialization (Borowitzka, 2018). It has been reported that the biomass and lipid production in microalgae are greatly influenced by different carbon sources and nutrient conditions (Sharma et al., 2016). Najafabadi, (2015) reported that the growth rate of the microalgae grown by using sodium acetate and sodium bicarbonate was much higher than that obtained under CO_2 enriched growth conditions. Bhatnagar et al., (2011) suggested that a mixotrophic mode of nutrition helps to obtain better biomass and lipid yield. Keeping these findings into consideration, the present study was conducted to analyze the impact of both mixotrophic (different C/P ratio) cultivation as well as phosphate nutrition on the growth, macromolecular content, and photosynthetic performance of *C. vulgaris*. In addition, FTIR spectra of *C. vulgaris* cells study was also used to analyze the cell composition including production of lipid under varying C:P ratios.

7.2 Materials and methods

7.2.1 Experimental design

C. vulgaris was cultured in 100 mL of BG-11 medium in a 250 ml conical flask. The microalga was grown in a culture room under the controlled conditions with a light intensity of 60 μmol photons/m^2/s and a light/dark cycle of 14/10 hours. The room temperature was maintained at 25 ±2^0C. For mixotrophic growth, the BG-11 medium (devoid of inorganic carbon and phosphate) was supplemented with organic carbon and inorganic phosphate in a different ratio. The concentration of organic carbon in each flask was fixed at 4 mM, whereas phosphate concentration was varied to maintain the different molar ratios of carbon: phosphorous (C:P ratio; 40:0, 40:1, 40:2, 40:3, 40:4, and 40:5). A system growing algae in BG11 media served as control. All the experiments were conducted in triplicate.

7.2.2 Estimation of biomolecules

The protein content of the microalgal cell suspension was estimated by following the method of Lowery et al.,(1951), modified by Herbert et al., (1971), using bovine serum albumin (BSA) as standard. The total carbohydrate content was determined using the phenol- sulfuric acid method as described by Dubois et al., (1956). Lipid analysis was performed gravimetrically based on a method given by Bligh and Dyer (1959). (Page no. 66-71)

7.2.3 Fourier transform infrared spectroscopy analysis (FTIR) Spectra

The *C. vulgaris* cells grown under different were harvested on 6th day and the cell biomass was dried overnight in an oven at 60°C. The dried biomass was grounded and

it was mixed with KBr (1:10, w/w). The FTIR spectra (4000–400 cm^{-1}) were used for calculation of total lipid (1740 cm^{-1}), Lipid/protein (1740/1650 cm^{-1}), lipid carbohydrate (1740/1040 cm^{-1}) ratio (Laurens and Wolfrum, 2011; Mayers et al., 2013) and the ratio of CH_3/CH_4 (2964/2924) to assess the change in the saturated and unsaturated fatty acids.

7.2.4 Scanning Electron Microscopy (SEM) coupled with EDS

Scanning Electron Microscope and energy dispersive X-ray system was used to study the morphological alterations and mineral status of the different elements in the algal cells. The harvested cell samples were prepared as described in chapter-III.

7.2.5 Fast chlorophyll fluorescence induction kinetics

The measurement of chlorophyll fluorescence induction kinetics (OJIP curve) at fixed excitation (450 nm) and emission wavelengths (650 nm) was done by using Aqua pen C. The photosynthetic parameters (quantum yield Fv/Fm, ET0/RC, TR0/RC, ABS/RC, PIabs) were derived from the OJIP curve described in chapter –III of materials and methods.

7.3 Results

7.3.1 Effect of organic carbon and phosphate (C:P ratio) on the cell constituents

The effects of varying C:P ratios were investigated as a potential metabolic engineering strategy to alter the biochemical composition of microalgae. The optical density (Fig.7.1 a) of the cultures measured at 665 nm increased with an initial increase in the C:P ratio. After the C:P ratio of 40:3, a decline in the optical density

was observed at C/P 40:4, as compared to BG-11 medium-grown cells (control). The protein content (Fig. 7.1 b) of *C. vulgaris* cell showed an increasing trend with an increasing C:P ratio from 40:0 to 40:4 when compared with the control (BG-11 grown cells). However, the protein content of cells at a C:P ratio of 40:4 was about two-fold higher than the protein level in the BG-11 grown cells (control). In comparison to BG-11 grown cells, the carbohydrate content (Fig. 7.3) in *C. vulgaris* cells initially increased with the addition of sodium acetate as a carbon source (C:P ratio 40:0), irrespective of the phosphate concentration. However, the level of carbohydrates started declining with an increasing C:P ratio as compared to C:P ratio of 40:0. TOC (Fig. 7.2.a) and lipid (Fig. 7.2.b) content in the microalga showed a significant increase in the values up to C:P ratio of 40:2, as compared to BG-11 grown control cells. However, further increases in the C:P ratio beyond 40:2 resulted in a gradual decrease in both TOC and lipid content. These results indicated that the mixotrophic growth of *C. vulgaris* was beneficial for an increase in biomass. However, the addition of phosphate to mixotrophic culture supported an increase in all the cell constituents (protein, lipid, TOC) except carbohydrate. There is a possibility that phosphate plays an important role in carbon fixation as well carbon allocation in the cell.

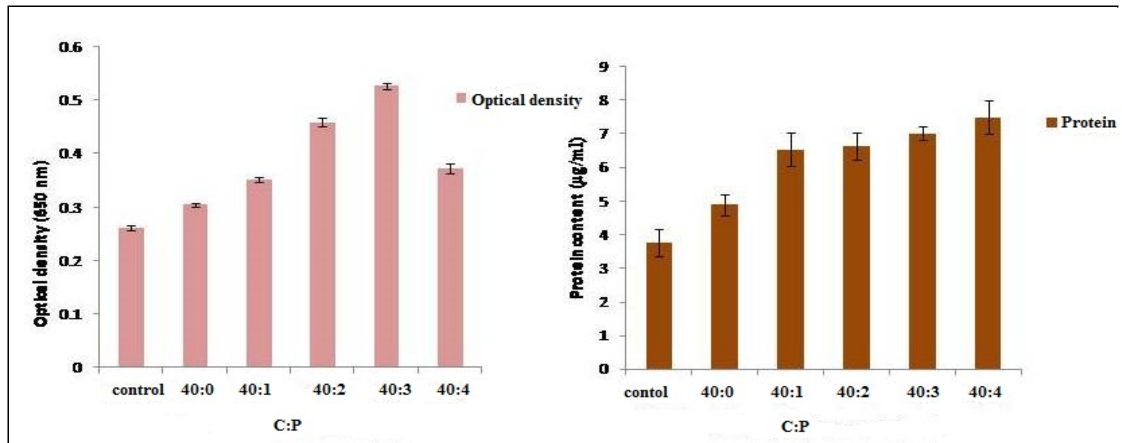

Fig.7.1 (1.a) Optical density of *C. vulgaris* in response to various C:P (40:0-40:4). **(1.b)-** Protein content of *C. vulgaris* in response to various C:P (40:0-40:4). Data are the mean of three replicates ± SD.

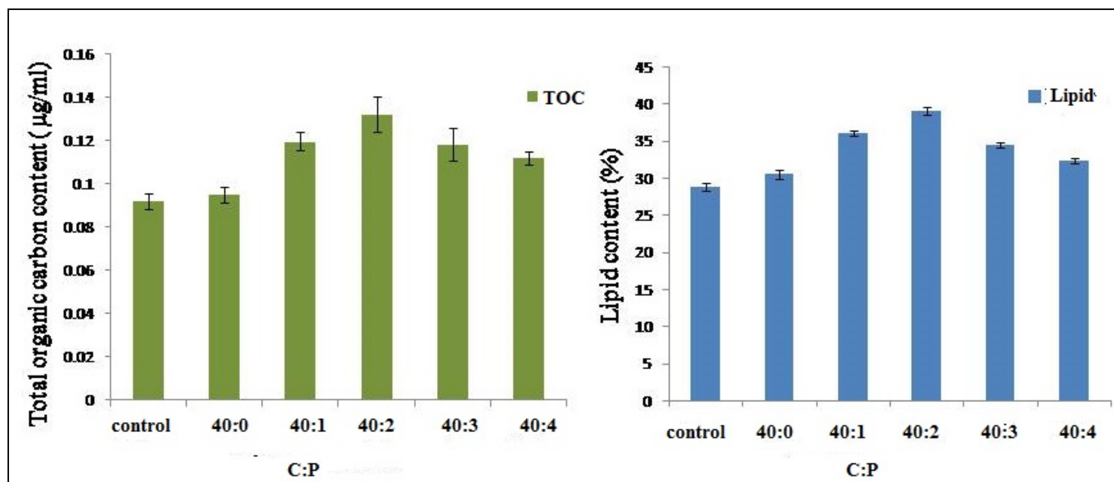

Fig.7.2 **(a)** Total organic carbon content of *C. vulgaris* in response to various C:P (40:0-40:4). **(b)-** Lipid content of *C. vulgaris* in response to various C:P (40:0-40:4). Data are the mean of three replicates ± SD.

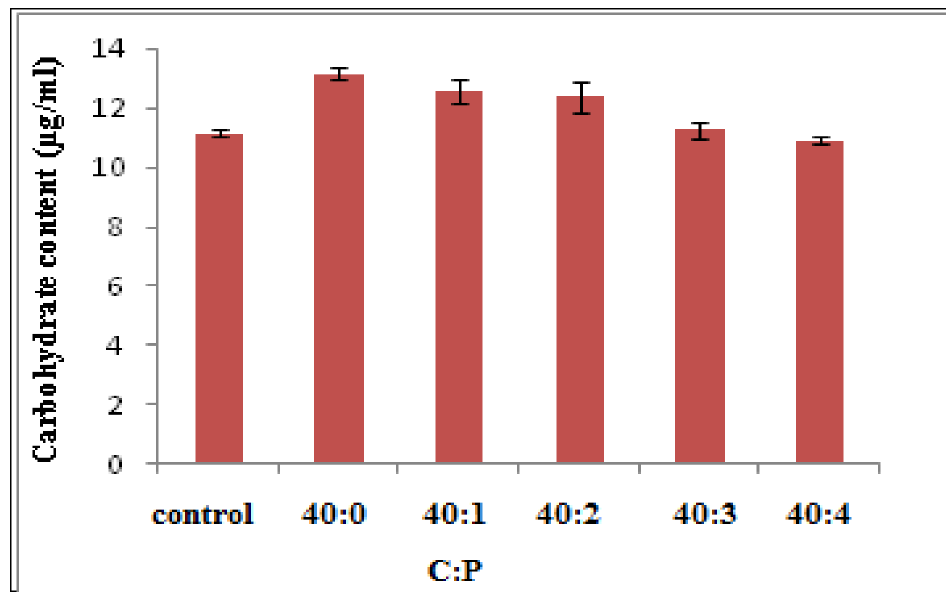

Fig.7.3 Carbohydrate content of *C. vulgaris* in response to various C:P (40:0-40:4). Data are the mean of three replicates ± SD.

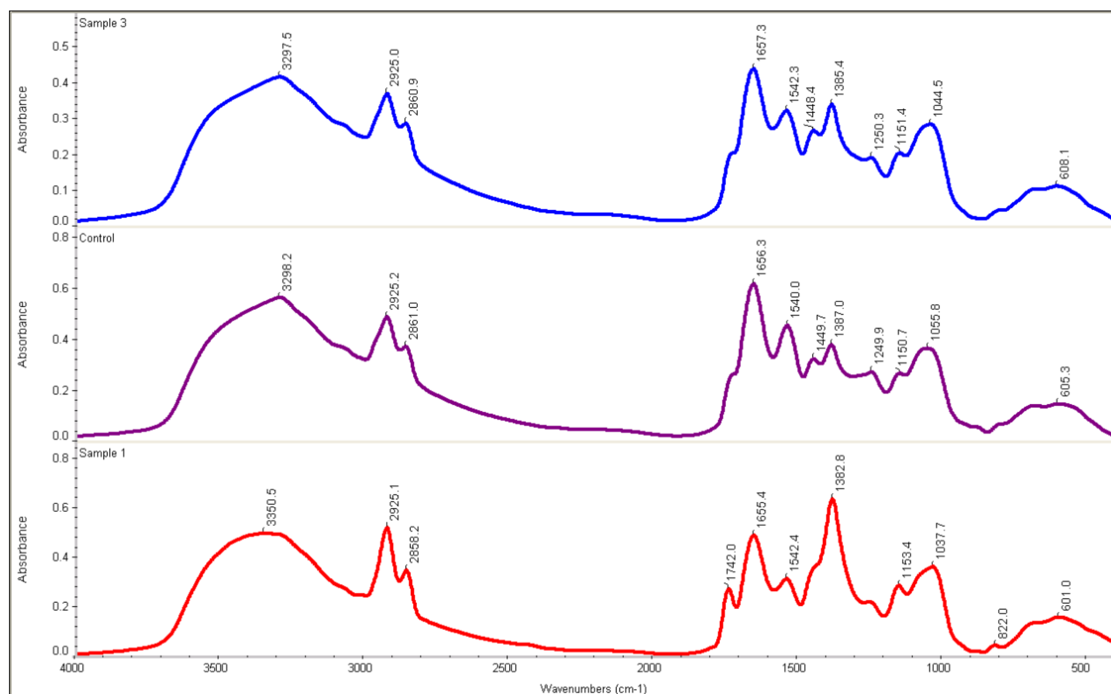

Fig.7.4 FTIR spectra (4000-500 cm^{-1}) of *C. vulgaris* cells grown in BG-11 medium (control) and medium with C:P ratio of 40:0 and 40:3.

Table:7.1 The total lipid (1740 cm^{-1}), a ratio of lipid/ carbohydrate (1740/1040 cm^{-1}), lipid/protein (1740/1650 cm^{-1}), and ratio of saturated/ unsaturated fatty acids (2964/2924 cm^{-1})were derived from the FTIR spectra (4000-500 cm^{-1}) of cell biomass of *C. vulgaris* grown in BG-11 medium and C:P ratio (40:0 and 40:3). The data were derived from the FTIR spectra (Fig. 7.4)

Mode of cultivation	Total lipid (1740)	Lipid/Carbohydrate (1740/1040)	Lipid/Protein (1740/1650)	CH_3/CH_4 (2964/2924)
BG- 11	0.80	0.72	0.40	0.76
40:0 (C:P)	1.00	0.83	0.58	0.66
40:3 (C:P)	0.90	0.64	0.39	0.83

7.3.2 Effect of C:P ratio on the FTIR spectra

The FTIR spectra (Fig. 7.4) of cell biomass grown BG-11 medium (control) and with different C:P ratio (40:0 and 40:3) showed variation in the absorption peaks of signature molecules, such as carbohydrate (1040 cm^{-1}), protein (1650 cm^{-1}), and total lipid (1740 cm^{-1}) content. The FTIR spectra of cell biomass of *C. vulgaris* (Fig.7.4) depicted major changes in the IR peaks at 2925 cm^{-1} and 1384 cm^{-1} wavenumbers associated with lipid and lipoproteins, respectively. A significant increase in the IR absorption peaks of the microalgal cell biomass grown under phosphate-deprived mixotrotphic culture suggested enhanced accumulation of lipid content as compared to phosphate sufficient mixotrophic culture. The results (Table 7.1) showed that the total lipid content of mixotrophic culture of *C. vulgaris* maintained with different C:P ratio (40:0 and 40:3) was higher than the control (BG-11 grown cells). However, cellular lipid content was maximum in the phosphate-deprived cells with a C:P ratio of 40:0. The results indicated that the addition of organic carbon was supportive to lipid accumulation in the microalga as compared to photoautotrophic growth of *C. vulgaris* in BG-11 medium (control). The lipid/carbohydrate (1740/1040 cm^{-1}) ratio

(0.89) was also higher in the organic carbon supplemented mixotrophic culture when compared with the BG-11 grown cells (control). The most significant observation was that the phosphate starved mixotrophic culture was more supportive to lipid accumulation than the phosphate sufficient condition of mixotrophic culture. Similarly, lipid/protein (1740/1650 cm^{-1}) ratio in the BG-11 grown cells was the lowest. Under the mixotrophic state of microalgal growth, the lipid/protein ratio was found to be significantly higher. However, the lipid/protein ratio in the phosphate-deprived mixotrophic culture was higher than the phosphate sufficient condition. These results suggested that higher lipid accumulation could be induced by the addition of organic carbon as well as phosphate deprivation. The addition of phosphate perhaps restored the normal physiological conditions in the cells leading to the better synthesis of protein and carbohydrate, which led to a decrease in the lipid/carbohydrate and lipid /protein ratios as compared to that under the phosphate starved condition. Further, results on the ratio of saturated/ unsaturated fatty acids (2964/2924 cm^{-1}) in the *C. vulgaris* cells was maximum in the phosphate sufficient mixotrophic culture, followed by BG-11 grown cells (control) and phosphate starved mixotrophic culture. Thus, it could be suggested that the maximum unsaturated fatty acids were synthesized in the phosphate starved mixotrophic culture, an essential ingredient required for better yield of biodiesel.

7.3.3 Effect of carbon and phosphate nutrition on the EDS analysis of elements

The EDS data obtained from the *C. vulgaris* cells grown in BG-11 medium and grown mixotrophically with varying ratios of C:P (40:0-40:3) exhibited variation in elements localized in the K shell (Fig. 7.5). The results of EDS data in BG-11medium grown *C.*

vulgaris cells showed the highest level of K and Mg elements (1.08 and 0.72 weight %, respectively) and the lowest level of ‘O’ and Na elements (27.89 and 0.68 weight %). The level of O and Na elements was higher (42.17 and 1.54 weight %, respectively) in the *C. vulgaris* cells grown mixotrophically without phosphate (C:P ratio, 40:0) and with phosphate (C:P ratio, 40:3) (40.69 and 3.03 weight %, respectively) when compared with that in the BG-11 grown cells. On the other hand, as compared to that in the BG-11 grown cell, the level of K and Ca elements were lower in the mixotrophic culture of microalga under both phosphate (P) starved (0.40 and 1.18 weight %, respectively) and P supplemented condition (0.49 and 0.13 weight %, respectively). The level of S element was not influenced by the presence and absence of phosphate (0.63 weight %) under the mixotrophic growth condition (C:P ratio, 40:0 and 40:3), but the level of S element (1.01 weight %) was about 80-90% higher in the BG-11 grown cells. The results indicated that as compared to BG-11 grown cells, the level of K and Mg diminished significantly under the mixotrophic growth condition, but showed an increasing trend with an increase in the phosphate concentration. On the contrary, the accumulation Na element in the mixotrophic culture was very high as compared to that in the BG-11 medium grown cells. The addition of phosphate to mixotrophic culture could further accentuate the accumulation of Na by 4.5 fold. Further, results indicated that the accumulation of Ca was significantly very high in the mixotrophic culture under the phosphate starved condition, but the accumulation of Ca was reduced with the addition of phosphate.

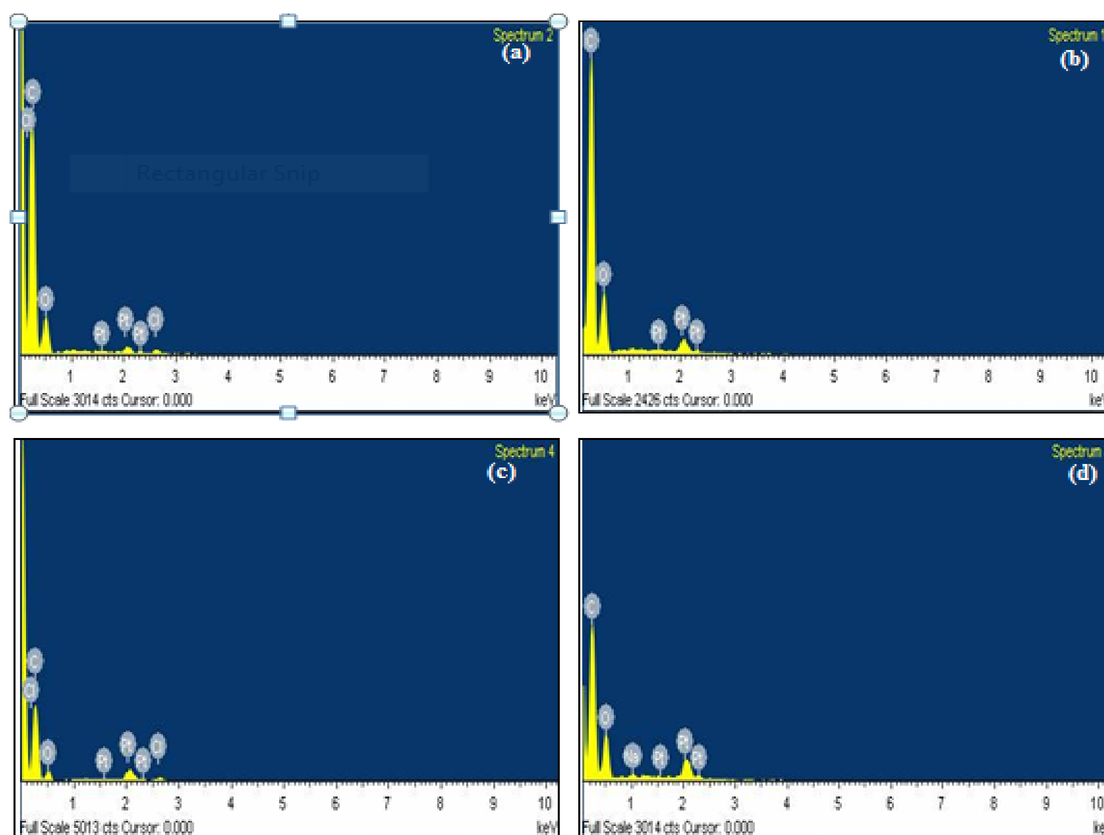

Fig.7.5 SEM-EDS (scanning electron microscope- energy dispersive X- ray Spectroscopy) results of *C. vulgaris* cell. **(a)** BG-11 grown cells, **(b)** C:P , 40:0, **(C)** C:P, 40:3, **(d)** C:P, 40:5.

7.3.4 Effect of varying C:P ratio on the photochemistry of PS II

The chlorophyll (Chl) fluorescence induction kinetics (OJIP curve) is considered to be a better tool to analyze the photosynthetic characteristics of microalgal cells. The initial fast rise of Chl fluorescence to 'O' denotes the initial level F_0 (also termed as ground fluorescence) and maximum Chl fluorescence level 'P' in the OJIP curve is termed as Fm, when the total QA (primary acceptor) in photosystem II (PS II) is reduced. The chlorophyll fluorescence induction kinetics was measured in dark-adapted cells of *C. vulgaris* cells treated with varying C:P ratios (40:0-40:5). The photosynthetic parameters presented in the (Table.7.2) were derived from the OJIP curve obtained by using a PAM fluorometer. The quantum yield (Fv/Fm) was found

to be highest (0.729) in the BG-11 grown cells (control) when compared with the quantum yield (Fv/Fm) values of the cells grown mixotrophically with different C:P ratios. The lowest quantum yield (0.667) was observed in the phosphate starved mixotrophic cells (C:P ratio of 40:0). The results indicated that phosphate sufficient condition was essential for a better quantum yield of PS II. The value of Fv/Fm is commonly used to evaluate the effect of 'stress' (Murchie et al., 2013), reflecting the high sensitivity of PSII to environmental stimuli. The NPQ of excited chlorophylls (1Chl*) denotes the dissipation of excess excitation energy trapped by the photosynthetic apparatus (Chukhutsina et al., 2014). The lowest NPQ value was found in the case of BG-11 grown cell (0.26), but the highest NPQ value (0.83) was recorded in the phosphate starved mixotrophic culture (C:P ratio, 40:0) of *C.vulgaris*. However, phosphate sufficient condition of mixotrophic culture (C:P ratio, 40:3 and 40:5) led to a sharp decrease in the NPQ value. These results demonstrated that the presence of phosphate was essential to prevent the build-up of the proton gradient across the membrane, which is considered to be the main driving force for non-phtochemical quenching of the Chl fluorescence (Ruban, 2017). Earlier it has been suggested that NPQ represents the fastest response of the photosynthetic membrane to stress conditions (Demmig-Adams et al., 2014). The relative fluorescence decrease (Rfd) value, represents the relation between both photochemical quenching and non-photochemical quenching. The results in the present investigation suggested that the NPQ was more predominant under the phosphate starved condition (C:P ratio of 40:0) when compared with the phosphate sufficient condition (C:P ratio of 40:3 and 40:5). The results revealed that the contribution of NPQ in the Chl fluorescence was significantly reduced in the presence of phosphate. The values of ABS/RC and ETo/RC, respectively, denoting the absorption of photons (ABS) and its subsequent

transfer of energy down the electron transport chain (ETo), are equalized concerning PS II reaction centres (RC). Significantly higher values of ABS/RC and ETo/RC in the phosphate starved mixotrophic culture (C:P ratio, 40:0), as compared to phosphate sufficient mixotrophic culture (C:P ratio, 40:3 and 40:5) or BG-11 grown culture, suggested that reduced number of active PS II centres might be contributing to higher values of ABS/RC and ETo/RC in the phosphate starved cells. Further results on PIabs, denoting photosynthetic performance index or vitality index of the *C. vulgaris* cells, showed that PIabs value of BG-11 grown cells (14.78) was about two-fold higher than the PIabs value (6.373) of phosphate starved mixotrophic culture (C:P ratio, 40:0). However, the addition of phosphate to mixotrophic cultures (C:P ratio, 40:3 and 40:5) resulted in a substantial increase in the PIabs values (15.69 and 18.60, respectively), which were higher than the BG-11 grown cells (control) as well as phosphate starved mixotrophic cultures (C:P ratio, 40:0).

A corollary of these results suggested that mixotrophic culture supplemented with a sufficient quantity of phosphate (as with C:P ratio, 40:3 and 40:5) was essential for optimum photosynthetic performance in mixotrophic cultures. Reduced photosynthetic performance in the phosphate starved mixotrophic culture was presumably on account of non-photochemical quenching and cessation of bioenergetic processes. As compared to BG-11 medium grown cells the photosynthetic efficiency of mixotrophic cultures was better, provided that the mixotrophic culture was supplemented with sufficient phosphate concentration.

Table: 7.2. The photosynthetic parameters (Fv/Fm, ABS/RC, ETo/RC, Rfd, NPQ, PIabs) were derived from the OJIP curve of C.*vulgaris* cells grown in the BG-11 medium (control) and mixotrophic condition with varying C:P ratio (40:0, 40:3, and 40:5).

Mode of cultivation	Fv/Fm	NPQ	PIabs	ABS/RC	ET0/RC	Rfd
BG-11	0.729±0.02	0.26±0.008	14.784±0.04	1.064±0.05	0.663±0.027	0.23±0.03
C:P (40:0)	0.667±0.031	0.84±0.02	6.373±0.034	1.292±0.021	0.693±0.033	0.46±0.032
C:P (40:3)	0.724±0.026	0.35±0.022	15.691±0.032	1.021±0.035	0.635±0.031	0.24±0.024
C:P (40:5)	0.727±0.028	0.33±0.042	18.604±0.027	1.100±0.042	0.708±0.045	0.19±0.027

7.4 Discussion

It has been reported that mixotrophic growth of microalgae helps to obtain better biomass and lipid yield (Bhatnagar et al., 2011; Choi and Yu, 2015). The mode of cultivation and nutritional conditions have been used to enhance the cell cycle, biomass, and lipid production in microalgae (Ratha et al., 2013). So far, nutrient starvation is considered as the main lipid inducer to improve the lipid productivity in green microalgae (Xin et al., 2011). The present investigation also revealed that mixotrophic growth of *C. vulgaris* with the support of organic carbon could provide better growth and synthesis of several biomolecules than the growth in the BG-11 medium. Since phosphate (P) is an essential nutrient and plays important role in the physiology of green algae (Chen et al., 2011) and regulates the cellular enzyme activity, metabolic pathways, including photosynthesis (Stigter et al., 2015). In the present study, results revealed that mixotrophic culture with increasing C:P ratio (40:0-40:4) supported an increase in all the cell constituents (protein, lipid, TOC) except carbohydrate with an increasing C:P ratio. Earlier workers have demonstrated

that phosphate plays an important role in energy metabolism (ATP synthesis) and carbohydrate metabolism (Yaakob et al., 2021). Therefore, an increase in lipid, protein, and TOC with the addition of phosphate indicated phosphate-dependent restoration of normal physiological conditions in the cells, leading to the better synthesis of important cell constituents. However, faster depletion of carbohydrates in the mixotrophic culture under the P sufficient condition could be on account of higher energy demands of the cells. Further, these observations were confirmed by the data on FTIR analysis of cell biomass grown in BG-11 medium and mixotrophic condition (with and without P). The lipid/carbohydrate and lipid /protein ratios in the mixotrophic culture were higher than the BG—11 medium-grown cells. The effect of mixotrophic culture on the accumulation of lipid content was more pronounced under the P-deprived condition. Further, the level of saturated fatty acids in the P starved mixotrophic culture was found to be higher than the BG-11 grown cells and phosphate sufficient mixotrophic culture. The results were also supported by the prominent IR absorption peak (1384 cm^{-1}) in the mixotrophic culture, associated with lipoprotein. Kozlowska-Szerenos et al., (2000) evaluated the effect of phosphate deprivation on the growth of *C. vulgaris* and suggested that growth declined by 30–40% in comparison to P sufficient condition, but there was no significant effect on the total chlorophyll. Recently, Galbraith & Martiny, (2015) revealed a consistent relationship between phosphorous and carbon content of phytoplanktons, which elaborates the understanding of how carbon metabolism is influenced under the P deficient condition. Several reports have shown that TAG levels in diatoms were greatly enhanced under P depleted conditions (Brembu et al., 2017) and the cellular response of phytoplanktons to low levels of P levels was the substitution of

phospholipids with non-phosphorus lipids and excess carbon flow towards the storage lipid.

The EDS results revealed that, unlike the BG-11 grown cells, the level of K and Mg elements diminished under the P starved mixotrophic growth condition, but showed an increasing trend with an increase in the phosphate concentration. On the contrary, the accumulation of Na and Ca elements in the mixotrophic culture was very high as compared to that in the BG-11 medium-grown cells. The addition of phosphate to the mixotrophic culture could further accentuate the accumulation of Na and diminished the level of Ca. These results suggested that elemental composition was determined by both the mode of cultivation as well as presence or absence of phosphate. There could be some correlation between these elements and the bioenergetics of the *Chlorella* cells. Earlier workers have suggested that the accumulation of Na, K, and Ca are related to the electrochemical gradient and proton gradient of the thylakoids and play an important role in the pH-dependent down-regulation of photosynthetic electron transport (Kramer et al., 2003).

Earlier findings have demonstrated that a reduced level of phosphate leads to a reduction in photosynthetic phosphorylation and photosynthetic electron transport by PS II (Liang et al., 2013). Benavente-Valdes et al., (2016) reported that photoautotrophic growth significantly increases the metabolic consumption of phosphorous by *C. pyrenoidosa*. But so far little effort has been made to understand the role of P in the regulation of photosynthesis in microalgae under the mixotrophic mode of cultivation. In the present investigation, the fast Chl fluorescence induction kinetics (OJIP curve) revealed that the quantum yield of PS II (Fv/Fm) was found to be the highest in BG-11 grown cells as compared to mixotrophic culture (C:P ratio,

40:0 and 40:4). The results suggested that P sufficient condition was essential for a better quantum yield of PS II (Fv/Fm). The Fv/Fm is an important photosynthetic parameter for the evaluation of the health of photosynthetic machinery (Murchie et al., 2013) and the sensitivity of PSII to environmental stimuli. The total flow of photons, absorbed by the pigments of the PSII antenna and normalized against the RC number (ABS/RC) and trapping of energy per reaction centre (RC) of PSII, denoted as TR_0/RC (Wang et al., 2012) showed the highest value in P starved mixotrophic culture but declined with increasing C:P ratio. This observation could be interpreted in terms of a reduced number of active PS II reaction centres under the P starved condition, not due to faster energy distribution. This assumption was further supported by higher values of NPQ and Rfd in *C. vulgaris* cells under the P deprived mixotrophic culture (C:P ratio, 40:0). NPQ of excited chlorophylls (1Chl*), denoting the dissipation of excess excitation energy trapped by the photosynthetic apparatus (Chukhutsina et al., 2014), is another important photosynthetic parameter to characterize the bioenergetics of photosynthetic electron transport. The relative fluorescence decrease (Rfd) ratio represents both the photochemical and non-photochemical quenching components (Lichtenthaler et al., 2005). A simultaneous increase in the NPQ and Rfdin the P starved mixotrophic culture indicated a rise in the proton gradient of the thylakoid membrane, which was reduced in the presence of P, perhaps due to phosphate-dependent ATP synthesis and dissipation of the proton gradient of the membrane. Further, it was observed that PIabs value, denoting the photosynthetic performance index or vitality index (Stirbet et al., 2018), was found to be about two-fold higher in BG-11 grown cells than the P starved mixotrophic culture (C:P ratio, 40:0). However, the addition of phosphate to mixotrophic cultures (C:P

ratio, 40:3 and 40:5) resulted in a substantial increase in the PIabs values, which were higher than that in the BG-11 grown cells (control).

The results obtained in the present investigation revealed that synthesis of important cell constituents, except carbohydrate, in the *C. vulgaris* cells (protein, lipid, and TOC) was better in the mixotrophic culture than the BG-11 grown cells; and to some extent, the number of cell constituents increased with increasing phosphate concentration. The data on FTIR and EDS analysis revealed that the macromolecular and elemental composition of *C. vulgaris* cells was dependent on the mode of cultivation and the presence of phosphate. The photosynthetic efficiency of mixotrophic cultures was better than the BG-11 grown cells, provided that the mixotrophic culture was supplemented with sufficient phosphate concentration. Reduced photosynthetic performance and macromolecular synthesis in the P starved mixotrophic culture of *C. vulgaris* was presumably on account of higher NPQ and cessation of bioenergetic processes, which could be improved with the addition of phosphate.

Chapter VIII

Effect of C:N ratio on mixotrophic cultivation of Chlorella vulgaris

Effect of C:N ratio on mixotrophic cultivation of *Chlorella vulgaris*

8.1 Introduction

Microalgae are sunlight-driven cell factories that convert carbon dioxide to various products such as lipids, carbohydrates, proteins, vitamins, and several other value-added products (Chisti, 2006). The three most important nutrients such as carbon, nitrogen, and phosphorus are essential requirements of the microalgae for cellular functions and biomass production (Dou et al., 2013). Though nitrogen, phosphorus, silica (for diatoms), and iron are the limiting nutrients for the growth of microalgae (Neenan, 1986), nitrogen limitation has a stronger effect and directly influences photosynthesis, energy collection due to loss of chlorophyll (chl) and causes disturbances in other metabolic functions. The discoloration of algal cells is a common response of cells to nitrogen limiting conditions, but the accumulation of carotenoids, polysaccharides, and certain fatty acids such as polyunsaturated fatty acids (PUFAs) occur in the microalgae (Becker, 1994). The nitrogen requirement of microalgal cells, depending upon the different algal groups, can range from 1% to 10% (Grobbelar et al., 2004). But type and concentration of nitrogen source is the most critical factor for accumulation of lipid and carbohydrate content in the microalgae (Chen et al., 2011; Chisti, 2007). A wide variety of studies have demonstrated that microalgae tend to allocate their carbon molecules to energy-rich lipids or carbohydrates only when they encounter nitrogen deficiency in the medium (Hu et al., 2008; John et al., 2011). Nitrogen starvation of the microalgal cells leads to the synthesis of lipid in the form of triglyceride fatty acids as carbon and energy storage (Van Vooren et al., 2012). Nitrogen deprivation of cells causes a sharp increase in the ratio of lipid/carbohydrate in the microalgal cells with a marked

decrease in the protein and chlorophyll content (Ho et al., 2012; Sun et al., 2014). Su et al., (2011) reported that nitrogen-deprived microalga *N. oculata* accumulated about 48% lipid content in the cells. The cultivation of *S. obliquus* CNW-N and *C. vulgaris* FSP-E strains under nitrogen deprived conditions is accompanied by a dramatic increase in the cell carbohydrate from 21 to 49% and from 15 to 51%, respectively (Ho et al., 2013). The *Scenedesmus* sp. grown under nitrogen or phosphorus starved conditions showed higher lipid content (30 % and 53 %, respectively) (Xin et al., 2010). The cell constituents in microalgae under nitrogen limiting conditions showed a strong influence on both the lipid and carbohydrate contents of many microalgae (Ho et al., 2012). It has been shown that nitrogen starvation of microalgal cells leads to an increase in the proportions of saturated (e.g., palmitic acid) and monounsaturated (e.g., oleic acid) fatty acids, which are the primary constituents of biodiesel (Siaut et al., 2011).

Microalgae have attracted worldwide attention as a potential source of triglyceride oils for making biodiesel (Chisti, 2007; Hu et al., 2008; Huang et al., 2010). Microalgae can be cultivated by using photoautotrophic, heterotrophic, and mixotrophic modes of growth (Miao and Wu, 2006). The photoautotrophic process requires only sunlight, carbon dioxide, water, and inorganic nutrients (Chisti, 2013), but the biomass and biofuel production under photoautotrophic growth is low due to limited penetration of light in the dense culture of algae. The biomass and biofuel production in microalgae under heterotrophic growth conditions, using organic carbon under dark conditions, also seem to be growth restrictive as there is a requirement of light for many metabolic processes of microalgae. It has been emphasized that organic carbon in the heterotrophic mode of cultivation initially stimulates the biomass and oil content in the microalgal culture but gets depleted in the long run (Miao and Wu,

2006; Liu et al., 2011). Glucose addition in the culture is known to promote the physiological changes in *C. vulgaris*, which strongly affects the metabolic pathway of carbon assimilation, cell size, and storage of carbon (Perez-Garcia et al., 2011), protein, chlorophyll, and other cell constituents (Arora et al., 2021). The heterotrophically grown *Chlorella zofingiensis* has been reported to contain a much higher level of triglycerides as compared to the photoautotrophically grown alga (Liu et al., 2010).

The carbon and nitrogen metabolism in microalgae are interlinked and share common resources for the proper growth of microalgal cells. The primary assimilation of inorganic nitrogen (ammonium) to form amino acids requires carbon skeletons in the form of α-keto-acids and energy in the form of ATP and NADPH to synthesize the amino acids glutamate, glutamine, and aspartate. Very small quantities of keto-acids were found in *Chlorella spp*. when grown photoautotrophically, but the level of keto-acids was much higher under the heterotrophic conditions or nitrogen starvation (Millbank, 1957). However, the presence of higher keto-acids under the heterotrophic mode of nutrition could be interpreted as a reduced rate of nitrogen assimilation under the dark condition due to the tight coupling of photosynthesis and nitrogen assimilation. Thus, the higher biomass and lipid production in microalgae under a mixotrophic mode of cultivation can be tried as it offers the opportunity to manipulate carbon sequestration as well as nitrogen nutrition. The organic carbon acetate, a common building block for the biosynthesis of organic molecules, is also a potential organic waste from waste-activated sludge Moreover, it has also been demonstrated that microalgae initially synthesize carbohydrates to provide short-term energy and glucose is the main monosaccharide which is a feedstock for alcohol fermentation (Wang et al., 2016). The present investigation was carried out to enhance the

carbohydrate and lipid productivity, using acetate as exogenous organic carbon and varying level of nitrate nitrogen. It was assumed that an initial increase in the cellular carbohydrate content, triggered by cellular availability of organic carbon and nitrogen limiting condition, without seriously compromising the biomass productivity, might help in enhancing the lipid productivity (Heredia-Arroyo et al., 2010; Liu et al., 2010; Cho et al., 2011). However, there is a lack of serious effort in the direction of using varying organic carbon and nitrogen (C:N) ratios for attaining higher biomass and lipid yield. The present investigation aims to study the biomass, cell composition, nutrient status, and photosynthetic attributes of unicellular microalga *C. vulgaris* in response to varying C:N ratios. It is expected that the study would shed light on the mechanism of cellular adaptation to nitrogen limiting conditions, which is crucial for achieving higher lipid productivity.

8.2 Materials and Methods

8.2.1 Experimental design

For the experiment, microalgae *C. vulgaris* was grown in the Erlenmeyer flask (250 ml) in the presence of BG-11 medium (Stanier et al., 1971) (pH 7.5±0.2). The cells were cultured in 100 mL of BG-11 medium in a 250 ml conical flask. The microalga was grown in a culture room under the controlled conditions with a light intensity of 60 µmol photons/m^2/s and a light/dark cycle of 14/10 hours. The room temperature was maintained at 25 ±2^0C. For mixotrophic growth, the BG-11 medium (devoid of inorganic carbon and nitrogen) was supplemented with organic carbon and inorganic nitrogen in different ratios. The concentration of organic carbon in each flask was fixed (4.0 mM sodium acetate), whereas nitrate-nitrogen concentration was varied to maintain the different molar ratio of carbon: nitrogen (C:N ratio; 1:0, 1:1, 1:2, 1:3 and

1:4). A system growing algae in BG11 media served as control. All the experiments were conducted in triplicate.

8.2.2 Estimation of biomolecules

The protein content of the microalgal cell suspension was estimated by following the method of Lowery et al.,(1951), modified by Herbert et al.,(1971), using bovine serum albumin (BSA) as standard. The total carbohydrate content was determined using the phenol- sulfuric acid method as described by Dubois et al., (1956). Lipid analysis was performed gravimetrically based on a method given by Bligh and Dyer, (1959). (Page no. 66-71).

8.2.3 Fourier transform infrared spectroscopy analysis (FTIR) spectra

The *C. vulgaris* cells grown under different C:N were harvested in the exponential phase. The dried biomass was grounded and used for FTIR analysis as described in chapter –III of materials and methods.

8.2.4 Scanning electron microscopy (SEM) coupled with EDS

Scanning Electron Microscope and energy dispersive X-ray system was used to study the morphological alterations and mineral status of the different elements in the algal cells. The harvested cell samples grown under varying C:N ratios were analyzed by using SEM coupled with an energy dispersive X-ray spectrometer (EDS) detector and plotting was done using EDS in conjunction with scanning electron microscopy (SEM) (JEOL, Japan; model JSM-6490LV).

8.2.5 Fast chlorophyll fluorescence induction kinetics

Chlorophyll fluorescence induction kinetics and Non-photochemical quenching (NPQ) in dark-adapted cells harvested after 48 hours of experiment were measured by Pulse Amplitude Modulated (PAM) fluorimeter (Aquapen-C AP 110-C, Photon Systems Instrument, Czech Republic). Calculations were done as described in chapter-III of materials and methods.

8.3 Results

8.3.1 Effect of different C:N ratios on growth and chlorophyll content

The mixotrophic growth of *C. vulgaris* was monitored in terms of absorbance of the culture at 665 nm for 10 days (Fig.8.1) under varying C:N (1:0, 1:2, 1:3, and 1:4) ratios. The cell growth of *C. vulgaris* cells in the presence of BG-11 medium (without exogenous organic carbon), under a similar set of growth conditions, served as control. Keeping the concentration of acetate carbon constant i.e., 4.0 mM in the growth medium under mixotrophic growth conditions, the nitrogen concentration was proportionately increased (C:N ratio of 1:1, 1:2, 1:3, and 1:4). The results revealed that the addition of acetate carbon during the growth of *C. vulgaris*, stimulated the growth, which proportionately increased with increasing C:N ratios (1:0, 1:1, 1:2, and 1:3), when compared with the control (BG-11 grown cells). However, the growth of *C. vulgaris* cells at C:N ratio of 1:4 slightly declined when compared with C:N ratio of 1:3, but the overall growth was about two-fold higher than that observed in the BG-11 grown cells. The results suggested that the growth of *C. vulgaris* was stimulated by both organic carbon and nitrogen sources, predominantly due to the contribution of organic carbon. The results on total chlorophyll in the microalga showed a gradual

increase in the total chlorophyll pigment with an increasing C:N ratio (1:0-1:1) as compared to BG-11 grown cells (control). A further increase in the C:N ratio (1:2, 1:3, and 1:4) exhibited a relative decline in the total chlorophyll content as compared to C:N ratio (1:1). However, the overall chlorophyll content at higher C:N ratios was comparatively similar to that in the BG-11 grown cells (control). These results indicated that unlike the growth response of microalga to different C:N ratios, the presence of organic carbon could essentially stimulate the synthesis of chlorophyll pigment, irrespective of the level of nitrogen.

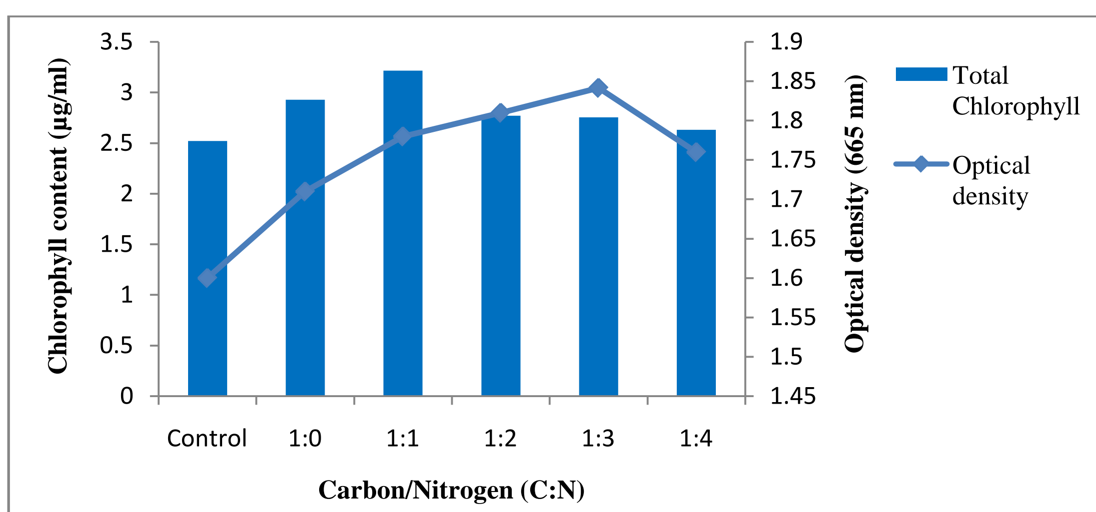

Fig.8.1- Effect of different C:N ratio (1:0-1:4) on the chlorophyll content (primary axis) and growth (secondary axis) of *C. vulgaris* cells. The cells were grown in BG-11 medium (without organic carbon) served as control.

8.3.2 Effect of different C:N ratios on protein and carbohydrate content

Protein and carbohydrate content of *C.vulgaris* cells was measured in response to varying C:N ratio (1:0-1:4) under mixotrophic growth conditions (Fig.8.2). The protein and carbohydrate content in *C. vulgaris* cells grown in BG-11 growth medium served as control. The results showed that initially, the protein content

increased with increasing C:N ratio (1:0 and 1:1) as compared to BG-11 grown cells (control). A further increase in the C:N ratio from 1:2 to 1:4 led to a slight decline in the protein content of *C.vulgaris* cells as compared to protein content in the cells obtained at C:N ratio of 1:1, which was still higher than the control cells (BG-11 grown cells). These results clearly showed an increase in the protein content due to the addition of an organic carbon source. However, the synthesis of protein in the cells was also stimulated due to the addition of different doses of nitrogen throughout the range of C;N ratios, as compared to the protein content in the control (BG-11 medium devoid of organic carbon), On the other hand, the carbohydrate content in the *C. vulgaris* cells gradually increased with increasing nitrogen content throughout the range of C:N ratios (1:0-1:4) as compared to BG-11 grown cells (control). There was about 25% increase in the level of carbohydrate at C:N ratio of 1:4, which was found to be the highest. The results demonstrated that the carbohydrate synthesis was enhanced due to an increase in the nitrogen component of the medium as well as exogenous addition organic carbon sources. Further, the results also revealed that the higher C:N ratio, which was unfavorable to the growth of *C. vulgaris* under the mixotrophic mode of cultivation, could also support the enhanced synthesis of carbohydrate content.

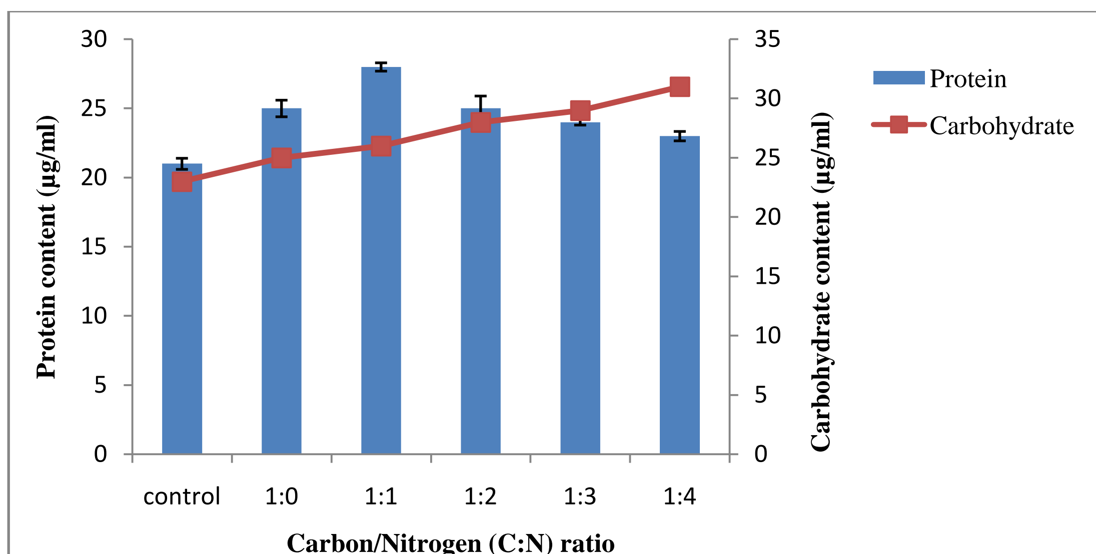

Fig.8.2 Effect of different C:N ratio (1:0-1:4) on the protein (primary axis) and carbohydrate content (secondary axis) of *C. vulgaris* cells. The cells were grown in BG-11 medium (without organic carbon) served as control. Data are the mean of three replicates ± SD.

8.3.3 Effect of different C:N ratios on TOC and lipid content

TOC and lipid content in the microalga *C. vulgaris* cells was measured in response to varying C:N ratios (1:0-1:4). The cells were grown in the BG-11 medium (devoid of organic carbon source) served as control (Fig.8.3). The results on TOC showed a gradual increase in the level of TOC under different C:N ratios up to ratio of 1:3. There was a slight decrease in the TOC content at C:N ratio of 1:4. At C:N ratio of 1:3, the TOC content was enhanced by about 80% as compared to BG-11 grown cells (control). The results clearly showed that the TOC level in the microalga was enhanced due to the addition of both organic carbon and nitrogen as evident from the elevated TOC content throughout the range of C:N ratios. On the other hand, the cellular lipid content in the *C. vulgaris* cells increased in response to C: N ratio up to a ratio of 1:3 as compared to control (BG-11 medium grown cells). A further increase

in the C:N ratio from C:N ratio of 1:2 to 1:4 showed a decline in the lipid content, which corresponded with the growth of *C. vulgaris* cells. Thus, the C:N ratio of 1:2 was the optimum condition with about 30% increase in the lipid content in the *C.vulgaris* cells as compared to the control cell (grown in BG-11 medium devoid of organic carbon). These results, thus, revealed that the addition of organic carbon source under the mixotrophic mode of cultivation stimulated the synthesis of lipid, irrespective of the level of nitrogen source, which gradually declined with an increase in the nitrogen sufficient condition as evident from the adverse effect of higher C:N ratios (1:3 and 1:4). Thus, the results suggested that organic source of carbon with limiting nitrogen nutrition was more favourable for lipid synthesis.

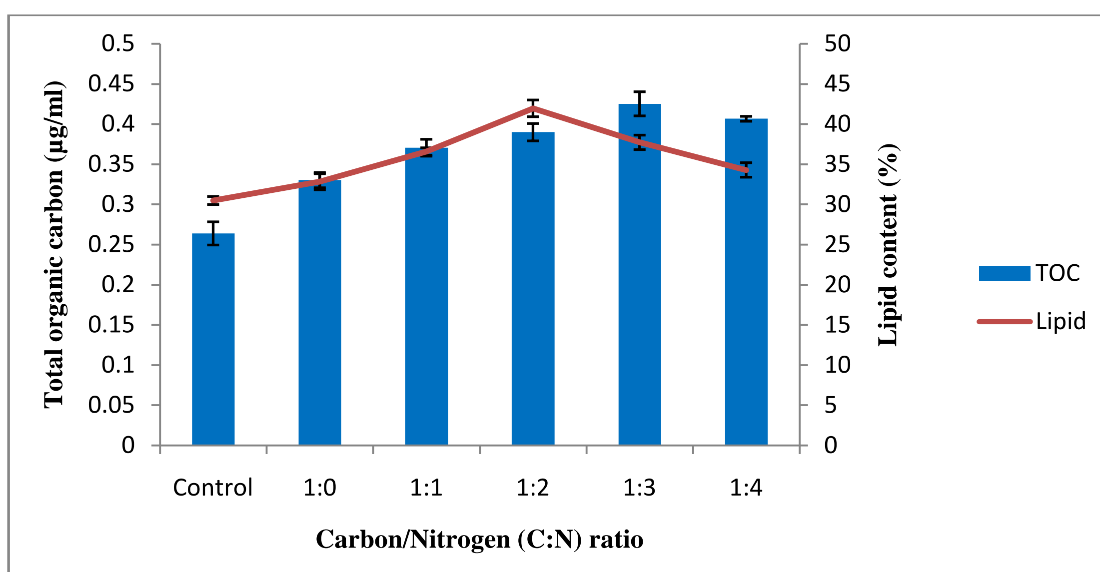

Fig.8.3- Effect of different C:N ratio (1:0-1:4) on the total organic carbon (primary axis) and lipid content (secondary axis) of *C. vulgaris* cells. The cells were grown in BG-11 medium (without organic carbon) served as control. Data are the mean of three replicates ± SD.

8.3.4 FTIR analysis of cell biomass of *C. vulgaris* cells grown in BG-11 medium (control) and different C:N ratios (1:0 and 1:3)

The FTIR spectra of cell biomass of *C. vulgaris* grown BG-11 medium (control) and medium with different C:N ratios (1:0 and 1:2) showed variation in the IR absorption peaks of signature molecules such as carbohydrate (1040 cm^{-1}), protein (1650 cm^{-1}) and total lipid (1740 cm^{-1}) content. The FTIR spectra of cell biomass of *C. vulgaris* (Fig.8.4) depicted major changes in the IR peaks at 2925, 1650, and 1040 cm^{-1} wavenumbers, associated with lipids, proteins, and carbohydrates, respectively. A significant increase in the IR absorption peaks of the microalgal cell biomass grown in BG-11 medium or C:N ratio of 1:2 (nitrogen sufficient condition) higher protein content (1650 cm^{-1}) and moderate carbohydrate content (1040 cm^{-1}). But the *C. vulgaris* cells grown under nitrogen-deficient condition at C:N ratio of 1:0 depicted higher IR absorption at wavenumbers 2925, 2960 and 1150 cm^{-1}, depicting the enhanced accumulation of lipid and carbohydrate, particularly the starch molecules (1150 cm^{-1}) than the cells grown in BG-11 and at C:N ratio of 1:2. Besides, the IR absorbance peak at 1385 cm^{-1} wave number, associated with lipoproteins in the cells, was found to be higher at C:N ratio of 1:2, followed by BG-11 grown cells. The absorbance peak in the *C. vulgaris* cells grown under the nitrogen deprived condition (C:N ratio of 1:0) was highly reduced, perhaps due to reduced synthesis of protein. The results in Table- 8.1 showed that the total lipid content of mixotrophic culture of *C. vulgaris* maintained at C:N ratio of 1:0 was the highest as compared to control (BG-11 grown cells) and C:N ratio of 1:2. The results suggested that *C. vulgaris* cells accumulate more carbohydrate and lipid under the nitrogen limiting condition, irrespective of organic carbon source. The lipid/carbohydrate (1740/1040 cm^{-1}) and lipid-protein (1740/1650 cm^{-1}) ratios were higher in the case of cells grown under

nitrogen deprived condition at C:N ratio of 1:0, when compared with the cells grown in BG-11 (control) and C:N ratio of 1:2. Perhaps the nitrogen sufficient condition restored the normal physiological conditions, which was no more stress condition where the storage of high energy carbon compounds was required. This assumption was corroborated by an enhanced accumulation of lipid and starch under the nitrogen-deprived condition. Further, results on the ratio of saturated/ unsaturated (CH_3/ CH_2) fatty acids (2964/2924 cm^{-1}) in the *C.vulagris* cells showed maximum saturated lipids in the BG-11 medium grown cells, followed by the cells grown at C:N ratio of 1:0 and 1:2. Thus, it could be inferred that the reduced ratio of CH_3/CH_2 was on account of enhanced synthesis of unsaturated fatty acids due to the addition of exogenous organic carbon for a mixotrophic mode of cultivation.

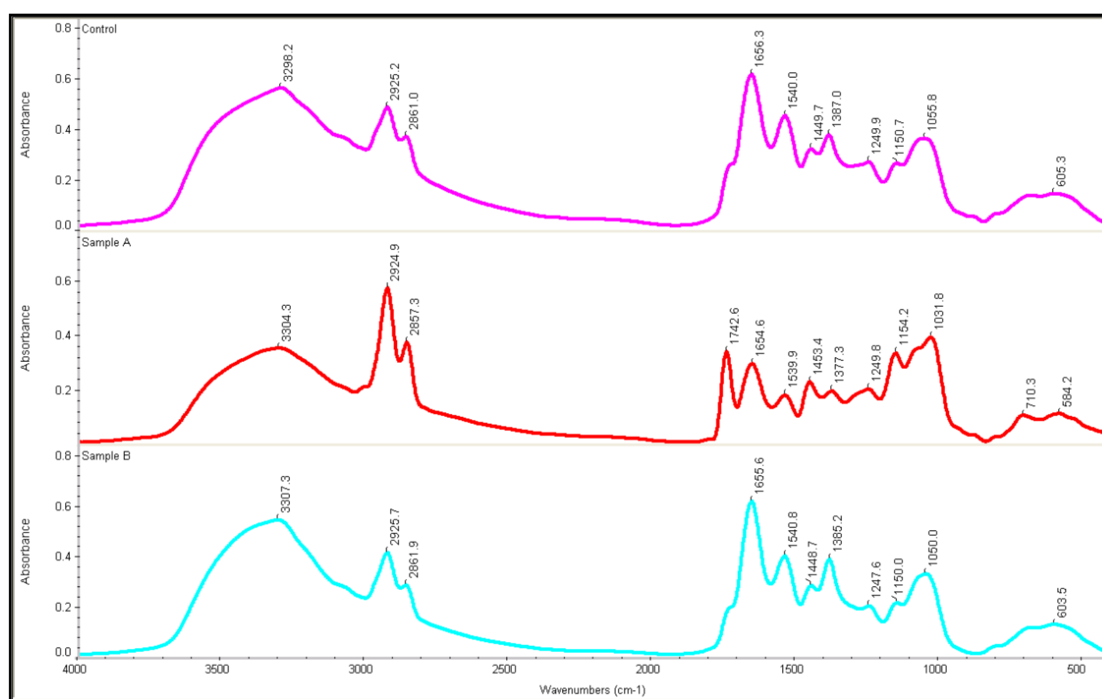

Fig. 8.4. FTIR analysis of cell biomass of *C. vulgaris* cells grown in BG-11 medium (control), in the medium supplemented with organic carbon without nitrogen source (sample A) at C:N ratio of 1:0 and in the presence of both organic carbon as well as nitrogen (sample B) at C:N ratio of 1:3.

Table. 8.1. The table depicts the relative content of the total lipid (1740 cm^{-1}), ratios of lipid/ carbohydrate (1740/1040 cm^{-1}), lipid/protein (1740/1650 cm^{-1}) and ratio of saturated/ unsaturated fatty acids (2964/2924 cm^{-1}). The data were derived from the FTIR spectra (4000-500 cm^{-1}) of cell biomass of *C. vulgaris* grown in BG-11 medium and different C:N ratio (1:0 and 1:2) as given in Fig. 8.4.

Mode of cultivation	Total lipid (1740 cm^{-1})	Lipid/Carbohydrate (1740/1040 cm^{-1})	Lipid/protein (1740/1650 cm^{1})	Saturated/ unsaturated lipids (2964/2924 cm^{-1})
BG-11 (Control)	0.70	0.77	0.43	0.76
C:N (1:0)	1.00	0.90	1.10	0.64
C:N (1:2)	0.5	0.55	0.29	0.58

8.3.5 EDS analysis of elements in *C. vulgaris* in response to different C:N ratios

The EDS data obtained from the *C. vulgaris* cells grown in BG-11 medium as well as under mixotrophic mode of cultivation with varying ratios of C:N (1:0-1:4) exhibited vast variations in elements localized in the K shell (Fig. 8.5). The results of EDS data at different C:N ratios (1:0, 1:2, and 1:4) showed an increasing trend in the weight % of K (from 0.76 to 1.12), Na (0.68 to 3.46), Ca (from 0.09 to 0.47) and Mg (from 0.80 to 1.22). However, the weight % of these elements was higher at C:N ratio of 1:4 when compared with BG-11 grown cells (control). However, the level of Na element was found to be several-fold higher in the cells at different C:N ratios when compared with the control (BG-11medium grown *C. vulgaris* cells). This could be attributed to the increasing concentration of nitrogen in mixotrophic culture. The lowest values of these elements at C:N ratio of 1:0 could be due to metabolic disorder in the absence of nitrogen and molecular interference of acetate organic carbon in the transport and accumulation of these essential nutrients. Further, the EDS results showed that level of C (weight %) was the highest in the BG-11 grown control cells, whereas the weight

% of C declined with increasing level of nitrogen and the lowest value of C was recorded at C:N ratio of 1:4 (49.9), which was about 32 % lower than that in the control. However, the weight% of P in the K shell increased due to the addition of acetate carbon as compared to control. Thus, it could be concluded that the weight percent of Ca, P, Mg, and Na increased under the nitrogen sufficient condition, while the C level declined. Thus, the mixotrophic cultivation of microalgae resulted in an enhanced accumulation of certain essential elements, particularly under the nitrogen sufficient condition, when compared with photoautotrophic growth in BG-11 medium.

(a)

Element	Weight%	Atomic%
C K	61.99	73.08
O K	27.89	24.69
Na K	0.68	0.42
Mg K	0.72	0.42
S K	1.01	0.45
K K	1.08	0.39
Ca K	0.27	0.10
Pt M	6.35	0.46
Totals	100.00	

(b)

Element	Weight%	Atomic%
C K	58.75	71.45
O K	25.89	23.63
Na K	4.11	2.61
Mg K	0.80	0.48
P K	1.05	0.50
S K	0.98	0.45
K K	0.76	0.29
Ca K	0.09	0.03
Pt M	7.56	0.57
Totals	100.00	

(c)

Element	Weight%	Atomic%
C K	51.36	64.72
O K	31.51	29.81
Na K	4.50	2.96
Mg K	0.66	0.41
P K	1.09	0.53
S K	1.03	0.49
K K	0.90	0.35
Ca K	0.12	0.04
Pt M	8.84	0.69
Totals	100.00	

(d)

Element	Weight%	Atomic%
C K	49.94	65.33
O K	27.35	26.86
Na K	3.46	2.36
Mg K	1.22	0.79
Al K	1.31	0.76
Si K	3.06	1.71
S K	0.95	0.47
Cl K	0.23	0.10
K K	1.11	0.45
Ca K	0.47	0.18
Fe K	0.56	0.16
Pt M	10.34	0.83
Totals	100.00	

Fig. 8.5- Effect of different C:N ratios on the photosynthetic parameters in *C. vulgaris* cells grown under mixotrophic conditions. (a) control (BG-11 grown) (b) 1:0 (C:N) (c) 1:2 (C:N) (d) 1:4 (C:N).

Fast chlorophyll fluorescence induction kinetics, using PAM fluorimeter, was measured in *C. vulgaris* cells grown under different C:N ratios (1:0-1:4). All the photosynthetic parameters were derived from the OJIP curve of the fluorescence induction kinetics of dark-adapted (for 10 minutes) cells (Table 2). The value of Fv/Fm in the dark-adapted cells is commonly used to assess the effect of the 'stress' condition (Murchie et al. 2013), reflecting the high sensitivity of PSII to environmental stimuli. Quantum yield (Fv/Fm) of PSII in *C. vulgaris* cells grown in BG-11 medium and at C:N ratio of 1:2 was found to be the highest (0.73) and the lowest value of quantum yield was under nitrogen deprived condition (C:N ratio of 1:0). Total photons absorbed by the pigment antenna of PSII and electron transfer of energy per reaction centre, denoted by ABS/RC and ET_o/RC, respectively (Wang et al., 2012) were found to be the highest at C:N ratio of 1:1 and the lowest value was under the nitrogen deprived condition at C:N ratio of 1:0. It is assumed that photosynthetic quantum yield and photosynthetic efficiency of PSII were reduced in the absence of nitrogen at C:N ratio 1:0. Earlier it has been reported that an increased level of reduced QA under the nitrogen starved condition could be due to a decline in the CO_2 fixation and reduced probability of electron sink (Strasser et al., 2004). The photosynthetic parameter PIabs is an indicator of plant vitality, which represents the overall photosynthetic performance index in photosynthetic organisms (Stirbet et al., 2018). The present results on PIabs in the microalga *C. vulgaris* revealed the highest PIabs value (17.2) at C:N ratio of 1:2, followed by that in the BG-11 grown cells (15.8). The lowest PIabs value (13.8) was obtained in the cell grown at C:N ratio of 1:0, irrespective of the organic carbon source. The results indicated that nitrogen nutrition has an important role in photosynthetic activity.

NPQ of excited chlorophylls (1Chl*) denotes the dissipation of excess excitation energy trapped by the photosynthetic apparatus (Chukhutsina et al., 2014). The present results showed the highest NPQ value (0.39) at C:N ratio of 1:1, followed by the NPQ (0.36) in the BG-11 grown cells. The lowest NPQ value was noted in the case of nitrogen starved cells (0.27) at C:N ratio of 1:0. A higher NPQ is an indicator of the protection efficiency of the cells against the light stress-induced damage to photosynthetic apparatus (Ruban, 2016). The present results indicated that non-photochemical quenching was in proportion to the quantum yield of the microalgal cells. The Relative fluorescence decrease (Rfd) ratio represents both the ratio of both photochemical and non-photochemical quenching, and it is also proportional to the net CO_2 assimilation rate (Lichtenthaler et al., 2005). The Rfd value in the *C. vulgaris* cells grown at C:N ratio of 1:1 was not only found to be the highest, but it was about two-fold higher than that observed in the BG-11 grown cells (control) as well as nitrogen starved condition at C:N ratio of 1:0. These results revealed that quantum yield (Fv/Fm), as well as photosynthetic performance (PIabs) of the microalga *C. vulgaris*, was found to be the best at C:N ratio of 1:2, which was better than that in the BG-11 grown cells (control). On the other hand, energy absorption (ABS/RC), electron transfer (ETo/RC) as well as non-photochemical protection efficiency (NPQ) in the photosynthetic apparatus were better at C:N ratio of 1:1. There is the probability that the absence of nitrogen (C:N ratio of 1:0) or higher level of nitrogen (C:N ratio of 1:4) even in the presence of organic carbon exerts an adverse effect on the photosynthetic machinery, but the overall photosynthetic efficiency was better at C:N ratios (1;1 and 1:2) with the low level of nitrogen. These observations were also corroborated by the Rfd value, which indicated higher net CO_2 assimilation (Lichtenthaler et al., 2005). The earlier findings have also demonstrated that

carbohydrate synthesis in microalgae was better under the nitrogen-sufficient condition.

Table-8.2- Photosynthetic parameters (Fv/Fm, NPQ, PIabs, ABS/RC, ETo/RC, and Rfd) were derived from the OJIP curve of fast chlorophyll fluorescence induction kinetics in the microalga *C. vulgaris* grown under varying C:N ratio. Data are the mean of three replicates ± SD.

C:N	Fv/Fm	NPQ	PIabs	ABS/RC	ETo/RC	Rfd
BG-11	0.73±0.022	0.36±0.027	15.8±0.008	1.06±0.01	0.66±0.023	0.23±0.025
1:0	0.63 ±0.031	0.27±0.035	13.8±0.005	1.04 ±0.025	0.54 ±0.043	0.23±0.016
1:1	0.66 ±0.042	0.39 ±0.04	14.5 ±0.023	1.47 ±0.036	0.73 ±0.02	0.46±0.023
1:2	0.73 ±0.035	0.29 ±0.023	17.2 ±0.026	1.42 ±0.071	0.64 ±0.033	0.24±0.025
1:4	0.71 ±0.025	0.28 ±0.038	14.5 ±0.032	1.13 ±0.053	0.63 ±0.041	0.19±0.038

8.4 Discussion

Mixotrophic cultivation of microalgae could greatly enhance microalgal growth with various nitrogen sources (Chen & Zhang, 1997; Chojnacka & Noworyta, 2004; Li et al., 2018). Nutrient limitation affects the growth and photosynthetic capacity of microalgae, and it severely restricts cell division and alters cell composition (Reitan et al., 1994). Ma et al., (1997) evaluated the growth of *C. zofingiensis* in semi-continuous culture with special emphasis on nitrogen and carbon metabolism and suggested that if nitrogen supply is limited in proportion to other elements, photosynthesis may continue but the resultant compounds will include a smaller proportion of nitrogen-rich components, greater amount of more energy-rich components such as lipids and carbohydrates. In the present investigation, microalgal growth was enhanced under the mixotrophic mode of cultivation up to C:N ratio of 1:3 (i.e., 4 mM of sodium acetate and 12 mM of sodium nitrate) as compared to BG-

11 medium grown cells (devoid of organic carbon), while chlorophyll and protein content showed increase up to C:N ratio of 1:1 i.e., an equimolar ratio of sodium acetate and sodium nitrate (4 mM, each). On the contrary, the cellular carbohydrate and TOC content in the *C. vulgaris* cells showed a rising trend with an increasing C:N ratio up to 1:4. However, the lipid content in the *C.vulgaris* cells showed a maximum increase only up to C:N ratio of 1:2. FTIR analysis of the cells at C:N ratio of 1:0 and 1:2 showed better accumulation of lipid in the nitrogen deprived mixotrophic culture when compared with BG-11 grown cells. The increased IR absorbance at 1740 and 1150 cm^{-1} wavenumber indicated higher accumulation of lipid and starch in *C. vulgaris* cells under the nitrogen deprived condition (C:N ratio of 1:0) when the protein synthesis is severely decreased as evident from reduced absorbance at 1650 and 1540 cm^{-1}. Earlier findings have also shown an opposite response of carbon and nitrogen metabolism, where enhanced carbohydrate accumulation in the microalgae was simultaneously accompanied by reduced synthesis of protein, particularly proteins of PSI and PSII reaction centres rather than cytoplasmic proteins. Similarly, Ma et al., (1997) suggested that if the nitrogen supply is limited in proportion to other elements, photosynthesis may continue but there would be a small proportion of nitrogen-rich components and a greater amount of more energy-rich components such as lipids and carbohydrates. However, ratio of saturated/ unsaturated (CH_3/ CH_2) fatty acids (2964/2924 cm^{-1}) in the *C. vulagris* cells showed a maximum level of saturated lipids in the BG-11 medium grown cells, followed by the cells grown at C:N ratio of 1:0 and 1:2.

Acetate is also known to be lipid-soluble and interferes with the membrane transport of phosphate and ATP synthesis. It has been reported that during mixotrophic cultivation of microalga in the presence of acetate as an organic carbon source for

three days, the consumption of acetate by cells was found to be slowed down because of the nitrogen limiting condition, suggesting that acetate assimilation would be correlated to nitrate nutrition under mixotrophic cultivation. This was perhaps the reason for an increase in the TOC and carbohydrate content in the *C. vulgaris* with an increasing C:N ratio. Earlier, it has been reported that the addition of an optimal amount of acetate in the mixotrophic cultivation could markedly improve the carbohydrate synthesis in the *Chlorella sorokiniana* NIES-2168, which was the highest value ever reported (Wang et al., 2016). Thus, the present results demonstrated that acetate could be a great inducer for both cell growth and carbohydrate synthesis, which was in agreement with previous reports (Chen et al., 2016), but nitrogen nutrition was also crucial for the synthesis of cell components and biomass production. The present results on EDS analysis of essential nutrients showed that the level of Ca, P, Mg, and Na increased under the nitrogen sufficient condition, while the C level declined. Thus, it was concluded that the mixotrophic cultivation of microalgae could also enhance the accumulation of certain essential elements, particularly under the nitrogen sufficient condition, which might be contributing to the increase in biomass production.

Marquez et al., (1993) and Hata et al., (2000) suggested that mixotrophic culture of algae involves simultaneous uptake of organic compounds as well as CO_2 as carbon sources for cell synthesis, indicating that photosynthesis continues to be functional. Whereas Martinez et al., (1997) suggested that in the mixotrophic cultivation of micoalgae, the photosynthetically derived energy mainly contributed to the growth and cell maintenance, while the organic carbon source was utilized for biomass production. It appears interesting to study the behavioral pattern of the photosynthetic machinery in *C. vulgaris* under the mixotrophic mode of cultivation, particularly in

the presence of acetate as an organic carbon source and nitrate as a nitrogen source. The quantum yield (Fv/Fm) of PS II in the dark-adapted cells has been commonly used to assess the effect of 'stress' condition (Murchie et al., 2013) and is considered as the most sensitive parameter of PSII against environmental stimuli. In the present study, Quantum yield (Fv/Fm) and photosynthetic performance index (PIabs) of PSII in *C. vulgaris* cells were found to be the highest at C:N ratio of 1:2, while both the parameters showed the lowest value at C:N ratio of 1:0 as compared to BG-11 grown cells (control). The photosynthetic parameter PIabs is an indicator of plant vitality, which represents the overall photosynthetic performance index in photosynthetic organisms (Stirbet et al., 2018). Similar to our results, previous reports have also shown the negative effect of higher ammonium nitrogen on the microalgal photosynthetic performance in the *Spirulina platensis* and *C. vulgaris* (Li et al., 2016; Markou et al., 2017).

Non-photochemical quenching (NPQ) of excited chlorophylls (1Chl*) denotes the dissipation of excess excitation energy trapped by the photosynthetic apparatus (Chukhutsina et al., 2014). The present results showed the highest NPQ value (0.39) at C:N ratio of 1:1. A higher NPQ value is an indicator of the protection efficiency of the cells against stress-induced damage to photosynthetic apparatus (Ruban, 2016). The Relative fluorescence decrease (Rfd) ratio is proportional to the net CO_2 assimilation rate (Lichtenthaler et al., 2005). The Rfd value in the *C. vulgaris* cells grown at C:N ratio of 1:1 was found to be about two-fold higher than that observed in the BG-11 grown cells (control). On the other hand, energy absorption (ABS/RC), electron transfer (ETo/RC) as well as non-photochemical quenching efficiency (NPQ) in the photosynthetic apparatus were better at C:N ratio of 1:1. The microalgal photosynthetic protection mechanism can be evaluated by NPQ value (Li et al., 2016;

Markou et al., 2017) which was found to be higher in the mixotrophic cultivation than the autotrophic mode of growth. However, the absence of nitrogen at C:N ratio of 1:0 or a higher level of nitrogen as at C:N ratio of 1:4 might be exerting an adverse effect on the photosynthetic machinery. The earlier workers have demonstrated that excess ammonium nitrogen had a negative effect on the specific energy fluxes ABS/RC, ETo/RC, and NPQ related to light energy absorption, conversion, and dissipation in both the autotrophic and mixotrophic cultivations (Li et al., 2018). There could be a probability of a fewer number of QA-reducing PSII reaction centres (RCs) with an increase in the size of some RCs.

Chapter IX

Summary and Conclusion

9.1 Summary and Conclusion

The impending danger of global warming and climate change is now visible in the world panorama. The high concentration of CO_2, the most important GHG, needs to be minimized. Although CCS methods have been worked upon to a large extent, they are unmanageable in terms of cost-effectiveness and their long-term environmental safety is a cause of concern. Bio-sequestration of carbon in organic, inorganic, or gaseous form using microalgae, the sun-driven sink of carbon has emerged as an alternative way of converting CO_2 into biomass. In both the natural and engineered systems the amount of carbon fixed in the form of biomass and lipid is highly influenced by nutrient availability and environmental factors. Literature reveals that significant work has been done on carbon sequestration via microalgae. Very few studies are available on the effect of physiological and physico-chemical factors on the photosynthetic apparatus and macromolecule of microalgae. Therefore, the present thesis is devoted to understanding the synergistic interaction among various physiological, physico-chemical factors and nutritional variations to develop a highly productive bio algae system, which is much needed for commercializing biofuel production.

In the present investigation, an attempt has been made to study the ability of *C.vulgaris* to withstand the various environmental stresses and nutritional alterations. The overall stress tolerance ability was exploited for carbon sequestration, biomass production, and improvement in lipid synthesis, which serves as a source of biofuel. The first objective of the work was to isolate a strain of microalga, which has the potential to sequester more carbon and could yield a better amount of biomass. The carbon sequestration was studied in three different forms of carbon, i.e, organic

carbon, inorganic and gaseous forms of carbon. The growth of microalgae under harsh environmental conditions such as varied pH conditions, excess CO_2 supply combined with high light intensity, different spectral quality of light, salinity stress, limited nitrogen nutrition, and phosphorous nutrition, was also optimized concerning photosynthetic efficiency, biomass, total organic carbon content, proline, and lipid content.

The present research work can be concluded as:

- The cells of *C. vulgaris* in response to the addition of $NaHCO_3$ (0-25 mM) showed the best growth and maximum concentration of all the biomolecules (protein, lipid and carbohydrate).
- The cells of *C. vulgaris* grown in the presence of NaCl concentrations (0-400 mM), with and without bicarbonate (20 mM) showed a higher level of growth, protein, and carbohydrate, suggesting an improved NaCl tolerance of microalga due to the presence of bicarbonate. However, total lipid and proline content showed an increase with the rising concentration of NaCl, particularly in the absence of bicarbonate.
- The addition of $NaHCO_3$ (20 mM) exhibited its antagonistic effect against the adverse effect of salinity on the growth, level of macromolecules except for proline. This was further confirmed by the SEM-EDS analysis of NaCl treated cells, exhibiting morphological variations as well as reduced accumulation of Na and Cl. The chlorophyll fluorescence induction kinetics revealed NaCl induced decline in the photosynthetic performance and quantum yield was mitigated by the presence of $NaHCO_3$, while non-photochemical quenching of chlorophyll fluorescence was enhanced.

- The present findings on microalga *C. vulgaris* showed a pH-dependent increase in the level of protein and carbohydrate content by the addition of bicarbonate, particularly between pH 7.5-8.5. However, the presence of $NaHCO_3$ enhanced the total organic carbon (TOC) and lipid content with increasing pH (6.5 to 10.5). These observations were supported by the FTIR analysis of cell constituents in response to pH as well bicarbonate.

- The photosynthetic parameters like Fv/Fm, Mo, ABS/RC, and ETo/RC showed optimum values at pH 8.5. However, these parameters were negatively influenced by the addition of HCO_3^-, particularly under alkaline pH conditions (pH 8.5-10.5). The values ABS/RC, NPQ, and qE values significantly increased with increasing alkalinity as well as with the addition of HCO_3^-.

- The effect of enhanced alkalinity, as well as bicarbonate, was significantly reversed by the uuncouplers and ptrotonophores, suggesting that the maximum adverse effect of both bicarbonate alkalinity was mediated by the high proton gradient across the membrane.

- Microalga *C. vulgaris* grown under different concentrations of CO_2 as well as light intensity (50-300 µmol photons/m2/s). The optimum growth of *C. vulgaris* was observed at 5% CO_2 and 150 µmol photons/$m^{2/}$s. The cell constituents like chlorophyll, protein, and carbohydrate in the cells at low light intensities were enhanced in the presence of excess CO_2 (5%) as compared to air-grown cell culture.

- The lipid content showed a light intensity-dependent gradual increase of up to 300 µmol photons/$m^{2/}$s, which was further increased in the presence of excess CO_2.

- The photosynthetic parameters like Fv/Fm, Fv/Fo, Mo, Vj, ABS/RC, and TRo/RC showed that excess CO_2 supported improved functioning of photosynthetic apparatus under low light (50 µmol photons/$m^{2/}$s) (LL). However, under the high light (HL) condition, the photosynthetic parameters indicated that the primary photochemistry of PSII was negatively influenced by the excess CO_2.

- The results showed a synergistic effect of CO_2 and high light intensity on the photochemistry of PSII as evident from was an overall decline in the photosynthetic performance index (PIabs), non-photochemical quenching (NPQ), and relative fluorescence decrease (Rfd) ratio in the presence of excess CO_2.

- The *C. vulgaris* cells supplemented with glucose, sodium acetate, and sodium citrate (2-20 mM, each) were grown photoheterotrophically for 16 days. The results revealed that microalga initially grew better in the presence of glucose up to 7th day, followed by a gradual decline, perhaps due to depletion of the substrate. The sodium acetate showed its best effect on the *C. vulgaris* up to 16th day as compared to control (without organic substrate).

- Effect of different molar C (Acetate, 4 mM): N (0-20 mM) ratio on the growth of microalga revealed maximum growth, Protein, and chlorophyll up to C:N ratio of 1:1. However, carbohydrate content linearly increased with increasing C:N ratio (1:0- 1: 4). The TOC and lipid content increased up to C:N ratio of 1:2 and 1:3.

- The total lipid and lipid/carbohydrate and lipid/protein ratio declined with increasing C:N ratio, indicating that N-straved condition was favorable for lipid synthesis. As evident from the FTIR results, with an increasing level of nitrogen, there is a substantial increase in the protein and carbohydrate content.

- The photosynthetic parameters Fv/Fm, ABS/RC, PIabs, ETo/RC, NPQ, and Rfd showed improvement in the photosynthetic performance with increasing of C:N. The highest values of NPQ and Rfd in absence of nitrogen source indicated the reduced photochemical activity and inhibition of photochemistry of PSII. The C:N molar ratio of 1:2 was found to better to maximum photosystem yield and electron transport. The overall photosynthetic was relatively uninfluenced by the presence of organic carbon when grown photoheterotrophically.

- Effect of different molar C (Acetate, 4 mM): P (0-0.5 mM) ratio on the growth of microalga revealed maximum growth, Protein, and chlorophyll up to C:P ratio of 40: 16. However, carbohydrate content linearly increased up to C:P ratio of 40:4. The TOC and lipid content declined with an increase in C:P ratio. As evident from the FTIR results, with an increasing level of nitrogen, there is a substantial increase in the protein and carbohydrate content, but not in the lipid.

- The photosynthetic parameters Fv/Fm, ABS/RC, PIabs, ETo/RC, NPQ, and Rfd showed improvement in the photosynthetic performance with increasing of C:P ratio. The highest values of NPQ and Rfd were observed in the P-starved starved cells, indicating reduced photochemical activity and inhibition of the photochemistry of PSII.

Thus, this study offers proof of the concept of mitigating carbon and simultaneous production of algal biomass, carbohydrates, and proteins for various potential applications. Therefore, based on the present findings further attempts should be made for large-scale cultivation of *C.vulgaris* in outdoor conditions, our findings on pH, salinity tolerance, light intensity, and CO_2 supply should be exploited for harvesting a better amount of biomass.

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APPENDIX

PUBLICATIONS

Research papers

- Nisha Yadav, D P Singh (2021). Photosynthetic efficiency and compositional alterations in microalgae *Chlorella vulgaris* in response to changes in the pH condition. *Vegetos. Springer*. 34(1), pp.127-137.

- Nisha Yadav, Neha Gupta, D P Singh (2021). Ameliorating effect of Bicarbonate on salinity induced changes in the growth, Nutrient status, Cell constituents and Photosynthetic attributes of microalga *Chlorella vulgaris. Bulletin of Environmental Contamination and Toxicology*. Pp.1-9.

Book chapter

- Nisha Yadav and D P Singh (2020). Microalgae and Microorganisms: Important Regulators of Carbon Dynamics in Wetland Ecosystem. In book: Restoration of Wetland Ecosystem: A Trajectory Towards a Sustainable Environment (pp.179-193).

Workshops and conferences attended

- Presented a poster entitled "Factors regulating carbon sequestration, lipid production and biomass in microalgae" in an International Conference on Strategies for Environmental Protection and Management (ICSEPM-2016) mini-symposium on Environmental Biotechnology, Biorefinery and Solid Waste Management (BRSI), organised by Jawaharlal Nehru University, New Delhi, 11-13.
- Attended a National symposium on "IPRs in Agricultural Research" organised by B.B. Ambedkar University, Lucknow, (U.P.) and U.P. Council of Agricultural Research, Lucknow (U.P.) on 30-31st August 2017.

- Attended a workshop on "Socio-environmental Dimensions of Rejuvenating River Gomti" on 23rd April 2018 organised by the Department of Environmental Science, BBAU, Lucknow in association with Lokbharti.
- Attended one-day seminar on "Environmental Sustainability: Present Scenario and Future Aspects" organised by Department of Environmental science, B.B. Ambedkar University, Lucknow, U.P. on 10th January, 2019.
- Attended a workshop on "Building National STI Policy System and Governance for Effective R&D Ecosystem" organised by DST-Centre for Policy Research, BBAU, Lucknow (U.P.), on 5th March 2019.
- Presented an oral presentation on "carbon dioxide sequestration efficiency and its impact on cell constituents of microalgae *scenedesmus vacuolatus* and *chlorella vulgaris*". In international conference on 'environmental sustainability: innovations, translational dimensions, and way forward held at B B A University, in Feb, 2020.

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Ameliorating Effect of Bicarbonate on Salinity Induced Changes in the Growth, Nutrient Status, Cell Constituents and Photosynthetic Attributes of Microalga *Chlorella vulgaris*

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Abstract

The cells of *Chlorella vulgaris* exhibited NaCl (0–400 mM) induced decrease in the growth, protein, chlorophyll, carbohydrate and total organic carbon, whereas total lipid and proline content increased with rising level of NaCl. Addition of $NaHCO_3$ (20 mM) exhibited antagonistic effect against the adverse effect of salinity on the growth, level of macromolecules except proline. The SEM–EDS analysis of NaCl treated cells exhibited morphological variations as well as reduced accumulation of Na and Cl due to the presence of $NaHCO_3$. The results on chlorophyll fluorescence induction kinetics revealed NaCl induced decline in the photosynthetic performance and quantum yield, while non-photochemical quenching of chlorophyll was enhanced, particularly at lower concentrations of NaCl. Addition of $NaHCO_3$ to NaCl treated cells exhibited further increase in the non-photochemical quenching values. Thus, these results demonstrated that adverse impact of NaCl on the *C. vulgaris* cells was significantly mitigated in the presence of bicarbonate.

Keywords Cell constituents · Fast chlorophyll fluorescence induction kinetics · Photosynthetic performance · Salinity stress · SEM–EDS · Sodium bicarbonate

Abbreviations

Fv/Fm	Quantum yield
NPQ	Non-photochemical quenching
qE	Energy dependent quenching
PIabs	Photosynthetic performance index

Microalgae are promising resource for biodiesel production as they exhibit higher growth rate, short generation time and higher lipid content, which confer an edge to microalgae and make them useful as green resource for biofuel production (Chisti 2008; Davis et al. 2011; Srinivasan et al. 2018). However, cell biomass and biochemical composition of the microalgal cells are generally controlled by various environmental and nutritional conditions (White et al. 2015; Pal et al. 2011). It has been reported that lipid production in microalgal cells is usually enhanced under unfavorable growth conditions (Benavente-Valdés et al. 2016; Xin et al. 2011), particularly under high light stress, UV stress, salinity stress and nutritional stress etc. (Paliwal et al. 2017; Pancha et al. 2015). Salinity stress is one of the most common environmental stresses which cause varying degree of damage to plants and algae, depending upon the interactive effect of other growth conditions (Haghjou et al. 2014). The salinity stress has been reported to induce an increase in the lipid content in a host of microalgal strains, including *Chlamydomona s*sp. JSC4 (Ho et al. 2014). Now the stress-based strategies in cultured microalgae are widely recognized as environment friendly approach to induce overproduction of lipids – a source of biodiesel (Behera et al. 2015). However, stress induced increase in the lipid content of microalgae is simultaneously accompanied by significant decline in the growth and biomass of microalgae.

Physiological response of microalgae to salinity stress is generally observed in terms of salt-induced changes in the anti-oxidative defense system, damage to proteins and DNA, ion toxicity, reduced photosynthesis and decrease in the membrane permeability of the cell (Parida and Das 2005; Yang et al. 2015). However, the microalgal cells can easily adapt to such stress conditions by bringing about changes in their physiology and biochemical processes (Asulabh et al.

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RESEARCH ARTICLES

Photosynthetic efficiency and compositional alterations in microalgae *Chlorella vulgaris* in response to changes in the pH condition

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Abstract

Present investigation on the effect of varying extracellular pH conditions (pH 6.5–10.5) on growth, accumulation of cell constituents and photosynthetic performance of microalga *Chlorella vulgaris* revealed that slightly alkaline pH (7.5–8.5) was the preferred pH range for growth, synthesis of protein and carbohydrate. Whereas total organic carbon (TOC) and lipid content in the microalga gradually increased with pH ranging ranging from 6.5 to 10.5. The FTIR results on total lipid (1740 cm^{-1}), lipid/carbohydrate (1740/1650 cm^{-1}) and lipid/protein (1740/1040 cm^{-1}) ratio in the cells grown at pH 6.5, 8.5 and 10.5 supported the above observation on the estimated amount of macromolecules. The chlorophyll fluorescence induction kinetics (OJIP), exhibiting various photosynthetic parameters, showed initial improvement in the photosynthetic efficiency between pH 6.5–8.5, followed by pH dependent declining pattern. Addition of HCO_3^- (20 mM) showed little effect on the photosynthetic electron transport. The energy dependent quenching parameters of chlorophyll fluorescence like ABS/RC, NPQ and qE, usually associated with the energy state of the membrane, registered an increasing trend with rising pH (pH 6.5–10.5). Use of energy inhibitors DNP, CCCP and electron acceptors/donors (methyl viologen and phenazine methosulphate) on ABS/RC, NPQ and qE suggested that pH dependent regulation of photosynthetic performance in *C. vulgaris* was tightly coupled with proton gradient of the membrane and that was reversed by the energy uncouplers like DNP and CCCP or electron acceptor like MV.

Keywords *Chlorella vulgaris* · pH effect · Photochemistry of PS II · Lipid production · Metabolic inhibitors

Abbreviations

PS II	Photosystem II
Fv/Fm	Quantum yield of PS II
Mo	Initial slope of chlorophyll fluorescence induction curve
ET0/RC	Trapping of energy flux per reaction centre
ABS/RC	Absorption of energy flux per reaction centre
NPQ	Non-photochemical quenching
qE	Energy dependent quenching
Rfd	Relative fluorescence decrease
PIabs	Photosynthetic performance index
DNP	2,4-Dinitrophenol
CCCP	Carbonyl cyanide *m*-chlorophenyl hydrazone
PMS	Phenazine methosulphate
MV	Methyl viologen

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Introduction

Microalgae are known to produce many bioactive compounds and are considered to be very promising resource for biofuel production. The algal biomass is potentially useful for many bioactive products used in the pharmaceutical and cosmetic industries (Bhattacharjee 2016). Major advantage associated with the microalgae, as compared to plants, is their metabolic plasticity which opens the possibility of modifying their biochemical pathways and cellular composition by manipulating the culture conditions (Aslan and Kapdan 2006). In microalgal biotechnology, production of algal biomass is limited due to various nutritional and environmental conditions such as salinity (Bartley et al. 2014), temperature (Van Wagenen et al. 2012) and pH (Moheimani and Borowitzka 2011). It has also been demonstrated that environmental conditions such as pH, light and temperature have been used to manipulate the cultivation and biomass production in algae (Richmond and Becker 1986). Various studies have demonstrated that variability in pH conditions of natural habitats is an important factor which drastically influences the growth, photosynthesis as well as solubility

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