Studies of CO_2 sequestration by *Chlorella vulgaris* in stirred tank batch type photobioreactor

Dissertation

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Submitted

by

Priyanka
(Regn. No. 601204021)

Under supervision of

Prof. P. K. Bajpai	Dr. Haripada Bhunia
Distinguished Professor	Associate Professor
Department of Chemical Engineering	Department of Chemical Engineering

Department of Biotechnology
Thapar University
Patiala-147004, Punjab

June 2014

Declaration

I, the undersigned, here by declare that the research work presented in the M. Tech dissertation entitled "**Studies of CO_2 sequestration by *Chlorella vulgaris* in stirred tank batch type photobioreactor**" has been carried out by me under the supervision and guidance of Prof. P. K. Bajpai and Dr. Haripada Bhunia, Department of Chemical Engineering, Thapar University, Patiala. Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

Priyanka
Priyanka
(Reg. No. 601204021)

Certificate

This is to certify that dissertation entitled, "**Studies of CO_2 sequestration by *Chlorella vulgaris* in stirred tank batch type photobioreactor**" submitted by Ms. Priyanka in partial fulfillment of the requirements for the award of M. Tech in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any Degree or Diploma.

PBajpai 27-06-2014
Prof. P. K. Bajpai
Distinguished Professor
Department of Chemical Engg.

Dr. Haripada Bhunia
Associate Professor
Department of Chemical Engg.

Dr. Dinesh Goyal
Head & Professor
Department of Biotechnology

27/6/14
Dr. S. K. Mohapatra
Dean, Academic Affairs
Thapar University, Patiala

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Priyanka

Priyanka

Abstract

Global warming is becoming a grim intimidation to the nature. The earth's temperature is increasing every year which may result in weather extremities, rise in sea levels, annihilation of species, glaciers retreat, and similar other calamities. The rise in global temperature is attributed primarily to the high and persistently increasing amount of carbon dioxide (CO_2) gases in the atmosphere. The techniques to lower the effects of CO_2 are classified as chemical reaction based strategy (abiotic) and biological mitigation method (biotic). Abiotic method captures CO_2 by reaction with chemical compounds before it is released into the atmosphere. But, the disadvantages of this method are that the chemical reactions are highly energy intensive and costly; and the problematic disposal of the wasted chemical compounds. On the other hand, biological mitigation is more favorable as it directly converts CO_2 into biomass. Microalgae are able to capture solar energy and CO_2 with an efficiency of 5-10 times greater than that of higher plants. This technique can help mitigate global warming and produce a large number of value-added products like pigments, carotenoids, proteins, carbohydrates, lipids, biodiesel etc. This dissertation describes the effectiveness of *Chlorella vulgaris*, used in a photobioreactor at different concentrations (0.04%, 5%, 10%, 15% and 20%) of CO_2 in Fogg's media with 10% (of total volume) inoculum. 10% is the CO_2 concentration at which most of the kinetic parameters as well as total CO_2 fixed and fixation rate is found to be maximum. The pH and CO_2 concentration for this culture were also optimized.

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Abbreviation

AMOP	Aquatic Microbial Oxygenic Phototrophs
ASU	Air Separation Unit
BOD	Biochemical Oxygen Demand
COD	Chemical Oxygen Demand
ECE	Energy Conversion Efficiency
GHG	Green House Gases
IGCC	Integrated Coal Gasification Combined Cycles
IPCC	International Panel On Climate Control
NADP	Nicotinamide Dinucleotide Phosphate
PBR	Photobioreactor
TAG	Triacylglycerol

CHAPTER 1

INTRODUCTION

The world is experiencing rise in temperature generally known as global warming. This variation of the climate system is dominated by the greenhouse gases. Global greenhouse gas emissions due to human activities have grown since industrialization **[1]**. Carbon dioxide is the most important anthropogenic greenhouse gas. Global increases in CO_2 concentrations are mainly due to the combustion of fossil fuels, representing about 75% of the total anthropogenic CO_2 emissions **[1]**. With strong economic growth and continued heavy reliance on fossil fuels, worldwide CO_2 emissions still continue an upward trend in the early decades of the 21st century.

Measured atmospheric concentrations of carbon dioxide are currently 100 ppm (parts per million) higher than pre-industrial levels. Natural sources of carbon dioxide are more than 20 times greater than sources due to human activity, but over periods longer than a few years natural sources are closely balanced by natural sinks, mainly photosynthesis of carbon compounds by plants and marine plankton. As a result of this balance, the atmospheric mole fraction of carbon dioxide remained between 260 and 280 parts per million (ppm) for the 10,000 years between the end of the last glacial maximum and the start of the industrial era [**2**]. According to fourth IPCC Assessment Report, **Figure 1.1** is showing the current atmospheric CO_2 concentration at 392 ppm [**3**].

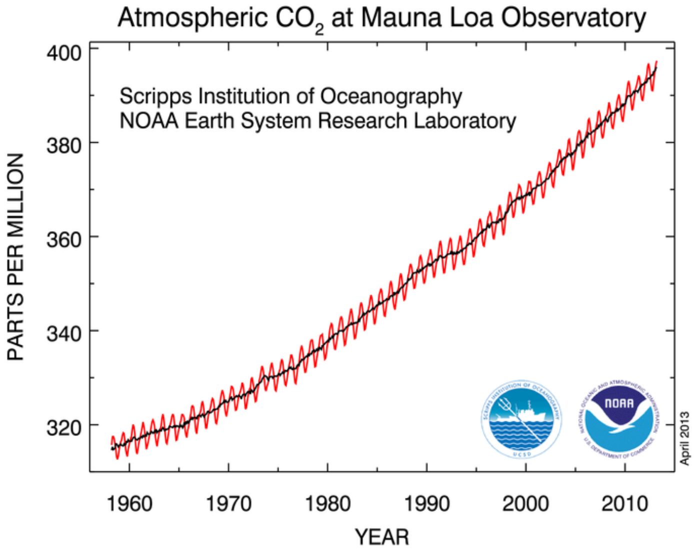

Figure 1.1: CO_2 concentration rise over the years [**3**]

The IPCC has concluded that the stabilization of the atmospheric CO_2 concentration requires CO_2 emissions to fall significantly below current levels to decline the impact of climate change **[1]**. So as individuals also we need to play some part from our side by following certain self-restraints and becoming little sensitive to our surroundings by following the below given methods **[4]**:

1. Reduction in consumption: the judicious use of energy may significantly change the present scenario. However, with ever increasing population the demand for energy does not seem to decrease in the future also.
2. Efficiency improvement i.e. switching from high-carbon (coal, petrol) to low-carbon fuels (ethanol).
3. Switching to renewable energy: this option is very promising and may prove to be the next generation fuel however a considerable scope of research is still needed to remove the obstacles pending at present.

The scientific community is currently examining potential approaches in order to reduce the anthropogenic contributions to global warming. Physico-chemical methods and conversion of CO_2 in to valuable product by microalgae are the sequestration options in the research and development panorama **[5]**. The extent of use will depend on factors such as cost, capacity, environmental impact, the rate at which the technology can be introduced and social factors such as public acceptance.

1.1 Research Objectives

The overall objective of the research is to capture CO_2 by microalgal sequestration that would help mitigate global warming. The specific objectives are:

- Optimization of pH and CO_2 concentration for maximizing growth, productivity, protein content and CO_2 fixation rate.
- Kinetic study of CO_2 sequestration at different CO_2 concentrations.
- Growth studies of microalga in dairy wastewater.

1.2 Dissertation Overview

This dissertation consists of five chapters. Chapter 1 contains a brief introduction to the research topic. Chapter 2 contains a more detailed literature review relevant to the research such as basic information of microalgae, their place and importance in the modern world, the factor effecting process of CO_2 sequestration, photo-bioreactor technology that is currently used for their proper cultivation, listed including descriptions of the different photo-bioreactor configurations and parameters monitored and their pros and cons. In chapter 3, materials and methods briefly discussed about the instrumentation and methodology involved. In chapter 4, results obtained are shown, analyzed and discussed. The final chapter (chapter 5) includes the conclusions that can be drawn from the results obtained as well as recommendations for future work.

CHAPTER 2

LITERATURE REVIEW

2.1 CO_2 Capture and Sequestration

2.1.1 Introduction to CO_2 capture

CO_2 sequestration involves the strategies which can be used to capture and store CO_2 for long duration. There are three basic approaches to CO_2 capture from fossil fuels shown in the **Figure 2.1** below:

i) Pre-combustion capture
ii) Post combustion capture
iii) Oxy-combustion capture

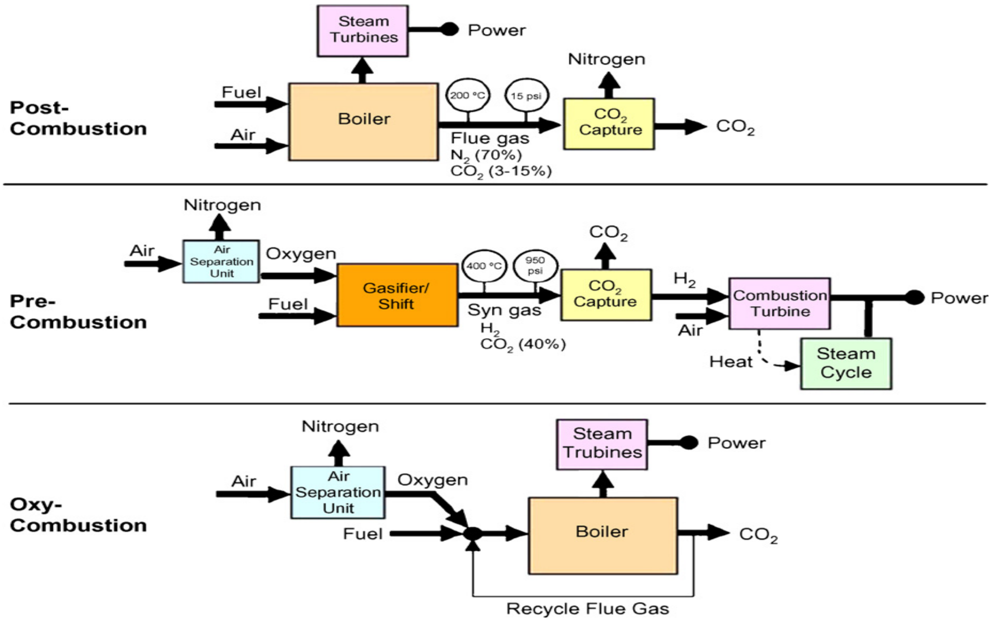

Figure 2.1: Block diagrams illustrating post-combustion, pre-combustion, and oxy-combustion systems [**6**]

(i) Pre-combustion carbon capture

In pre-combustion CO_2 capture, the CO_2 is recovered from some process stream before the fuel is burned. To the extent that the concentration and pressure of the CO_2 containing stream can be increased, the size and cost of the capture facilities can be reduced. This has led to efforts to develop combustion technologies that inherently produce concentrated CO_2 streams or CO_2 containing streams at high pressure, for which there are existing, capture processes [**6**].

(ii) Post-combustion CO_2 capture

Post-combustion capture involves the removal of CO_2 from the flue gas produced by combustion. It has the greatest near-term potential for reducing GHG emissions, because it can be retrofitted to existing units that generate two-thirds of the CO_2 emissions in the power sector [**6**].

(iii) Oxy-combustion

An alternative to capturing carbon from fuel gas or flue gas is to modify the combustion process so that the flue gas has a high concentration of CO_2. In the most frequently proposed version of this concept, a cryogenic Air Separation Unit (ASU) is used to supply high purity oxygen to a PC-fired boiler. The water is easily removed by condensation, and the remaining CO_2 can be purified relatively inexpensively. Conditioning of the flue gas consists of drying the CO_2, removal of O_2 to prevent corrosion in the pipeline, and possibly removal of other contaminants and diluents, such as Ar, N_2, SO_2 and NO_x [**6**].

2.1.2 Methods for CO_2 sequestration

Based on above mentioned approaches following methods given in the figure below are being employed worldwide to capture CO_2. All but one method is of biological nature as it involves utilization of microorganisms. All the methods have their own pros and cons thus they can be used in combination where their best properties can be used to get the best outcome. **Figure 2.2** gives the diagrammatic representation of these methods.

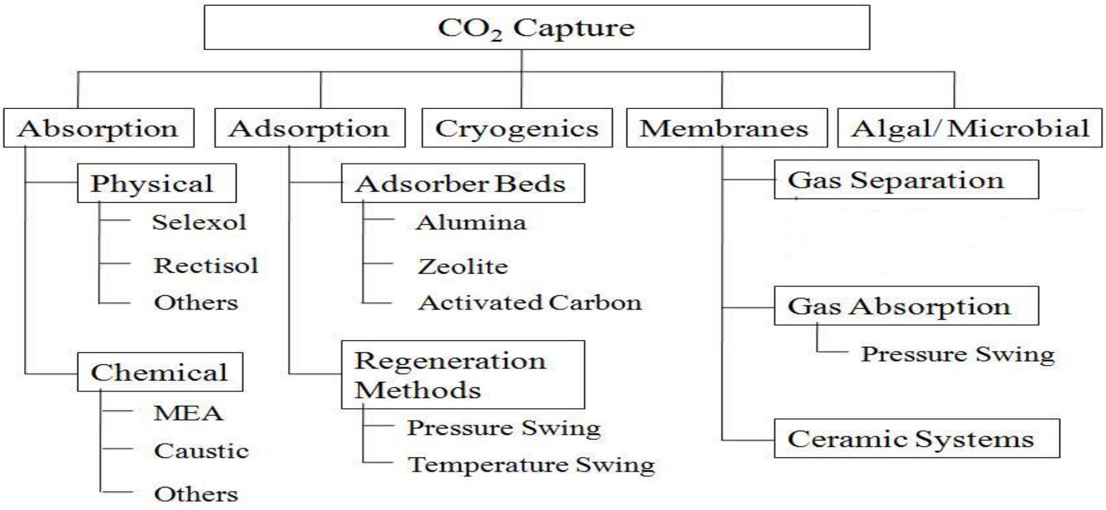

Figure 2.2 Major carbon capture and sequestration technologies [**7**]

(i) Cryogenic distillation

Cryogenic distillation is an air separation process, where gaseous components of a mixture are separated by condensation. Cryogenic method can capture CO_2 in a liquid form, being easier to transport and storage. CO_2 and H_2O are separated from flue gas on the basis of differences in dew and sublimation points [**6**].

(ii) Electrochemical pumps for separation of CO_2 from flue gas

The molten carbonate and aqueous alkaline fuel cells have been studied for use in separating carbon dioxide from both air and flue gases. Operation of the molten carbonate fuel cell in a closed circuit mode (with application of an external emf) will result in the transport of carbonate ions across the membrane. The molten carbonate electrochemical separator requires oxidizing conditions for the formation of carbonate from carbon dioxide, and is less applicable for direct separation of CO_2 from fuel gases. Sulfur dioxide present in flue gas poisons the cell, resulting in sulfate formation. There are also severe problems with electrolyte segregation and electrode degradation in the severe high temperature flue gas environment [**6, 8**].

(iii) Chemical looping

Chemical looping is a novel method to facilitate carbon dioxide separation from flue gases. The oxygen for combustion of the fuel is provided by a regenerable metal oxide catalyst. The use of lattice oxygen from a metal oxide catalyst as the oxidizing agent, in lieu of gas phase oxygen from air, results in a flue gas that is not diluted by nitrogen. This results in a flue gas that is concentrated in carbon dioxide. Because air is not used in the combustion of the fuel, NO_x emissions are low [**6, 8**].

(iv) Membrane separation of CO_2

The development of a membrane separator for the selective removal of CO_2 in the presence of CO, H_2, H_2O, and H_2S (fuel gas) or N_2, O_2, H_2O, SO_2, NO, and HCl (flue gas) would be of tremendous economic value. A membrane material which either allows the selective transport (diffusion) or selective exclusion of CO_2 is desired. Membranes can separate carbon dioxide from a gas stream by size exclusion or by chemical affinity. A large body of research has been conducted on the properties of carbon dioxide selective membranes based upon inorganic materials such as zeolites, alumina, carbon, and silica. Membranes are needed for successful application to flue or fuel gas mixtures [**8**].

(v) Absorption (i.e. solvent scrubbing)

This is a well-established CO_2 capture system primarily used in the chemical and oil industries. Physical absorption is temperature and pressure dependent with absorption occurring at high pressures and low temperatures. Chemical absorption of CO_2 from gaseous streams such as flue-gases depends on acid–base neutralization reactions using basic solvents. Some of the preferred solvents for CO_2 removal are amines. The released CO_2 is compressed and the regenerated absorbent solution is recycled to the stripper column [**6, 9**].

(vi) Pressure swing adsorption (PSA)

This technique employs high pressure of the IGCC to enhance the extent of CO_2 adsorption. When CO_2 passes over the adsorption tower, it gets adsorbed on the adsorbent to much greater extent than it should in the normal conditions. Moreover, the thermal energy losses of the PSA are much smaller than other adsorption and absorption methods. However, the actual operating condition and optimum size is still unknown [**10**].

(vii) Biological method

Photosynthetic solution of carbon dioxide fixation

Physico-chemical means of CO_2 sequestration has disadvantages, having high costs associated with it there by need to develop the suitable technologies. Capturing, transporting and storing CO_2 are also very expensive processes. Biological method of CO_2 sequestration is an alternative to physical methods.

The concept of photosynthetic conversion to fix carbon dioxide using bacteria or micro-algae is quite promising as the byproduct it may result will be oxygen, which lay the very foundation of human existence. Cyanobacteria or micro-algae have been suggested to perform the role of photosynthesis. In order to promote uniform growth of the organisms, the distribution of photosynthetic photon flux light in the wavelength range of 400–700 nm needs to be delivered to the bioreactor.

2.2 Microalgae for CO_2 Sequestration

Algae are a large and diverse group of simple, typically autotrophic organisms, ranging from unicellular to multicellular forms. The largest and most complex marine forms are called seaweeds. They are photosynthetic like plants and simple because they lack the many distinct organs found in land plants. Some unicellular species rely entirely on external energy sources and have limited or no photosynthetic apparatus. All algae have photosynthetic machinery ultimately derived from the cyanobacteria, and so produce oxygen as a byproduct of photosynthesis. Algae can be classified into two types based on their sizes, microalgae and macroalgae. Microalgae are microscopic photosynthetic organisms (less than 2 µm in diameter). However, macroalgae are found in both marine and freshwater environments. Biologists have categorized microalgae in a variety of classes, mainly distinguished by their pigmentation, life cycle and basic cellular structure [**11**].

The microscopic image of *Chlorella vulgaris,* which was used in our study, is shown in **Figure 2.3**. It has been found that this strain of microalga can vary in diameter from 4-5µm.

Figure 2.3 Microscopic views of algal cells at 100 X magnification **[10]**.

i) The diatoms (Bacillariophyceae): These algae dominate the phytoplankton of the oceans, but are also found in fresh and brackish water. Approximately 100,000 species are known to exist. Diatoms contain polymerized silica (Si) in their cell walls. All cells store carbon in a variety of forms. Diatoms store carbon in the form of natural oils or as a polymer of carbohydrates known as Chrysolaminarin **[11]**.

ii) The green algae (Chlorophyceae): These are also quite abundant, especially in freshwater. They can occur as single cells or as colonies. Green algae are the evolutionary progenitors of modern plants. The main storage compound for green algae is starch, though oils can be produced under certain conditions.

iii) The blue-green algae (Cyanophyceae): Much closer to bacteria in structure and organization, these algae play an important role in fixing nitrogen from the atmosphere. There are approximately two thousand known species found in a variety of habitats [**12]**.

iv) The golden algae (Chrysophyceae): This group of algae is similar to the diatoms in pigmentation and biochemical composition. They have more complex pigment systems, and can appear yellow, brown or orange in color. Approximately one thousand species are known to exist, primarily in freshwater systems. The golden algae produce natural oils and carbohydrates as storage compounds.

All algae comprise of the proteins, carbohydrates, fats and nucleic acids. While the percentages vary with the type of algae, there are algae types that are comprised of up to 40% of their overall mass by fatty acids that could be extracted and converted into biodiesel [**13]**.

2.2.1 Photosynthesis

Photosynthesis can be defined as process by which plants and other autotrophic organisms convert CO_2 along with water in the presence of light energy into reduced organic compounds such as carbohydrates, proteins and lipids that can be used to fuel the organisms' activities. By product of this reaction is released as oxygen. Process of photosynthesis is performed by most of plants, algae and cyanobacteria. These are called as photoautotroph. Most of the energy necessary for life on earth is supplied by photosynthesis and atmospheric oxygen levels are also maintained by it.

Photosynthesis process comprise of two reactions out of which first is light dependent in which ATP and NADPH generates and the second one is light independent in which ATP and NADPH used up generally known as Dark cycle [**14]**.

Photosynthetic pigment

The solar energy required for photosynthesis is captured by photosynthetic pigment molecules. Different type of pigments, described as photosynthetic pigment, participate in this process. The major photosynthetic pigment is chlorophyll and accessory pigments are carotenoids and phycobillins.

Concept of pigment system

In all natural photosynthetic systems, pigment molecules are bound to proteins forming pigment-protein complexes, called as photosystem. The pigment systems have two components: photochemical reaction center and antenna complex. Photochemical reaction center carries out photochemical reaction. In all photosynthetic organisms, the reaction center contains the special pair of chlorophyll a molecule associated with specific proteins that participate in photochemical reactions. Antenna complex consist a number of distinct pigment-protein complexes known as light harvesting center. In higher plants, each light harvesting center consists of various pigment molecules (chlorophyll a, chlorophyll b and carotenoids) bound to proteins. The antenna complex captures light and feed it to reaction center by a process called as resonance energy transfer. The size of the antenna complex varies considerably in different organisms, generally 200 to 300 chlorophylls per reaction center in higher plants.

Photosystem are of two types in oxygenic photosynthetic organisms such as plants and microalgae on the basis of their light absorption maxima by reaction center chlorophyll molecules [**14]**.

Photosystem I

Photosystem I **(PS I)** is driven by light of wavelength 700 nm. The chlorophyll a at the photochemical reaction center complex of **PS I** is called as **P700**. **PS I** is found almost exclusively in non-appressed membranes of grana thylacoid and stroma thylacoid

Photosystem II

Photosystem II **(PS II)** is driven by light of wavelength 680 nm. The photochemical reaction center chlorophyll a of **PS II** is called as **P680**. **PS II** is located predominantly in appressed membranes of grana thylacoid

Stages of photosynthesis

Photosynthesis is a two step process: one stage is dependent on light and another is independent of it.

Light reaction

The light dependent reaction occurs in the grana of chloroplast which require direct light energy to make NADPH and ATP that are used in the dark reaction. A process of formation of ATP from ADP and inorganic phosphate by utilizing light energy is called photophosphorylation. The overall equation for the light-dependent reactions under the conditions of non-cyclic electron flow in green plants is:

$$2H_2O + 2NADP^+ + 3ADP + 3P_i + \text{light} \rightarrow 2NADPH + 2H^+ + 3ATP + O_2$$

Z scheme

Light dependent reactions in plants takes place in chloroplast and use light energy to synthesize ATP and NADPH. The light-dependent reaction has two forms: cyclic and non-cyclic. In the non-cyclic reaction, the photons are captured by chlorophyll and other accessory pigment capture photons in light-harvesting antenna complexes of **photosystem II**. When a chlorophyll molecule obtains sufficient excitation energy from the adjacent antenna pigments, an electron is transferred to the primary electron-acceptor molecule, **Pheophytin**, through a process called photo induced charge separation. An ATP synthase enzyme uses the Chemiosmotic potential (generated due shuttling of electrons through electron transport chain) to make ATP during photophosphorylation, whereas NADPH is a product of the terminal redox reaction in the Z-scheme. The electron enters a chlorophyll molecule in **photosystem I**. A second electron carrier accepts the electron, which again is passed down to electron acceptor whose reduction potential is more. Hydrogen ions are moved by energy created by the electron acceptors, across the thylakoid membrane into the lumen. Co-enzyme NADP, is reduced by an electron, has functions in the light-independent reaction. The cyclic reaction differs with non-cyclic, in the form that it generates only ATP, and no reduced NADP (NADPH) are created. The cyclic reaction takes place only at **photosystem I**. Once the electron is displaced from the photosystem, the electron is passed down the electron acceptor molecules and returns to **photosystem I**, from where it was emitted, hence the name cyclic reaction [**14**].

Water photolysis

The NADPH is the main reducing agent in chloroplasts, thus its production leaves chlorophyll oxidized. Plastocyanin replace electrons, lost from chlorophyll in **photosystem I**. However, since **photosystem II** includes the first steps of the Z-scheme, an external source of electrons is required to reduce its oxidized chlorophyll *a* molecules. Water is that source electrons in green-plant and Cyanobacterial photosynthesis. Two water molecules are oxidized by four successive charge-separation reactions by **photosystem II** to yield a molecule of diatomic oxygen and four hydrogen ions [**14**].

Dark reaction

The light independent reaction occurs in the stroma of chloroplast when the product of light reaction, NADPH and ATP, are used to make glyceraldehydes 3-phosphate (a triose phosphate) from reduction of carbon dioxide.

Calvin cycle

Calvin cycle is known as reductive pentose pathway. It can be divided in to three phases carboxylation, reduction and regeneration of Ribulose 1, 5-bisphosphate (RuBp). The overall light independent reaction in green plant is represented as:

$$3\ CO_2 + 9\ ATP + 6\ NADPH + 6\ H^+ \rightarrow C_3H_6O_3\text{-phosphate} + 9\ ADP + 8\ P_i + 6\ NADP^+ + 3\ H_2O$$

In carboxylation phase CO_2 is incorporated in to a five carbon sugar Ribulose 1, 5-bisphosphate and produces two molecule of 3-phosphoglycerate. The enzyme which catalyzes this reaction is Ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco). In reduction phase, the presence of ATP and NADPH from the light-dependent stages, 3 phosphoglycerate is reduced to glyceraldehyde 3-phosphate (G3P). To continue the process most of G3P produced is used to regenerate RuBp. The net production of fixed carbon in the Calvin cycle is one molecule of glyceraldehyde 3 phosphate. The other five glyceraldehyde 3-phosphate are processed in the remainder of the Calvin cycle to regenerate three molecule of RuBp. The intermediate of pathway include three, four, five and seven carbon sugars. The sugars produced during carbon metabolism yield carbon skeletons that can be used for other metabolic reactions like the production of amino acids and lipids [**14**].

2.2.2 Advantages of microalgae over terrestrial plants

Several aspects of aquatic marine oxygenic phototrophs (AMOP) physiology are relevant for evaluating their possible integration into renewable biofuel. Below mentioned factors describe how microalgae are better than other terrestrial plants for CO_2 sequestration and production of other industrially useful products.

(i) Superior solar energy yields

AMOPs have 5-10 times higher solar energy yields than more advanced terrestrial plants. It is mainly attributed to greater intrinsic solar energy conversion efficiency (ECE), ratio between the useful output of an energy conversion mechanism and the input, at 3–9% versus a theoretical maximum of 2.4% for C3 crops and 3.7% for C4 crops. In addition to that terrestrial plants have multiple photo protection mechanisms that differentially protect terrestrial plants by diverting absorbed light to heat. AMOPs are more resistant to water and thermal stresses [**15**].

(ii) Lack of recalcitrant biopolymers

AMOPs do not require structural biopolymers like cellulose, lignin, hemi-cellulose etc. which are essential for support and strength of higher plant (e.g. trees). This eliminates the need for pretreatments to breakdown cellulytic products and lowers the reactor temperature of their subsequent fermentation [**15]**.

(iii) Metabolic and ecological diversity

AMOPs exhibit enormous ecological, genotypic, and metabolic diversities with over 4000 distinct species of cyanobacteria and a comparable number of unicellular algae classified thus far. This diversity allows selection of genera/species that are adapted for growth in locally available aquifers (marine, hypersaline, thermophilic and freshwater), or have morphological features that allow cost-effective harvesting (filamentous, buoyant, or aggregate), or possess anaerobic metabolisms that enable production of hydrogen, ethanol, and/or organic acids by auto fermentation before final biomass conversion/utilization **[15]**.

(iv) Biosynthetic control of chemical composition by nutrient and environmental stresses

AMOPs direct majority of photosynthetic reductants into metabolic pathways that synthesize the most amenable bioenergy precursors starch, glycogen, and lipids rather than cellulose and lignin. This offers a potential processing simplification and gain in the overall energy yield. They can alter their composition under nutritional or environmental stresses. Using these techniques, protein content can be converted to energy storage compounds such as carbohydrates (starch, glycogen, polysaccharides) or higher energy lipids (fatty acids, monoacylglycerols, diacylglycerols and triacylglycerols) in a species dependent manner [**15]**.

(v) Water usage and harvesting

Seawater is natural water source for many AMOPs, which is plentiful and can be substituted by deep saline aquifers in the interior or nutrient-laden agricultural wastewater, while terrestrial biofuel crops require freshwater **[16]**. The volume of water required is lower than that required to grow maize at an equivalent weight of corn grain, eight-fold less than crop cultivation on fertilized land. Methods available for harvesting algae from broth include centrifugation, filtration, flocculation, and gravity sedimentation [**17]**. In order to coagulate the microalgal cells, polymer flocculants can bring the cells together via physical linking, called bridging. Sometimes, effective flocculation is achieved simply by changing the pH values (between 11 and 12) of the algal broth without adding extra flocculants [**18]**. Unfortunately, flocculation is not effective for harvesting in large scale open-pond systems or with oceanic cultivation.

Filtration is satisfactory for recovering relatively large microalgae/cyanobacteria, such as *Spirulina*, but unsuitable to recover the small species, such as *Chlorella* and *Scenedesmus* **[18]**.

2.2.3 Factors affecting microalgal growth

For proper microalgal growth an optimum environment is required, which depend on the below mentioned factors. However, if we change these factors we may observe different outcomes which may be beneficial for us in some other ways.

(i) Temperature effect

Ratio of O_2 to CO_2 solubility increases with the temperature causing significant amount of O_2 fixation by Oxygenase activity of Rubisco. In addition Rubisco affinity for CO_2 also decreases on increasing temperature. According to an investigation a unicellular cyanobacterium species i.e. *Synechococcus* elongates when bubbled with different concentrations of CO_2 at different temperatures drop in pH at 52˚C with 60% CO_2 was comparable to a drop in pH at 25˚C with 20% CO_2 showing that temperature dependent solubility of CO_2 provides an advantage to thermophilic algae to tolerate a higher concentration of CO_2 **[19]**.

(ii) pH effect

The pH of the culture medium is an important factor that significantly affects the growth of the algae and can be influenced by dissolving CO_2 and SO_x from the flue gas. With elevated CO_2 concentrations, pH drops down to pH 5 by this way $H_2O + CO_2 \rightarrow\rightarrow HCO_3^- + H^+$ and with higher SO_x concentrations even down to pH 2.6 have been reported [**20]**.

(iii) NO_x and SO_x in the flue gas

NO_x and SO_x influence the growth of the microalgae. *Chlorella* species cannot grow under higher combination of SO_x and NO_x because it causes drastic decrease in pH [**21]**. It was found by researchers that adding certain amounts of sodium sulphite instead of gaseous SO_2 to the medium results in a slower growth at 50 mM and cell death at 250 and 500 mM **[20]**.

(iv) Light provision

For the CO_2 fixation and biomass production optimum light intensity is necessary. For the microalgal productivity light has become the limiting factor if not provided in adequate amount. If cells got exposed to longer period with high light intensity this causes photoinhibition. From engineering point of view, geometry of reactor can reduce photoinhibition in micro algal suspension. Fernandes et al. studied the effect of circular and plan geometry in light penetration and found that Flat-plate is best PBR [**21]**.

(v) Mixing rate

Uniform mixing of nutrient and better distribution of light over cells can be achieved by the help of proper mixing that helps (1) in air bubble dispersion (2) increasing mass transfer from air bubbles to the liquid and then to cell (3) supply of nutrient component to the cell (4) prevents sedimentation of nutrient components. Gas under pressure which is supplied through sparger provides smaller bubble size and better gas distribution. This helps by increasing the productivity in tubular photo-bioreactor up to 40% [**22]**.

(vi) Photobioreactor design

Efficient light distribution in the whole region of culture in photobioreactor helps increasing growth rate. Many specially designed photobioreactors have been tried having better light system to distribute the intense light for efficient CO_2 sequestration and biomass formation. Internally illuminated airlift photo-bioreactor has designed by Su et al. **[23]** to study the light distribution for maximizing the growth efficiency of photosynthetic cells. Flat plate photobioreactor was constructed by Zijffers et al. **[24]** in which the sunlight is focused on the top of the bioreactor by linear frensal lenses and vertical plastic light guides were used for capturing it, reflected internally in these guides, and then distributed into the photobioreactor compartment. Sunlight can be better distributed and more light utilization was found with this design.

Different photobioreactors like Vertical tubular photobioreactor, Flat panel photobioreactor, Horizontal tubular photobioreactor, Helical type photobioreactor, Stirred tank photobioreactor, Hybrid type photobioreactor are used now a days. Cultivation of algae also requires developing a suitable photobioreactor having features like higher S/V ratio, mixing, mass transfer, scalability and ease of operation. A single photobioreactor is not good enough to have all the merits. Airlift reactor seems the most promising for the CO_2 sequestration. However, integrated type of reactor will help in ease of scalability [**25]**.

Membrane-type photobioreactors (PBRs) have high CO_2 removal efficiency but they have operational problems, such as membrane fouling and high design and operational costs. Therefore, tubular and bubble column-type PBRs are commonly applied for use in algal photoreactors **[26-28]**.

Basic design consideration of bioreactor: As basic disadvantage of each large fermenter is inability of design to provide an adequate supply of CO_2 and to remove metabolic heat efficiently. For solving this, L/D ratio should be kept about one for an efficient gas and algal cell contact [**29]**. As agitation is also required, design of impeller should be such as according to Shuler and Kargi **[30]**.

Diameter (D_i) =0.3 diameter of fermenter (D)

Width (W_i) =0.2 D_i

Length (L_i) =0.25 D_i

(vii) Volumetric mass transfer coefficient (k_La)

Determination of k_La in bioreactor is essential in order to establish its aeration efficiency. k_La and cell growth rate varies in the different region of liquid flow. Liquid flow region in photobioreactor can be divided into laminar flow, turbulent flow and transition zone depending upon gas velocity and rate of agitation. Change in k_La is achieved by changing (1) aeration rate (2) stirring speed (3) using feed back technique. If rate of aeration is very high, it causes shear stress which is possible reason for the fall in specific growth rate. Zhang et al. did the comparative analysis of k_La in different photobioreactors with different percentages of CO_2 and determined that requirement of k_La increases with decrease in the concentration of CO_2 in the inlet gas stream to meet the CO_2 demand of microalgal cells [**31**].

(viii) O_2 accumulation

The water splitting activity of **photosystem II** (P680) present in grana lamella of thylakoid is responsible for the oxygen evolution during photosynthesis. Overall biochemistry of photosynthesis for formation of 1 molecule of glucose leads to produce 6 molecules of O_2, by this it is concluded that as the concentration of O_2 rises along with increase in biomass leads to oxygenation activity of Rubisco process is known as photorespiration (waste process). Ambient factor for oxygenation activity of Rubisco are O_2, CO_2 and temperature **[30]**. Toxic effects are there of higher amount of oxygen in the algal culture as photo-bleaching and photosynthetic efficiency got reduced. So an efficient degassing system is provided in order to remove formed O_2.

(ix) Nutrient requirements

In addition to the carbon, nitrogen is the most important element that is required for microalgal nutrition and, as a constituent of both nucleic acids and proteins, nitrogen is directly associated with the primary metabolism of microalgae. Phosphorus is the third most important nutrient for microalgal growth, and should be supplied to significant excess as phosphates because not all phosphorus compounds are bioavailable (e.g. those combined with metal ions). In the case of marine microalgae, seawater supplemented with commercial nitrate and phosphate fertilizers is commonly used for production of microalgae. Nevertheless, trace species, such as metals (Mg, Ca, Mn, Zn, Cu and Mb) and vitamins, are typically added for effective cultivation [**4]**. Lipids contain twice the energy stored per C atom than do carbohydrates, which translates directly into a twofold increase in fuel energy content. Depletion of nitrate, silicate or phosphate from the growth medium has been shown to produce two fold to three fold increase in the relative amounts of starch (glycogen in cyanobacteria) or lipids in many AMOPs. Most AMOPs are rich sources of proteins when grown under nutrient replete conditions [**32]**. Different species of microalgae and cyanobacteria are listed in **Table 2.1**

Table 2.1 Notable species of microalgae and cyanobacteria **[32]**

S. no.	Species	Requirement	Advantages
1	*Chlorococcum*	Temp 15-27 °C, pH 4-9	CO_2 uptake 70%, densely culturable, doubling time 8 hrs.
2	*Chlorella*	Temp 15-45 °C, pH 3-7	CO_2 uptake 60%, doubling time 2.5–8 hrs, High growth ability, High temp. tolerance, Dispersible
3	*Euglena gracilis*	Temp 27 °C, pH 3-5	High amino acid content, Good digestibility (effective fodder),Grows well under acidic conditions, Not easily contaminated
4	*Viridiella*	Temp 15–42°C, pH 2–6	Accumulates lipid granules inside the cell High temp. and CO_2 tolerance
5	*Anabaena*	Temp 25-35 °C, pH 7-7.5	Temp resistant,N_2 fixation
6	*Enteromorpha clathrata*	Temp 24–33 °C,pH 7.5 - 8.0	Very little agitation is needed
7	*Spirulina platensis*	Temp 35 – 37 °C,pH 8.3 - 11.	Very tolerable to pH change.

2.3 Culture Systems

Two distinctive culture systems have been proposed for CO_2 sequestration with microalgae. One is the open pond system, and the other is the closed photobioreactor system. There is on-going discussion regarding whether the open pond system or the closed photobioreactor system would be better for CO_2 sequestration. Apparent advantages for utilizing the open pond system are low initial and operational costs. On the other hand, an advantage for the photobioreactor system has a higher potential productivity due to better environmental control and harvesting efficiency. For an open pond system, the size of the area needed to assimilate significant amounts of CO_2 is being criticized. However, there are already existing large-scale open pond systems.

2.3.1 Open system

Carbon dioxide from various industrial sources (power plants, chemical industries) can be converted to biomass using algal mass culture pond systems. The main advantage of using pond systems is that the technology is very well known and various commercial systems already exist. Algal pond systems are currently the most economical method to produce biomass on a large scale.

The products obtained from algal mass culture can be of very high value (for example, pharmaceutical or food grade *Spirulina* or *Chlorella*). However for an open pond system, the size of the area needed to assimilate significant amounts of CO_2 is being criticized. For example, the largest single algal production systems, developed by the Sosa Texcoco Co. near Mexico City is 900 ha [**32]**.

Outdoor systems are significantly limited with regard to cell growth due to several environmental issues, such as significant vapor losses, CO_2 diffusion to the atmosphere, varying temperatures and light utilization, as well as the threat of contamination and pollution. Also, another disadvantage of open pond systems is their lower productivity due to unreliable periods of sunlight and insufficient light transmission because of the high layer thickness of ponds, unless a sunlight collection device is used [**33]**.

2.3.2 Closed systems

Several studies have demonstrated that using microalgae for CO_2 fixation in photobioreactors (PBRs) is an effective and promising method [**14]**. In comparison with open systems, closed PBRs are characterized via regulated and well-controlled cultivation, with the additional benefits of low contamination risk, high CO_2 fixation efficiency, high metabolic flexibility, and controllable hydrodynamics. Such systems usually have a larger surface area (high surface/volume ratio) exposed to the light source to reduce the shadow effect, which is one of the main causes of the inhibition of microalgae in open systems. However, the scaling up of most closed PBRs with a high tube length faces some serious limitations related to light utilization, biomass circulation, mass transfer (CO_2/O_2), and the control of growth parameters. Consequently, the widespread view that PBRs are more effective than open ponds has not yet been proven with a large-scale system.

The criteria for a good microalgal-CO_2 fixation PBR system are good mixing, gas transfer, and light distribution [**33, 34]**. Various closed photobioreactors are currently used for microalgae cultivation, including (1) vertical tubular systems, (2) plate-type systems, and (3) column systems. Normally, column PBR systems are relatively low-cost and tubular PBR systems are the most attractive for large-scale outdoor cultivation [**34]**. Some previous studies claim that air-lift PBRs, which are the vertical tubular-type, can achieve the most CO_2 fixation efficiency because of their relatively better circulation and mass transfer. In addition, bubble column PBRs are often thought to be superior because of their well-defined flow patterns and circulation times through the use of risers and down-comers. However, the circulation time in airlift PBRs is too slow to reduce the photo-inhibitory effects [**35]** which directly influence the cell density and CO_2 fixation ability. In addition, it was demonstrated that most light is reflected directly by small bubbles, which adversely influence the photosynthetic efficiency, when using air-lift PBRs. It was also found that refined bubble column PBRs were similar to airlift PBRs due to their designed principle. Although tubular PBR systems are often considered the most suitable for effective microalgal-CO_2 fixation systems, their reactor sizes and length are limited by excess O_2 removal, CO_2 depletion, parameter control and high cost. Therefore, closed tubular PBR systems are difficult to scale up indefinitely, and rely on the multiplication of reactor units [**36**]. **Table 2.2** shows the major differences and advantages of both systems over each other.

Table 2.2A brief comparison of open and closed systems for microalgae cultivation [**36]**

Characteristics	**Open systems**	**Closed systems**
CO_2 fixation ability	Low	High
Biomass productivity	Low	High
Specific growth rate	Low	High
Contamination risk	Extremely high	Low
Evaporation losses	High	Low
Photosynthetic efficiency	Low	High
Surface area	Low	Extremely high
Process control	Difficult	Easy
Operation cost	Low	High
Scale-up	Easy	Difficult

2.4 Photobioreactor

Although the term ‘photobioreactor’ has been applied to open algal ponds and channels, it is best reserved for devices that allow monoseptic culture which is fully isolated from a potentially contaminating environment. The available photobioreactor configurations are numerous but most may be classified into one of two types: either tubular devices or flat panels. These can be further categorized according to orientation of tubes or panels, the mechanism for circulating the culture, the method used to provide light, the type of gas exchange system, the arrangement of the individual growth units, and the materials of construction employed. Design and scale-up methodologies for photobioreactors are poorly developed. Irrespective of the specific reactor configuration employed, several essential issues need addressing (i) effective and efficient provision of light (ii) supply of carbon dioxide while minimizing losses (iii) removal of photosynthetically generated oxygen that may inhibit metabolism or otherwise damage the culture if allowed to accumulate (iv) sensible scalability of the photobioreactor technology [**37]**.

2.4.1 Tubular photo-bioreactors

Tubular photo-bioreactors consist of long thin tubes arranged in different geometrical patterns (helical, straight tubes) to optimize irradiance from a point light source (sun). Generally liquid growth medium is circulated in these tubes by air bubbling and by injection of air into one end of the system and degassed at the other end. Experiments have shown that a large-scale tubular photo-bioreactor has failed and the main reason attributed for its failure was the large dissolved oxygen in the system. Hence a system should not at any stage be over saturated with oxygen as this would cause algae to shutdown photosynthesis and growth [**33, 38]**. It was also reported that tubular photo-bioreactors are difficult to build and maintain, and have limited scalability.

2.4.2 Mechanically stirred photo-bioreactors

Mechanically stirred photo-bioreactors use baffles to stir the growth medium to attain a mass transfer of air/CO_2 into liquid. A drawback of the stirred medium is if stirred vigorously the algae cell wall would be damaged by the high fluid shear forces [**38]**. If it is stirred slowly, eddy currents will not be established that move the algae toward the light source thereby decreasing the efficiency of light available for the photosynthetic process and also reducing mass transfer of nutrients from the air/CO_2 to the liquid in the systems.

2.4.3 Airlift photo-bioreactors

Airlift photo-bioreactors are basically a column divided into two parts, air/CO_2 is bubbled through only one side of the partition which causes a liquid current pattern to develop with the air bubble side called the riser and other part called the down comer as shown in **Figure 2.3**. These bioreactors are extensively investigated for fermentation process and wastewater treatment but have not been looked at as are placement for the popular tubular photo-bioreactors until recent times. The airlift photo-bioreactor characterized by high mass transfer, good mixing with low shear stress, low energy consumption, high potentials for scalability, easy to sterilize, readily tempered, good for immobilization of algae, reduced photo-inhibition [**34]**. However, the main limitations are the small illumination surface area and decrease of illumination surface area upon scale-up. It has become clear that biological carbon sequestration and hydrogen production technologies have been poorly studied in the airlift photo-bioreactors and are in their infancy of development [**36]**.

2.4.4 Bubble column photo-bioreactors

As shown in **Figure 2.3** Bubble column bioreactors are vertical columns either cylindrical or rectangular filled with growth medium and air is bubbled through a sparged system installed at the bottom. These systems have the highest gas hold up rates which mean they have the best mass transfer compared to other systems. A modified version of these bubble column bioreactors is porous membrane reactors, which have efficient aeration, give smaller bubbles and pressure drop across the membrane is low compared with other rigid sparged bubble column reactors. These characteristics are achievable at high gas flow rates with low energy costs [**39]**.

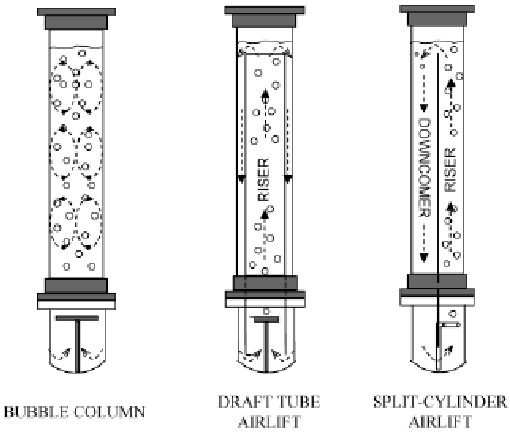

Figure 2.3 Configurations of bubble column and airlift photobioreactors [**32]**.

CHAPTER- 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microalga and culture medium

The culture of *Chlorella vulgaris*, a photoautotroph and fast growing microalga, was obtained from Science Technology and Entrepreneur's Park (STEP), Thapar University, Patiala. The micro-algae particle cells are unicellular, green colored with minimal cluster formation.

Fogg's media was used for the maintenance of the culture as well as subculturing for maintaining the stock. Composition of this solution is given in **Table 3.1**.

Table 3.1 Fogg's medium composition

Ingredients	Concentration
Potassium nitrate	0.5 g/L
Di-potassium hydrogen phosphate	0.2 g/L
Magnesium sulphate	0.2 g/L
Calcium chloride	0.1 g/L
A5 micronutrient solution	1 mL/L
Fe-EDTA stock solution	1 mL/L

To make up the Fe-EDTA stock solution, 26.1 g EDTA was dissolved in 286 mL water that has 19 g KOH. Then 24.9 g of $FeSO_4.7H_2O$ was dissolved in 500 mL water. Iron sulphate solution was added slowly to the potassium EDTA solution along with aeration and stirring. The pH rises to about 7.1 and the solution became wine red and very little precipitation occurred. Final volume was made up to 1 L and stored in a brown bottle in dark.

The A5 micronutrient solution, as the name suggests contains traces of metallic ions in the form of salts and other minerals. Certain ions and salts are needed in minute amounts as they are used as cofactor by enzymes for growth of algae. Composition of this solution is given in **Table 3.2**.

Table 3.2 A5 micronutrient composition

Ingredients	Concentration (g/L)
Boric acid	2.86
Manganese chloride	1.81
Zinc sulphate	0.22
Sodium molybdate	0.018
Copper sulphate	0.08

All chemicals were of AR grade and obtained from Himedia.

3.2 Instruments/ Equipment

3.2.1 Set-up for subculturing

The set-up for subculturing included a light source of 85 Watts cool white fluorescent light of CFL, and a temperature controlled orbital shaker. Flasks containing the media as well as the inoculum were placed in the orbital shaker. Cultures were maintained at 27ºC and 110 rpm. Illumination was provided by white fluorescent light and luminous intensity was kept around 5000 lx. The cultures were incubated till the stationary phase of the growth cycle was reached.

3.2.2 Set-up for CO_2 fixation

Set up for the CO_2 fixation experiments consisted of two light sources (luminous intensity was kept 5000 lx) and a Bioflo® / Celligen® 115 Bioreactor (New Brunswick Sci., USA) of 7.5 L capacity with working volume of 5.5 L containing inoculum and media and a CO_2/air gas supply. CO_2 was provided from a compressed CO_2 gas cylinder obtained from Lalit Gases, Patiala. Air was supplied through an oil-free air compressor of Apcon, India. Schematic diagram of the experimental setup is given in **Figure 3.1 (a)**. The gas flow was controlled with the help of a needle valve and rotameter. The total process flow diagram is shown in **Figure 3.1 (b)**.

3.2.3 UV-Vis spectrophotometer

Absorbance of algal cell suspension was taken at regular intervals, at 680 nm by a Lambda 35 UV/Visible Spectrophotometer (Perkin Elmer, USA).

3.2.4 Microscope

Images of live algal cells were captured using a Nikon eclipse 50 i microscope at 40X and 100X magnifications.

3.2.5 Centrifuge

Remi, R- 8C, BL centrifuge was used for phase separation of micro-algal cells and growth media.

3.2.6 Autoclave

Autoclave obtained from Equitron, India was used for sterilization of media and bioreactor.

3.2.7 pH meter

pH of media was determined by using pH meter (Thermo Scientific, Orion 5-Star model) and adjusted with the help of 1 N HCl and 1N NaOH. The pH probes (of pH meter and of bioreactor) were calibrated using buffers of pH 7 and 9.2.

3.2.8 Lux meter

Lux meter was obtained from Mastech, India, model MS 6610 to monitor the light intensity provided to the algal culture. Light intensity plays a remarkable role in the culture photosynthetic organisms. Hence it affects the biomass yield of *C. vulgaris*.

3.2.9 Laminar air flow chamber

Laminar Air Flow chamber from Thermodyne Pvt. Ltd., Faridabad was used for transferring the culture in sterile conditions.

3.2.9 Organic elemental analyzer (CHNS analyzer)

CHNS analyzer (Thermo scientific (UK) Flash 2000**)** was used to determine the concentration of carbon, nitrogen, sulphur and hydrogen in the algal samples.

3.2.10 BOD incubator and Thermo reactor

BOD incubator (Aar Kay Enterprises, Ambala cantt.) was used to incubate oxitop respirometer systems (OxiTop IS 6, IS 12) for 5 days to determine BOD. Thermo reactor (model 2025 D) was used for sample digestion for COD analysis.

3.3 Procedure

3.3.1 Subculturing/maintenance

Subculturing was carried out with 10% inoculum (mid exponential phase) in shake flask containing 200 mL of Fogg's media in temperature controlled orbital shaker at 27°C and 110 revolutions per minute (rpm) stirring for 5 days. The flask was plugged with cotton balls. Self-dissolved carbon dioxide from air was the only carbon source available here in order to avoid the growth of other heterotrophic bacteria. Culture was grown under continuous illumination of 5000 lx [**40**], which was provided with the help of 85 Watt CFL. Initial pH of the media was set at 7.2±0.2. The culture was subcultured after every two weeks to maintain fresh stock.

3.3.2 Autoclaving

Before starting a batch, sterilization of the photobioreactor is must. For this purpose, autoclave was employed as nothing was better than moist heat which kills not only microorganisms but also their spores. However, prior to autoclaving, photobioreactor was initially washed with chromic acid and then rinsed with soap solution and water. Photobioreactor was unassembled for this purpose. After washing, photobioreactor was allowed to air dry and then it was reassembled. Probes in photobioreactor were cleaned up using ethanol. Afterward, media was added to the photobioreactor and pH was set at 7.2±0.2 (pH was set before autoclaving). All the pipes which were dipped in media were tightened up using stoppers to prevent any escape of media during autoclaving.

Opening of every pipe was further plugged using cotton balls and aluminum foil. Water level was checked in the autoclave before starting. DO probe, pH probe and impeller shaft were covered with their respective caps. Photobioreactor was autoclaved at 121°C and 15 psi for half an hour. Removal of air from the autoclave was ensured before autoclaving.

3.3.3 Inoculum

10% inoculum was used and culture was always taken in their mid exponential phase as the number of cells is more as well as cells divides actively during this period. Before inoculation, the head plate of the photobioreactor was sterilized using Bunsen flame along with the ethanol. Funnel used for this purpose was also autoclaved.

3.3.4 Microalgal culture conditions experimental set up for CO_2 fixation experiment

Bioflo® / Celligen® 115 Bioreactor (New Brunswick Sci., USA) of 7.5 L capacity with working volume of 5.5 L was used. The temperature of 27±1˚C was measured with a sensor Resistance Temperature Detector (RTD) and maintained with the help of chiller and heating jacket. The pH of 7.2±0.2 was measured with the help of pH probe. Illumination of 5000 lx was provided using two 85 Watts CFLs. The mixture of air and CO_2 was sparged through a ring sparger at 0.5 vvm (volume gas/ volume liquid/ min). The flow of gas mixture at 2.75 L/min was set using the rotameter. Agitation of 200 rpm was provided through a Rushton disk type impeller for proper distribution of nutrients as well as breaking of the air bubbles to improve the mass transfer of CO_2. The CO_2 and air mixture was passed through micropore filter with 0.2-µm pore size prior to injection in photobioreactor. Similarly outlet of air was also guarded by similar filter to ensure that no algal cells can leave the photobioreactor. The outlet was installed with exhaust condenser (at low temperature i.e. 15°C) so that medium cannot evaporate from the photobioreactor. Baffles were provided in the bioreactor to ensure proper mixing by creating turbulence.

3.3.5 Gas mixture

In this experiment of biomitigation of CO_2, pure CO_2 from a cylinder with a preheater (to prevent choking at low temperature) and zero air through an oil free compressor (Apcon, India) were mixed. CO_2 concentration was varied in mixture during every batch but the aeration rate was fixed at 0.5 vvm.

3.3.6 pH optimization

The culture of *Chlorella vulgaris* was grown in Fogg's media and its growth was observed under different pH regime i.e. 6, 7, 8 and 9. However, other conditions i.e. light intensity; CO_2 concentration and agitation were kept constant. The experiment was performed for 120 hours (5 days). 10% freshly growing culture was inoculated in 200 mL Fogg's media and growth was measured in terms of optical density (O.D.) at 680 nm. The light intensity was kept at 5000 lx and temperature was maintained between 27±1˚C. Cultures were shaken gently thrice a day to avoid clumping and accelerate the growth process.

3.3.7 Estimation of dry weight of biomass

Aseptically collected samples were filtered using Whatman's No. 1 filter paper, washed and dried at 60ºC overnight to obtain the dry weight of the biomass [**40**]**.** Optical density of *Chlorella* for the corresponding day was measured using UV/visible spectrophotometer. Biomass was taken by this method only for five days and after that optical density was converted to dry weight using a calibration curve.

3.3.8 Determination of kinetic parameters

The biomass values were plotted against time to construct the growth curves. From the exponential phase, the specific growth rate μ (1/d) was calculated according to the Equation (3.1)

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} ----(3.1)$$

where, X_2 and X_1 are the dry biomass weight (g/L) at time t_2 and t_1 respectively. From the different μ values, the maximum specific growth rate μ_{max} (1/d) was determined. The cell doubling time was estimated by Equation (3.2)

$$t_d(d) = \frac{\ln 2}{\mu_{max}} \quad ------------ (3.2)$$

The maximum biomass obtained was designated as X_{max} (g/L). The biomass concentration ΔX (g/L) over cultivation time Δt was calculated as $\Delta X = X_t - X_0$. The overall biomass productivity $P_{overall}$ (g/L/d) was calculated using Equation (3.3)

$$P_{overall} = \frac{\Delta X}{\Delta t} ----(3.3)$$

where, X_t is the biomass concentration at time t and X_o is the initial biomass concentration at inoculation time (t_o). P_{max} (g/L/d) was designated as the maximum productivity.

3.3.9 Determination of CO_2 utilization efficiency

The initial biomass concentration of inoculum and maximum biomass concentration achieved in the bioreactor was designated as X_0 and X_t (g/L) respectively.

Thus, the CO_2 fixation rate F_c (g/L/d) was calculated according to Equation (3.4)

$$F_c = \frac{P_{overall} * 0.5 * 44}{12} ----(3.4)$$

where, 0.5 is taken as carbon content of dried biomass which is calculated with the help of CHNS analyzer, 12 (g/mol) and 44 (g/mol) presents the molecular weight of carbon and CO_2, respectively [**41**].

3.3.10 Biomass analysis

(i) CHNS analysis

Elemental analyses of total nitrogen, carbon and sulfur was done to determine carbonate and organic carbon and to get some idea of the composition of the organic matter, based on total organic carbon/total nitrogen [C/N] ratios.

Freeze-dried and crushed samples were weighed (5-10 mg) and mixed with an oxidizer (vanadium pentoxide [V_2O_5]) in a tin capsule for the CHNS analysis, which was then combusted in a reactor at 1000°C. The sample and container melt, and the tin promote a flash combustion in oxygen enriched atmosphere. A constant flow of carrier gas (helium) that passes through a glass column packed with an oxidation catalyst of tungsten trioxide (WO_3) carries combustion products CO_2, SO_2, and NO_2 and a copper reducer, both kept at 1000°C. At this temperature, the nitrogen oxide was reduced to N_2. The N_2, CO_2, and SO_2 were then transported by the helium to a 2-m-long packed column (Poropak Q/S 50/80 mesh) and quantified with a Thermal Conductivity Detector (set at 290°C).

(ii) Protein Estimation

Protein was estimated with the help of Lowry method [**40**] but before it, samples were homogenized along with lysis buffer containing 0.5 M Tris HCl, 8 M Urea, 5% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) ß mercepto-ethanol. Final pH was adjusted to 6.8 and then centrifuged at 4°C for 20 minutes at 10,000 rpm. After centrifugation supernatant was taken up and Lowry method was followed.

3.3.11 Determination of growth kinetics using logistic equation

Logistic equation was used to determine the growth kinetics of algae. It is a good choice for explaining the growth curve as it does not use substrate term for explaining the entire growth profile of the algae. X v/s t gives a sigmoid variation of X as a function of t and it can explain the entire growth profile (lag, exponential, and stationary phase) of the culture satisfactory [**42**]. Equation 3.5 is the logistic equation.

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{X_{\max}}\right) \text{-------------------} (3.5)$$

Where X is the dry cell weight (g/L) $X_{\max}$ is the maximum dry cell weight (g/L) and k is the apparent specific growth rate (1/d) for this strain. On integrating with the boundary conditions $X(0) = X_0$ and rearranging Equation3.5, it can be written as Equation 3.6 where the initial biomass concentration X_0 is in (g/L).

$$X = \frac{X_{\max}}{1+\left(\frac{X_{\max}}{X_0}-1\right)e^{-kt}} \text{ ------------------------ } (3.6)$$

This may be further rewritten in the form of Equation (3.7)

$$y = \frac{a}{1+be^{-kt}} \text{ ------------------- } (3.7)$$

where $X_{\max}$ is 'a' and $\left(\frac{X_{\max}}{X_0}-1\right)$ is 'b'. These constants were determined by fitting the experimental data in Equation3.7 in OrigiPro 8.0 using curve fitting tool. A confidence bound of 95% was taken into consideration to find the fit.

$$\text{b=}\left(\frac{X_{\max}}{X_0}-1\right)\text{; a= } X_{\max} \text{ (g/L) and } X_0 = \frac{a}{1+b}$$

3.3.12 Microalgae grown in dairy wastewater

Culture of *Chlorella vulgaris* was grown in dairy waste (obtained from Verka plant, Sangrur, Patiala) for 5 days at 27°C in orbital shaker in shake flask to study the effect of wastewater on growth of microalga. Sample of dairy waste was first filtered and then autoclaved before it was used to grow microalga. After 5 days of experiment, algal sample was centrifuged and supernatant was taken for BOD measurement in oxitop respirometer systems having pressure sensors for BOD testing and measurements in a closed system. Microorganisms in the sample consume the oxygen and form CO_2 which was absorbed by NaOH, creating a vacuum which can be read directly as a measured value in mg/L of BOD.

The OxiTop® BOD instrumentation heads have an AutoTemp function. If the sample temperature is too cold, the start of BOD measurement is automatically delayed (by at least 1 hour) until a constant temperature has been reached.

Apart from the automatic storage of 5 measured values (1 value per day), further measured BOD values can be read at all times during or after the period of 5 days, it permits the tracking of check values or measurements over longer periods. The OxiTop BOD Instrumentation measurement system also offers improved controllability and simple operation

COD

Supernatant obtained after centrifuging (at 10,000 rpm for 10 minutes) *Chlorella* sample was diluted 20 times in distilled water and mixed thoroughly. Then 5 ml of sample was added to 15 mL of distilled water in a glass tube. After that, 10 mL of 0.25N $K_2Cr_2O_7$ solution and 20 mL of concentrated H_2SO_4 were also added in the glass tube. Then it (glass tube) was kept in the thermoreactor at 150°C for 2 hours. Finally, the glass tube was cooled and sample was taken in cuvette for its spectrophotometric analysis.

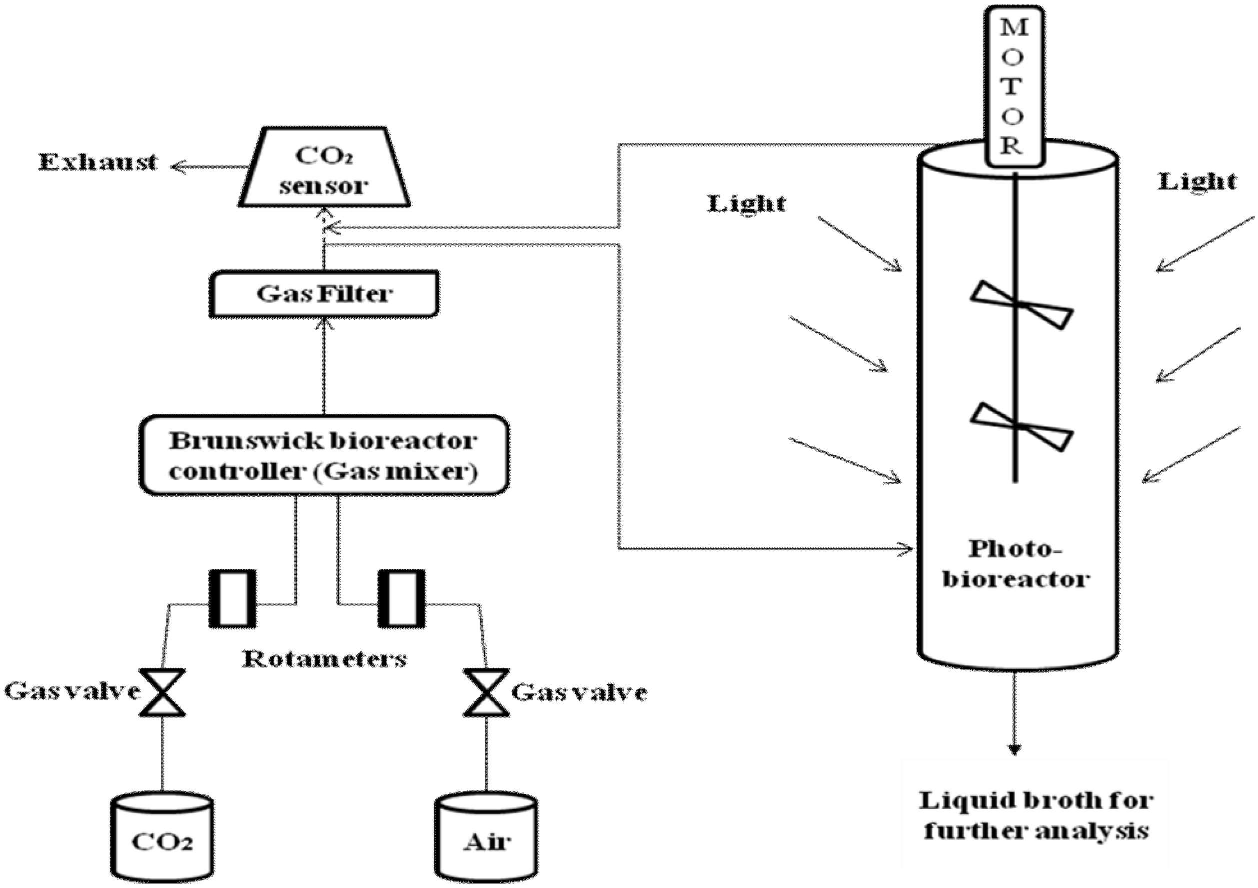

Figure 3.1(a) Schematic diagram for CO_2 fixation experimental set up

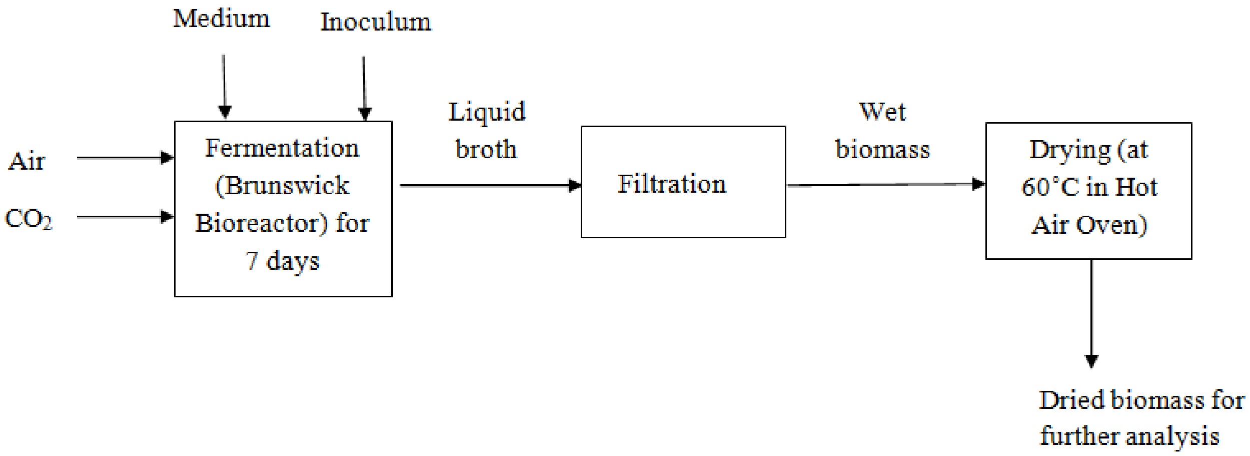

Figure 3.1(b) Process flow diagram of CO_2 fixation experiment

CHAPTER 4
RESULTS AND DISCUSSION

4.1 pH Optimization

pH of media is an important factor which significantly affects the growth of the algae. The variation in pH affects the solubility and availability of nutrients, enzyme activity, and transport of substrates across plasma membrane and electron transport in respiration and photosynthesis. So, pH was optimized for the microalga *C. vulgaris.*

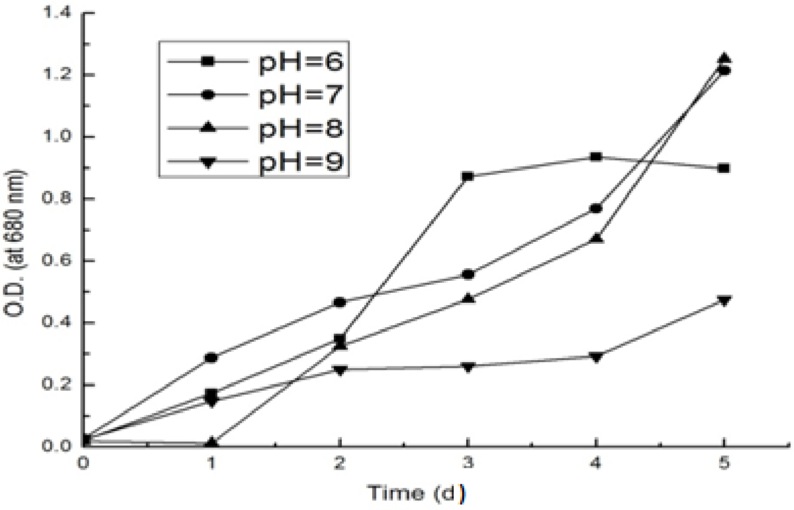

Figure 4.1 Growth patterns of alga at different pH

The growth patterns under various pH regimes, varying from 6 to 9 are given in **Figure 4.1**. Best growth was observed at pH 7, where biomass was found maximum and lag phase was found to be of minimum span. The organism was found to grow well between pH 7 and pH 8. So, a pH shift above 6 was found not to negatively affect the growth. But, when the pH was lower than 6 or higher than 8, the growth was noticeably reduced. When *C. vulgaris* was cultured at pH 9, the growth started ceasing after 5, whereas at pH 7 and 8, growth continued and was still increasing. The growth pattern was similar in the pH range 7-8. The pH shift in media, which was due to non-controlling of pH, did not seem to affect the growth. Maximum growth is found in between 6-8 pH but at 6 pH lag phase is found to be longer and at 8 growth was found slow in comparison to 7. So the best pH is found to be 7.

During when experiment was carried out in photobioreactor the initial pH of media was observed around 8 and it was made around 7 manually with the help of 1N HCl before inoculation. However on the first day pH was found to decrease sharply. It was noted that presence of culture did not contribute to lowering the pH, it was the effect of CO_2. Since similar effect was observed in control experiment of pH optimization with culture and without CO_2 here it was found that pH was rising along with growth.

But in photobioreactor decrease in pH was found proportional to increase in CO_2 concentration; however pH varies in window of 5.8 to 6.5.

4.2*Chlorella vulgaris* Dry Weight Calibration Curve

Figure 4.2 shows the calibration curve of O.D. v/s algal biomass concentration. Growth concentration was measured in terms of biomass dry weight (g/L). Biomass can be more easily measured by using absorbance than by measuring cell dry weight directly. So, the relationship between optical density and cell dry weight for *C. vulgaris* was established by linear regression. It was found that optical density precisely predicted the dry weight (R^2=0.988). Hence, O.D. values were used to calculate the biomass.

$$y = 0.393\,x - 0.01;\ R^2 = 0.998$$

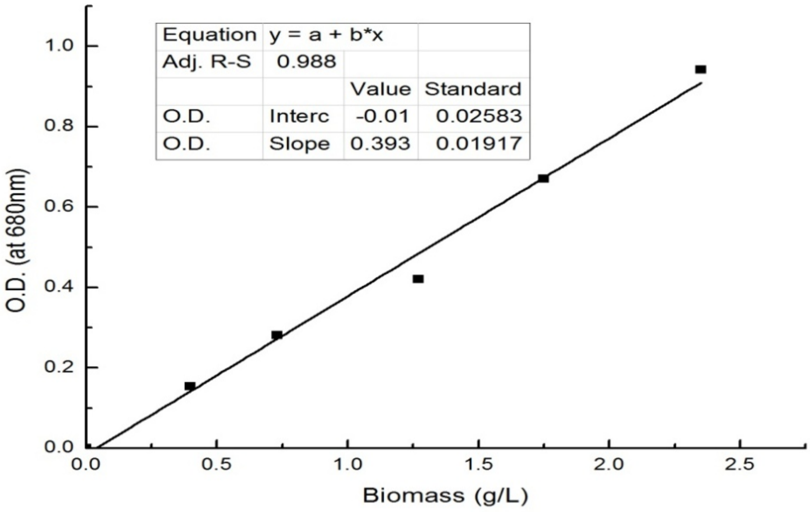

Figure 4.2 Calibration curve of O.D. v/s algal biomass concentration

The value x is biomass concentration (g/L). The value of y is optical density of algal biomass sample measured by UV-visible spectrophotometer at 680 nm.

4.3 Growth Kinetics for *Chlorella vulgaris*

Figure 4.3 shows the growth pattern of the algal cells at different CO_2 concentrations. Growth was measured in terms of biomass dry weight (g/L). *Chlorella* showed the maximum biomass growth of 7.96 g/L at input of 10% CO_2 concentration and it corresponds to 13.81 g/L of CO_2 sequestered. It is established from the **Figure 4.3** that biomass concentration increases with increase in CO_2

concentration. It follows an upward trajectory up till 10% CO_2 concentration and then starts declining. It can be clearly concluded that biomass growth is higher at 10%. At different CO_2 concentrations 0.04%, 5%, 10%, 15% and 20%, maximum biomass concentration corresponds to 0.705 g/L, 5.78 g/L, 7.96 g/L, 7.66 g/L and 7.42 g/L respectively.

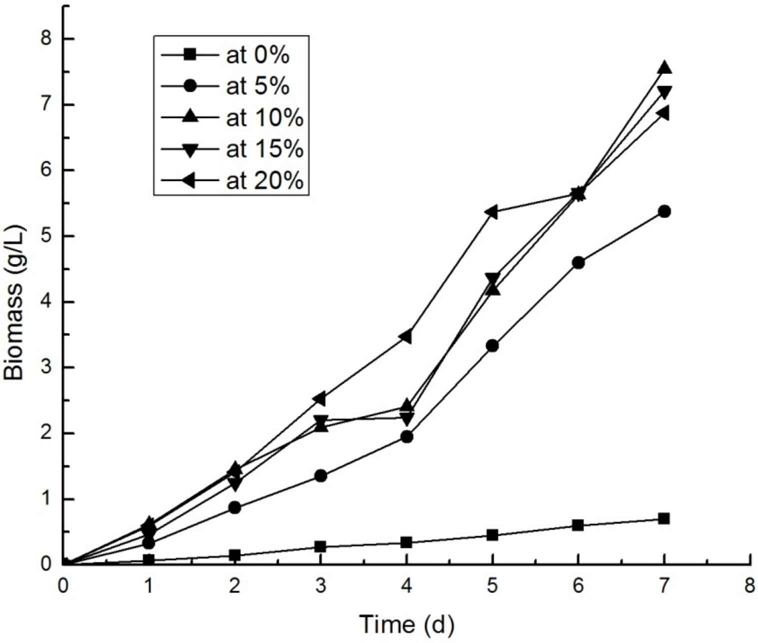

Figure 4.3 Biomass v/s time curve for microalga at different CO_2 concentrations

The X_{max} value of *Chlorella vulgaris* after 168 hr was found to be 7.96 g/L at 10% CO_2 concentration and the values of μ_{max}, productivity and doubling time (t_d) were calculated as 0.311 l/d, 1.078 g/L/d and 2.22 d respectively**.** It must be understood that it is very unlikely to see stationary phase in autotrophs, however their growth does get affected by depletion of limiting growth factors. As the A5 micronutrient which had used for Fogg’s media has a maximum life span of 2 months only after that it affects the growth of microalgae.

4.4 Total CO_2 Utilized

To determine the potential of using the selected algal strain for carbon dioxide sequestration, the algal cells were cultured at different CO_2 concentrations. Runs were carried out for 7 days in Brunswick photobioreactor with Ruston disk type impeller. The inoculum used was pre-adapted to CO_2. Carboxylase activity of ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco) catalyzes the CO_2 fixation reaction in microalgae. However, at lower concentration of CO_2, Rubisco shifts towards oxygenase activity making them ineffective for the CO_2 fixation. The microalgae have developed carbon concentrating mechanism (CCM) which helps in increasing intracellular CO_2

concentration for the smooth functioning of carboxylase activity of Rubisco. Activation of CCM leads to expression of carbonic anhydrase (CA) enzyme. The activity of CA causes alkaline environment outside the cell as it transports hydroxide ions outside the cell in association with the capture of H^+ ions for the interiors of the thylakoid membranes [**43]**. This may be the reason for the rise in pH when microalga was not sparged with CO_2.

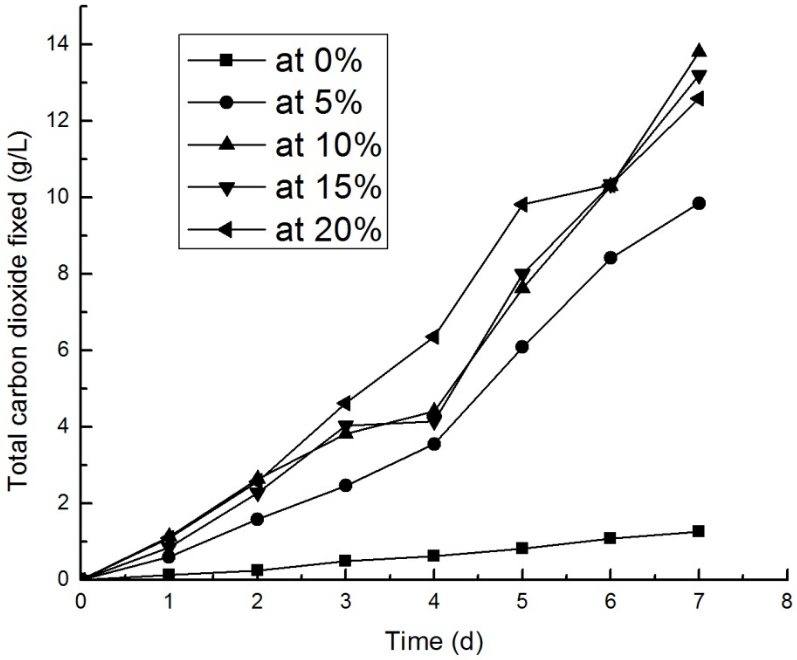

Figure 4.4 Total CO_2 utilized v/s time curve of microalga

Figure 4.4 shows the CO_2 fixed for all CO_2 concentrations 0.04%, 5%, 10%, 15% and 20%. Conditions like illumination, temperature, pH and agitation were kept constant at 5000 lux, 27±1°C, 7±0.2, and 200 rpm respectively. *Chlorella* showed the maximum CO_2 fixation i.e. 13.81 g/L at input of 10% CO_2 concentration. It follows an upward trend up till 10% CO_2 concentration and then starts declining. The reason for this is fall in pH due to rise in CO_2 level. Through influencing on the distribution of inorganic carbon species along with it pH has an indirect effect on the growth and photosynthesis of microalga [**44]**.When CO_2 level increased, *Chlorella* preferred aggregative growth form (like bio-floc or biofilm) than individual suspended form that's why at higher CO_2 level fixation as well as growth is declining.

4.5 Kinetic Parameter Studies

Biomass (X) values at different times and exponential regression were used to calculate the maximum specific growth rate (μ_{max}) during the logarithmic phase. CO_2 fixation rates and captured CO_2 amounts continue to rise as reaction proceeds. This indicates that algal cells are still functioning at an optimal level. 10% is the CO_2 concentration at which all the kinetic parameters, total CO_2 fixed and fixation rate were found to be maximum. But it is clear from the above figures that higher the concentration of CO_2 more is the growth of microalga till 10 % but at 15 and 20% CO_2 growth is less in comparison to 10%. Flue gas approximately contains 9-15% carbon dioxide which can be utilized by the microalga for their growth and subsequently removing carbon dioxide from the environment, so it is evident from these results that we can directly use flue gas for the growth of microalga.

Table 4.1 Effect of CO_2 concentration on kinetic parameters of the algal strain

CO_2 concentration (%)	**μ_{avg} (1/d)**	**Productivity (g/L/d)**	**t_d (d)**	**CO_2 fixation rate (g/L/d)**	**Total CO_2 fixed (g/L)**
0.04	0.238	0.099	2.91	0.1812	1.27
5	0.271	0.768	2.55	1.405	9.85
10	0.311	1.078	2.22	1.972	13.81
15	0.305	1.03	2.27	1.885	13.20
20	0.374	0.982	1.85	1.79	12.59

In this experiment we have carried out 5 batches including blank. In every succeeding batch the inoculum from previous batch was taken, as a result with every transfer of inoculum the lag phase of algal growth was significantly reduced. As depicted in **Table 4.1,** maximum carbon dioxide fixation rate of *Chlorella vulgaris* was calculated to be 1.972 g/L/d at 10% CO_2 concentration by Equation 3.4. Continuous rise in fixation of CO_2 coincides with increase in biomass concentration. CO_2 fixation rate and maximum CO_2 fixed in 2nd batch of 168 hours at 10% CO_2 concentration were 1.972 g/L/d and 13.81 g/L respectively. The μ_{avg} and doubling time were found best at 20% CO_2 concentration. Monodispersivity ensured maximum mass transfer possible. Conditions were decided carefully to enhance every positive parameter of growth which is well reflected from all the above mentioned parameters.

4.6 Biomass Analysis

(i) CHNS analysis

Table 4.2 Shows the percentage of carbon content which remains same till 15% CO_2 concentration, i.e. 46%. However, decline in carbon content was observed at 20% CO_2. The carbon fixation decline clearly indicates that at higher CO_2 concentrations *Chlorella* was not able to utilize carbon dioxide properly but capable of doing so in flue gases in which CO_2 concentration is 9-15 %. The lower specific growth rate at 15% CO_2 level may be due to non-representative biomass determination and this caused lower biomass measurement at 20% CO_2 level. However, the concentration of nitrogen remained approximately same which clearly indicates that high CO_2 concentration does not affect much on protein content of *Chlorella*.

Table 4.2 Elemental profile of algae at different CO_2 concentration

Sample	**CO_2 concentration (%)**	**N %**	**C %**	**H %**	**S %**
1	5	7.63	46.39	6.901	0.549
2	10	7.34	46.99	6.625	0.495
3	15	6.16	46.40	6.633	0.551
4	20	5.77	35.90	5.405	0.740

The carbon content obtained from CHNS analysis was used in Equation 3.4 for calculation of CO_2 fixation rate F_c (g/L/d) and studied further. Nitrogen content of microalgae generally varies but is usually between 4 and 8% of the cells (dry weight basis) depending on physiological state and nutrient limitation [**45**] and we have observed the same from our readings that means the nutrient composition provided was best for growth of microalga. The C/N ratio also increased significantly in cultures exposed to the highest CO_2 concentrations, indicating the cycle focuses more on carbohydrate production than on protein up to 15% CO_2 concentration but C/N ratio significantly decreased at 20% CO_2. S% is found to be less which is best for growth as it gets converted in to sulphide which is poisonous for microalga as its higher amount decreases pH drastically.

It has been established by other investigators that 1 unit of biomass produced consumes 1.83 units of CO_2 **[41].** We have also used this in our studies and also calculated carbon content fixed in algae by CHNS analysis which supports the fact that it is around 50%.

(ii) Protein estimation

Table 4.3 presents the protein content of the microalga studied. A high amount of proteins was observed in *C. vulgaris* at 5% CO_2 concentration. For that a calibration curve is plotted i.e. O.D. v/s protein concentration (μg/mL).

$$y = 0.0020\,x - 0.01;\ R^2 = 0.996$$

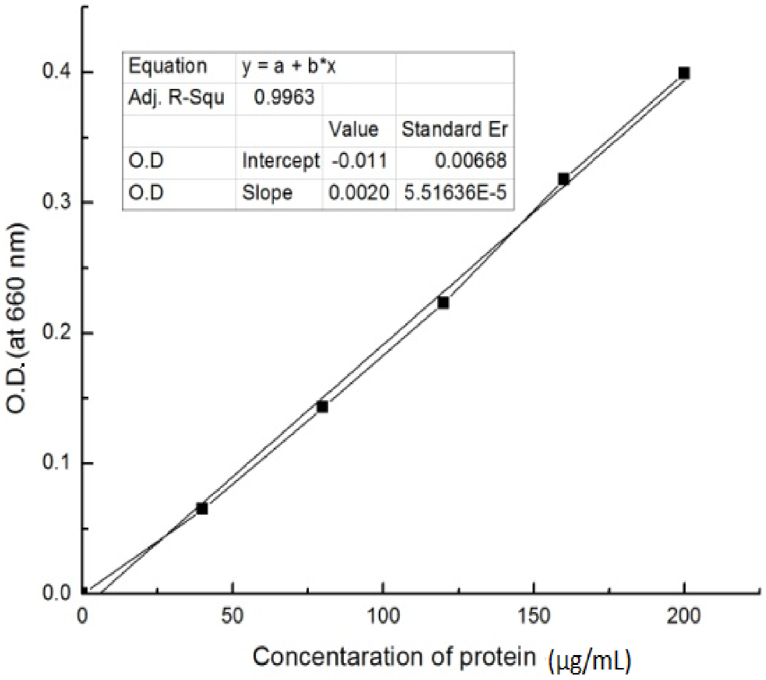

Figure 4.5 Calibration curve of O.D. v/s protein concentration (µg/mL)

All the samples were kept at 1/20th dilution**.** According to **Figure 4.5,** straight line equation y= 0.0020x - 0.011 was obtained where y = O.D. and x = amount of protein obtained.

Table 4.3 Parameters calculated for protein estimation

CO_2 concentration (%)	**O.D. (at 540 nm)**	**Amount of protein (g/L)**	**% of protein**
5	0.218	2.291	42.56
10	0.317	3.28	41.21
15	0.303	3.14	40.99
20	0.280	2.91	39.21

As shown in **Table 4.3,** protein content was found to be maximum at 5% CO_2 concentration. A good similarity was observed between the data obtained by Lowery method and CHNS analysis. We can compare that at 5% CO_2 concentration in CHNS analysis N (%) content was found to be maximum supporting the fact that protein formation machinery is more functional at 5% CO_2.

4.7 Predicting Biomass Productivity Behavior

Logistic equation as given below was used to determine the growth kinetics of alga which was predicted with the help of OriginPro 8.0 using curve fittineg tool.

$$X = \frac{X_{\max}}{1 + \left(\frac{X_{\max}}{X_0} - 1 \right) e^{-kt}}$$

Figure 4.6 shows predicted cell growth profile of *Chlorella sp.* in stirred photobioreactor at different percentage of CO_2. Experimental data is fitted with logistic equation. X_{max} and k obtained from predicted logistic model are given in **Table 4.4**. Data points were average of experimental runs, here in **Figure 4.6** line represents the predicted and symbols represent the experimental data.

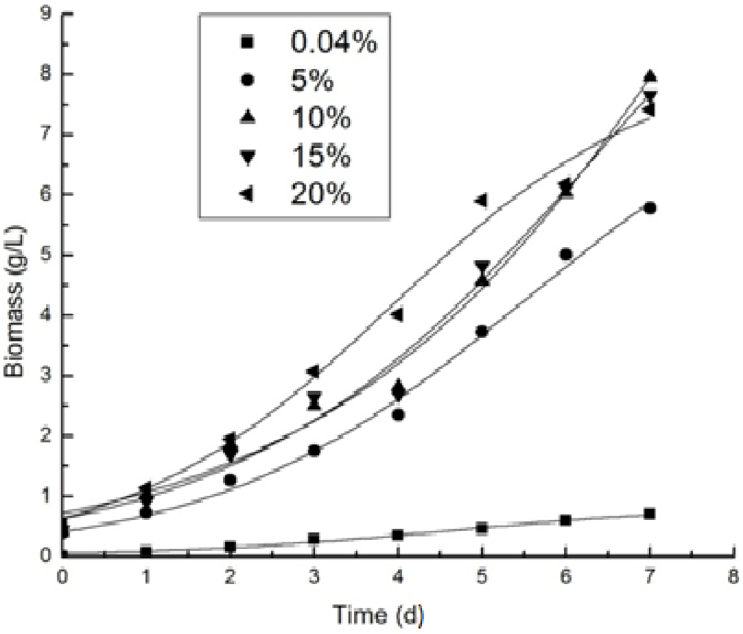

Fig 4.6 Predicted cell growth profile of algal cells at different CO_2 concentrations

Table 4.4 shows predicted values of logistic model for maximum biomass and apparent specific growth rate with their R^2 values at different CO_2 concentration in stirred photobioreactor.

Table 4.4 Predicted values of logistic model

CO_2 % (v/v)	**c = k (1/d)**	**a = X_{max} (g/L)**	**R^2**
0.04	0.6068	0.8572	0.98678
5	0.5397	8.4882	0.99285
10	0.4027	21.5593	0.98777
15	0.4677	13.6075	0.98078
20	0.6399	8.3020	0.98811

The curve fitting was found to be in good agreement with experimental values as the R^2 values were approximately equal to 0.98 (**Table 4.4**). From the predicted curve, we can also find the values of constant k and X_{max}. The pattern of X_{max} rise and fall was found to be precisely similar to experimental values of X_{max}. As Rubisco enzyme is working for CO_2 fixation, its activity is dependent on concentration of CO_2, O_2 and pH value. At 0.04% CO_2, due to less amount of substrate, it shifts towards its oxygenase activity and at very high concentration of CO_2 (15% and 20%) substrate inhibition is responsible for less biomass production in comparison to 10%. The best curve fitting for our data is non-linear as it minimizes the sum of squared residuals.

4.8 Growth Studies in Dairy Wastewater

As the mode of algal cultivation influences the economy of whole process, the present study is focused more on this decisive step. For this purpose, culture of *Chlorella vulgaris* was grown in dairy wastewater for 5 days at 27°C in orbital shaker in shake flask to study the effect of wastewater on growth of microalga. It can be observed from **Figure 4.7** that alga grew at much higher rate in wastewater than in regular medium.

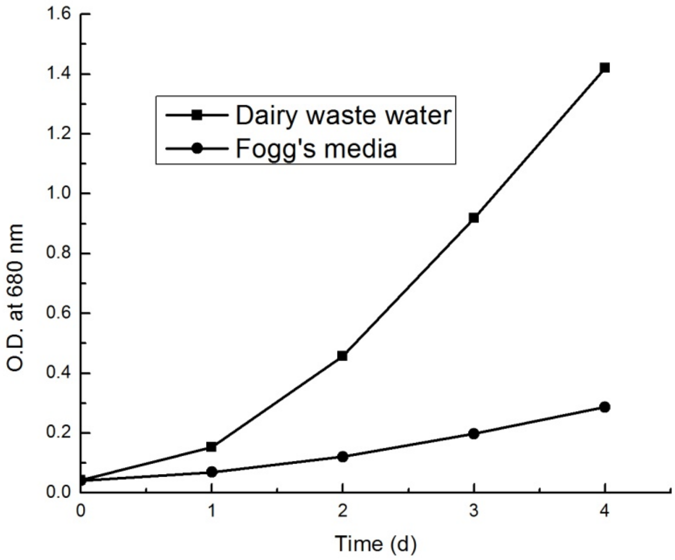

Fig 4.7 Growth profile of microalga in dairy wastewater and synthetic media

It can also be observed that dairy wastewater is a better source for growth of microalga in comparison to Fogg's media. Here, we can directly correlate growth of microalga with optical density.

COD and BOD

Table 4.5 shows that decline in value of COD and BOD after inoculating the wastewater with *Chlorella.* Alga utilized organic compounds present in wastewater along with atmospheric CO_2.

Table 4.5 COD and BOD values after inoculation with *Chlorella vulgaris*

Sample (wastewater)	**BOD (mg/L)**	**COD (mg/L)**
Without filtration	2500	3100
After filtration and autoclaving	2300	2400
After 5 days of inoculation	96	120
Control (Fogg’s media)	35	44

It was observed that dairy wastewater will be the most scalable and economic medium for alga cultivation. Alga cultivation in wastewater provides an opportunity to achieve double benefits. One, it results in cost-effective treatment of wastewater and second, it is easy availability of nutrients in the medium. It also results in lower costs of algal biomass production as well as much higher growth than control media.

CHAPTER 5

CONCLUSIONS AND RECOMENDATIONS

5.1 Conclusions

CO_2 fixation rates and captured CO_2 amounts continue to rise as reaction proceeds. This indicates that algal cells are still functioning at an optimal level at higher concentrations of CO_2. 10% is the CO_2 concentration at which all the kinetic parameters as well as total CO_2 fixed and fixation rate are found to be maximum. The conclusions obtained from our studies can be summarized as follows

- *Chlorella vulgaris* grows best at pH 7.
- Maximum average specific growth rate is 0.374 (1/d) at 20% CO_2 concentration.
- Maximum carbon fixation rates and CO_2 fixed of *Chlorella vulgaris* are1.972 g /L/day and 13.81g/L respectively, at 10% CO_2 concentration.
- Maximum protein content, 42.56%, was measured at 5% CO_2 concentration. Value the data is supported by the CHNS analyzed N% which is 7.63%.
- The curve fitting was found to be in good agreement with experimental values as the R^2 values of biomass fitted (at all CO_2 concentrations) were approximately equal to 0.98.
- It was observed that dairy wastewater was the most scalable and economic medium for alga cultivation

5.2 Recommendations

1. As *Chlorella* was found tolerant to high CO_2 concentrations (15 - 20%)**,** it can be directly used to sequester the CO_2 of flue gases in which CO_2 concentration is 9-15%.

- Instead of using commercial CO_2 gas cylinder, we can directly use flue gases as a source of CO_2 which will decrease the cost of separation of CO_2.

2. Additionally, instead of using Fogg's media we can use dairy wastewater for growth and maintenance of microalga which will be best for commercial purpose such as

- Decreasing the cost of media employed for maintenance of microalga.
- Decreasing the BOD and COD of dairy wastewater and will help in wastewater treatment which we have observed after performing the experiments (replacing media with dairy wastewater).

3. Photobioreactor used had fixed S/V ratio. Bringing change in the dimensions may enhance its performance.

4. In the current research, effect of pH and CO_2 concentrations were studied. However, factors like illumination, media, temperature etc. are also need to be optimized for further improvement of the process.

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