Studies of CO_2 sequestration by *Anabaena viriabilis* in stirred tank batch type photobioreactor

Dissertation

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in
Chemical Engineering

Submitted

by

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Declaration

I, the undersigned, hereby declare that the research work presented in the M. Tech. dissertation entitled "**Studies of CO_2 sequestration by *Anabaena viriabilis* 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.

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Certificate

This is to certify that dissertation entitled, "**Studies of CO_2 Sequestration by *Anabaena viriabilis* in stirred tank batch type photobioreactor**" submitted by Mr. Abhishek Sharma in partial fulfillment of the requirements for the award of M. Tech. in Chemical Engineering at Thapar University, Patiala is an authentic work carried out by him 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.

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Abstract

Global warming is posing serious threat to our environment. Malignant manifestations of this phenomenon are weather extremities, rise in sea levels, annihilation of species, glaciers‘ retreat, and similar other calamities. Increasing amount of carbon dioxide (CO_2) gas is mainly responsible for global warming. Although chemical methods are currently being employed, but rays of hope lies with biological methods only to curb increasing CO_2 concentration. Currently large scale use of biological method is still subject to research. However with improving photobioreactor design and identification of promising strains (of microalgae and cyanobacteria) has strengthen the confidence of researchers. Microalgae and cyanobacteria are most suitable candidates to serve the cause as they 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 *Anabaena viriabilis*, used in a photobioreactor at different CO_2 concentration i.e. 0.04%, 5%, 7.5%, 10%, 12.5%, and 15%, in BG (-N) 11 media with 10% (of total media volume) inoculum. CHNS analysis has shown that with increasing CO_2 concentration the composition of cyanobacteria in regard with carbon varies. At 10% CO_2 concentration all the kinetic parameters as well as total CO_2 fixed and fixation rate were found 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

1.1 Introduction

The world is experiencing rise in temperature generally known as global warming. Global warming today is matter of great concern for us. Rise in average surface temperature of earth, leading to alteration in precipitation pattern, rise in sea level, and melting of glaciers are some of the malignant environmental problems that may be caused by this phenomenon **[1]**. Global warming is mainly attributed to an increase in the atmospheric concentration of greenhouse gases (GHGs), particularly carbon dioxide (CO_2), tropospheric ozone, methane, and chlorofluorocarbons **[2]**. However, it is CO_2 which contributes the most to global warming out of all GHGs. Global greenhouse gas emissions due to human activities have grown since industrialization. Industries related to electricity generation, cement, natural gas processing, iron and steel manufacturing and combustion of municipal solid waste are the major contributors of atmospheric CO_2 because of their dependence on carbon sources like coal, oil, natural gas for fulfilling their energy requirement **[3].** Global increase in CO_2 concentration is mainly due to the combustion of fossil fuels, representing about 75% of the total anthropogenic CO_2 emissions **[4]**. 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.

It is a matter of great surprise that the natural sources of carbon dioxide emission like volcanic eruptions, geological fissures 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 by plants and marine planktons. As a result of this balance, the atmospheric carbon dioxide remained between 260 and 280 parts per million for the 10,000 years between the end of the last glacial maximum and the start of the industrial era **[5].** Measured atmospheric concentrations of carbon dioxide are currently 100 parts per million (ppm) higher than pre-industrial levels. Increasing concentration of atmospheric CO_2 from past 50 years is depicted in

Figure 1.1. According to fourth international panel on climate control (IPCC) Assessment Report, the current atmospheric CO_2 concentration is at 392 ppm **[6]**. The concentration of CO_2 in the atmosphere in 2006 varied between 360 and 390 parts per million by volume (ppmv) during the year and features a continuous increase year after year **[7]**. It is predicted that by the end of 2050, CO_2 levels will reach above 450 ppm in the atmosphere if this growing trend left unchecked, which will have severe impact on sea levels, global climate patterns, and survival of many species and organisms **[8]**.

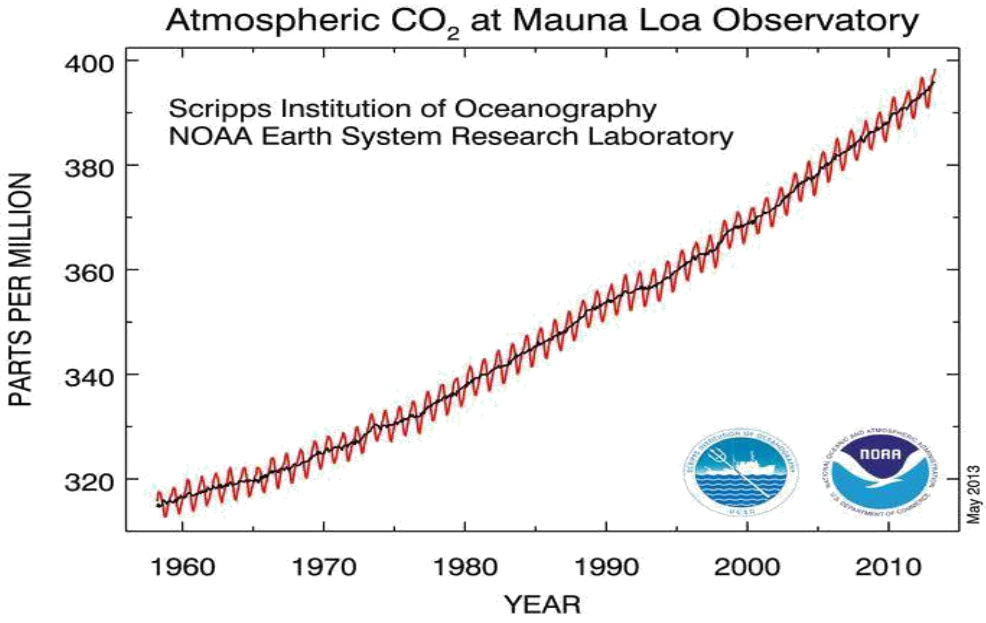

Figure 1.1: Rise in concentration of CO_2 in past fifty years **[9]**

For the stabilization of the atmospheric CO_2 concentration, CO_2 emissions should fall significantly below current levels to decline the impact on climate change. Different approaches may be followed in order to reduce the rising CO_2 concentrations:

i. Increasing efficiency in electricity generation and its optimum use.

ii. Expanding use of renewable energy sources such as wind, solar, biomass, geothermal and other alternate energy sources, including nuclear power

iii. Judicious use of energy may significantly change the present scenario.

iv. Capturing carbon dioxide emissions at fossil-fueled (especially coal) electric generating plants and permanently sequester the carbon.

The first three approaches need public acceptability to change their consumption needs and willingness to invest in ways that may be costly but will lead to more inhabitable planet. On the contrary the success of fourth approach directly depends upon the research. The scientific community is currently examining potential approaches in order to reduce the anthropogenic contributions to global warming. Processes for the carbon capture and sequestration are not yet available at commercial scale, but different technologies are being developed and assessed at pilot scale **[10]**. Currently, new technologies including chemical, photochemical and biological processes to transform the carbon dioxide into valuable products are in the research and development stage. However, whatever the technology used to capture CO_2 from flue gases, a social rejection of the storage of such an enormous amount of flue gases is to be expected. In any case, there will be an increase in the final cost of the energy. Moreover the release of byproducts that may be difficult to handle latter is also a matter of concern in case of physico-chemical methods. As an alternative to this scenario, the carbon dioxide can be used as feedstock for the

production of valuable products, which allows obtaining profit, thus reducing the cost of the energy **[11]**.

Carbon dioxide can be biologically converted into organic matter by photosynthetic microorganisms like microalgae and cyanobacteria, and this organic matter can be transformed latter into valuable products as bioethanol, biofuel, hydrogen, amino acids, etc. **[12]**. It is known that microalgae and cyanobacteria are unique, valuable and one of the most ecological variable groups of microorganisms on earth. They are the most productive transformers of CO_2 into O_2, reaching biomass production yields up to five times higher than those of the most efficient higher plants crops **[13]**. Nevertheless, this is not currently being done at industrial scale because of the high cost of producing cyanobacteria. It is now the right time to use this natural sentinel to fight against the growing menace of growing atmospheric carbon dioxide. 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.

1.2 Research Objectives

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

- Optimization of pH and CO_2 concentration for maximizing growth, productivity and CO_2 fixation rate.
- Kinetic study of CO_2 sequestration at different CO_2 concentrations.
- Study of growth of *Anabaena* in wastewater.

1.3 Dissertation Overview

This dissertation consists of five chapters. **Chapter 1** contains a brief introduction to the research topic. **Chapter 2** contains literature review relevant to the research. It gives insight to the work done by other investigators. It gives information related to *Anabaena*, different CO_2 mitigation approaches, advantages and requirement of cyanobacteria in general, and about photobioreactors.

In Chapter 3, information about materials (instruments), methods (experimental procedure) and assaying approaches used is given. **Chapter 4**, comprise of results obtained and their analysis. 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 Introduction to CO_2 Sequestration

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 as shown in **Figure 2.1**:

i. Post combustion capture

ii. Pre-combustion capture

iii. Oxy- combustion capture

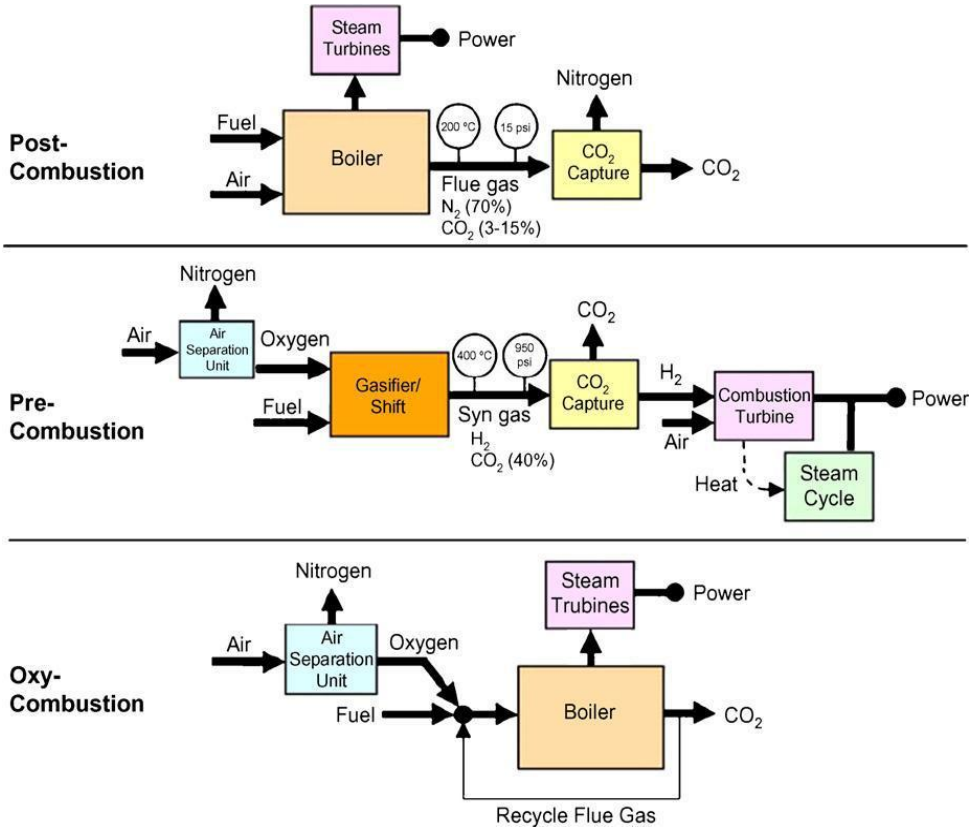

Figure 2.1: Block diagrams illustrating post-combustion, pre-combustion, and oxy-combustion systems **[14]**

i. Post-combustion CO_2 capture

Post-combustion capture involves the removal of CO_2 from the flue gas produced by combustion. Air is used by existing power plants, out of which almost four-fifth is nitrogen, for combustion and generates a flue gas that is at atmospheric pressure and typically has a CO_2 concentration of less than 15% (v/v). Thus, the thermodynamic driving force for CO_2 capture from flue gas is low (CO_2 partial pressure is typically less than 0.15 atmosphere), creating a technical challenge for the development of cost effective advanced capture processes. Despite of this difficulty, post-combustion carbon capture has the greatest near-term potential for reducing the emission of greenhouse gases (GHG), because it can be retrofitted to existing units that generate two-thirds of the CO_2 emissions in the power sector **[14]**.

ii. 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 **[14]**.

iii. Oxy-combustion

An alternative of capturing carbon from fuel gas or flue gas is to modify the combustion process so that the flue gas has high CO_2 concentration. A promising technology for accomplishing this is oxy-combustion, in which the fuel is burned with nearly pure oxygen (greater than 95%) mixed with recycled flue gas. A cryogenic air separation unit (ASU) is used to supply high purity oxygen to a pulverized coal-fired boiler. This high purity oxygen is mixed with recycled flue gas prior to combustion or in the boiler to maintain combustion conditions similar to an air fired configuration. This is necessary because currently available materials of construction cannot withstand the high temperatures resulting from coal combustion in pure oxygen. For a new unit, it should be possible to use smaller boiler equipment due to increased efficiency. The main attraction of this process is that it produces a flue gas which is predominantly CO_2 and water. 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 **[14]**.

2.2 Methods of CO_2 sequestration

Based on above mentioned approaches, following methods given in the **Figure 2.2** given 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 can be used in combination where their best properties can be used to get the best outcome **[14]**.

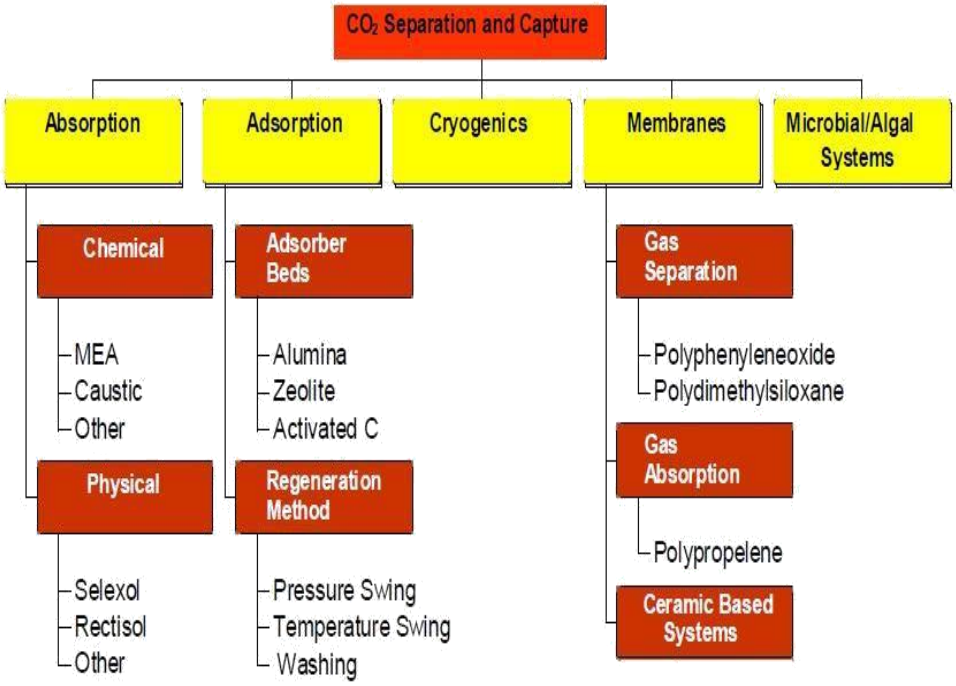

Figure 2.2: Major carbon capture and sequestration technologies **[15]**

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, which is easy to transport and store. CO_2 and H_2O are separated from flue gas on the basis of differences in dew and sublimation points **[14]**.

ii. 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, NOx emissions are low **[14, 16]**.

iii. 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) can be of tremendous economic value. 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 **[16]**.

iv. 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 **[14, 17]**.

v. Pressure swing adsorption (PSA)

This technique employs high pressure of the Integrated Coal Gasification and Combined Cycles (IGCC), in which coal is gasified with O_2 to produce CO, H_2O, CO_2 and H_2. 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 **[18]**.

vi. Biological methods

Photosynthetic solution of carbon dioxide fixation

The biological process of photosynthesis can be utilized to stabilize CO_2 and produce valuable by-products under a controlled environment. Photosynthetic biomass harvest used with CO_2 capture and storage can yield net removal of CO_2 from the atmosphere because the CO_2 put into storage comes from biomass which has absorbed CO_2 from the atmosphere or stationary sources as it grew. The overall effect is referred to as _negative net emissions‘ **[19]**.

The native aquatic photosynthetic microorganisms uptake CO_2 efficiently because they can grow rapidly and their biomass production yields are up to 5.4–10 fold greater than those of the most efficient higher plants crops **[20]**. Cyanobacteria and microalgae have been assumed as the suitable candidates to convert CO_2 into organic matter biologically. Not only can they lead to the production of commercial products like bioethanol, carotenoids etc., these photosynthetic microorganisms are useful in bioremediation applications and as nitrogen fixing biofertilizers **[21, 22]**.

2.3 Cyanobacteria for CO_2 Sequestration

Cyanobacteria are photosynthetic prokaryotes that capture sunlight for energy using Chlorophyll a and various accessory pigments. They are almost ubiquitous and are found in lakes, ponds, springs, wetlands, streams, and rivers, and they play a major role in the nitrogen, carbon, and oxygen dynamics of many aquatic environments. Not only do they live as free living organisms, symbiotic associations with more complex biota has also been observed; for example, the nitrogen-fixing

species *Anabaena azollae* forms a symbiotic association with the floating fern *Azolla*, which is widely distributed in ponds and flooded soils.

Cyanobacteria were formerly classified as blue-green algae because of their algal-like appearance, their possession of chlorophyll rather than bacteriochlorophyll, and their photosynthetic production of oxygen by a two-photosystem process as in algae and higher plants. Ultrastructural studies, however, clearly show that the Cyanobacteria are prokaryotic; that is, they lack nuclei and other organelles and they have a peptidoglycan cell wall that is typical of gram-negative eubacteria. Modern-day cyanobacteria include some 2000 species in 150 genera and 5 orders, with a great variety of shapes and sizes. **Table 2.1** lists the five orders of cyanobacteria with their characteristics and examples.

Table 2.1: The five orders of cyanobacteria recognized in the classic botanical taxonomic scheme **[23]**

Serial no.	Order	Characteristics	Illustrative genera
1	Chroococcales	Coccoid cells that reproduce by binary fission	*Aphanocapsa*, *Microcystis*
2	Pleurocapsules	Coccoid cells, aggregates or pseudo-filamants that reproduce by baeocytes	*Pleurocapsa*
3	Oscillatoriales	Uniseriate filaments, without heterocysts or akinetes	*Lyngbya*, *Microcoleus*
4	Nostocales	Filamentous cyanobacteria that divide in only one plane, with heterocysts	*Anabaena*, *Calothrix*
5	Stigonematales	Division in more than one plane; true branching and multiseriate forms; heterocysts	*Stigonema*, *Fischeralla*

Ecologically, there are three major groups in the aquatic environment:

i. Mat-forming species

Cyanobacteria are a common component of the periphyton (the ensemble of microorganisms attached to submerged surfaces), forming crusts and films over rocks, plants, sand, sediments, and other substrates. In many environments, these biofilms accumulate from millimeters to centimeters in thickness as vertically structured, microbial mats that form a benthic layer at the bottom of the water column, or that detach and float at the surface.

ii. Bloom-forming species

Bloom-formers tend to be found in warm, stable, nutrient-rich lakes and are largely absent from the polar and alpine regions. Three genera are particularly common and often co-occur: *Anabaena*, *Aphanizomenon*, and *Microcystis*. Some lakes contain a meta-limnetic bloom of *Oscillatoria*.

iii. Picocyanobacteria

Picocyanobacteria are the smallest of cyanobacteria cells, often around one thousandth of a millimeter in diameter; however, they typically occur in high concentrations. Two of the genera *Synechococcus* and *Prochlorococcus* are so widely distributed in the open ocean that they are probably the most abundant photosynthetic cell types in the biosphere. They are readily observed by epifluorescence microscopy, and increasingly by flow cytometry, and comparative studies have shown that they contribute a large proportion of total photosynthetic biomass in oligotrophic lakes. Their high surface-to-volume ratio confers an advantage in low nutrient conditions, and their small size can also be an advantage for light capture because of a lack of internal shading (‗package effect') **[23]**.

2.4 Photosynthesis: CO_2 Fixation Route

Photosynthesis as we can define as multi-step process by which plants and other autotrophic organisms convert carbon source, generally CO_2 along with water in the presence of light energy, normally from the sun, into reduced organic compounds that can be used to fuel the organism's activities. Oxygen is released as a byproduct. Most plants, algae and cyanobacteria perform the process of photosynthesis, and are called photoautotroph. Photosynthesis maintains atmospheric oxygen levels and supplies most of the energy necessary for life on Earth.

Cyanobacteria are among the very few groups that can perform oxygenic photosynthesis and respiration simultaneously in the same compartment, and some cyanobacterial species are able to fix nitrogen. This combination of metabolic pathways is unusual and this metabolic flexibility may be responsible for the evolutionary hardiness of the cyanobacteria and their ability to thrive under a wide range of conditions. Photosynthesis and respiration require electron transport pathways that to a large extent are catalyzed by protein complexes in membranes. Cyanobacteria utilize several redox active components in thylakoids for both photosynthesis and respiration, including the plastoquinone (PQ) pool, the cytochrome b_6f complex, and the soluble electron carriers in the lumen.

Photosynthetic electron flow

The photosynthetic electron transport chain in cyanobacteria is essentially identical to that in plants. Photosystem II (PS II) uses light energy to split water and to reduce the PQ pool. Electrons are transported from the PQ pool to the cytochrome b_6f complex and from there to a soluble electron

carrier (may be plastocyanin or cytochrome *c553*) on the luminal side of the thylakoid membrane. Either of these soluble one-electron carriers can reduce the oxidized PS I reaction center chlorophyll, P700 $^{+}$. This oxidized form of the reaction center chlorophyll is formed by a light-induced transfer of an electron from PS I to ferredoxin (Fd) and eventually to NADP. Reduced NADP can be used for CO_2 fixation. Photosynthetic electron transfer leads to a proton gradient across the thylakoid membrane. In PS II, protons are released into the lumen upon water splitting, and protons formed upon plastoquinol oxidation by the cytochrome b_6f complex are released into the lumen as well. The proton gradient across the thylakoid membrane is used for ATP synthesis by the ATP synthase in the thylakoid; this ATP may be applied for CO_2 fixation and for other cell processes **[24]**. **Figure 2.3** gives the diagrammatic representation of intersecting photosynthetic and respiratory electron transport pathway.

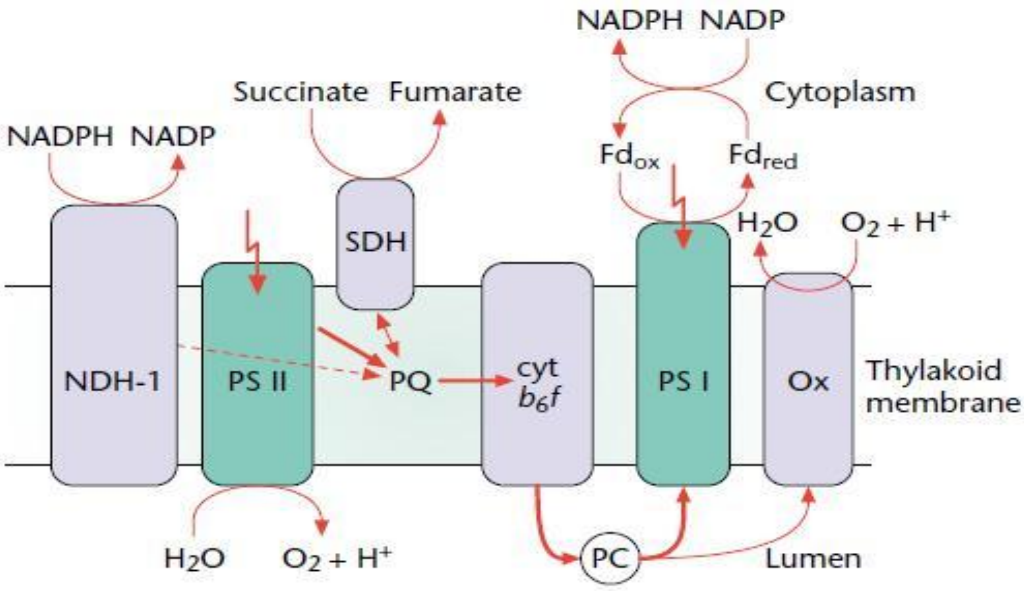

Figure 2.3: Schematic representation of the intersecting photosynthetic and respiratory electron transport pathways in thylakoid membranes of the cyanobacterium **[24]**

One important difference between cyanobacteria and plants is that the stoichiometry of PS I and PS II in at least some species of cyanobacteria is much larger than 1, whereas in higher plants an equal amount of PS I and PS II is the rule. An abundance of PS I may guarantee a rather oxidized PQ pool in the light, which is important to minimize photodamage [24]. Moreover, the high amount of PS I may serve to compete effectively with cytochrome oxidase for electrons when light is available, thus maximizing the number of electrons that can be used for CO_2 fixation.

2.5 Advantages of Cyanobacteria 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

cyanobacteria 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 **[20]**.

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 plants (e.g. trees). This eliminates the need for pretreatments to breakdown cellulytic products and lowers the reactor temperature of their subsequent fermentation **[20]**.

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 **[20]**.

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 **[20]**.

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. The volume of water required is lower than that required to grow

maize at an equivalent weight of corn grain, eightfold less than rape cultivation on fertilized land **[20]**.

2.6 Factors Affecting Cyanobacterial Growth

For proper cyanobacterial growth, an optimum environment is required, which depends 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. Light provision

Light is the basic energy source for phototrophic microorganisms. The intensity and utilization efficiency of the light supplied are thus of crucial importance in bioreactors. The photosynthetically active radiance is normally assumed to be in the wavelength range of 400–700 nm **[26, 27].** Light intensity decreases deeper within the culture medium, especially in high density cultures; hence, the issue of optical depth, which measures the proportion of radiation absorbed or scattered along a path through a partially transparent medium, should be considered in cyanobacterial bioreactor design **[26].** Experimental investigations reveal that the increase in light duration is directly proportional to increase in number of cultivated cyanobacteria as well as the increase in the light intensity. The ratio of light to dark (or low-intensity light) periods in a cycle is crucial for cyanobacterial productivity. A reduction in biomass production was observed in parallel with the reduction in light period duration **[28]**. Exposure to high intensity of light may lead to photo-inhibition due to formation of free radicals leading to toxic H_2O_2 formation **[28]**. Ideally the range of light intensity for high algal growth is from 4000-6000 lx **[29]**.

ii. Carbon uptake

Biological CO_2 fixation can be carried out by higher plants and cyanobacteria, yet the latter possess a greater ability to fix CO_2. The tolerance of various cyanobacterial species to the concentration of CO_2 is variable. Atmospheric CO_2 levels (0.0392% (v/v)) are not sufficient to support the high cyanobacterial growth rates and productivities needed for full-scale biofuel production. Waste gases from combustion processes, however, typically contain >15% (v/v) CO_2; this percentage indicates, in principle, that combustion processes will provide sufficient amounts of CO_2 for large-scale production of cyanobacteria **[26]**.

iii. Oxygen generation

Another specific issue of photobioreactors is the accumulation of photosynthetically generated oxygen that may inhibit metabolism or otherwise damage the culture if allowed to accumulate, especially when the rate of photosynthesis, which often correlates with the rate of CO_2 transfer, is high. Most solutions to this problem rely on the use of a degasser (or gas exchange unit), where dissolved oxygen can be released. However, to attain effective separation between the gas and

liquid phases, the path through the degasser should be such that the smallest bubbles have sufficient time to disengage from the liquid **[27]**.

iv. Gas transfer

Gases introduced into bioreactors serve a number of purposes in cyanobacterial cultivation, including: supply of CO_2 as sources of carbon for biomass primary and secondary metabolism, provision of internal mixing, which avoids nutrient concentration gradients; promotion of exposure of all cells to light, while minimizing self-shading and phototoxicity, control of pH by assuring dissolution of CO_2 and avoiding gradients thereof; and stripping of accumulated dissolved oxygen, hence reducing its toxicity to cyanobacteria. Among the various alternatives, bubbling CO_2-enriched air into the bottom of the bioreactor with bubble diffusers has been the most frequently used approach. Moderate overall transfer efficiencies (13–20%) can be achieved by this mode of gas delivery **[26]**.

v. Mixing rates

Mixing is a key parameter for acceptable performance of photobioreactors. Low mixing rates hamper gaseous mass transfer and might even permit biomass settling. In either case, poor mixing leads to emergence of stagnant zones, where light and nutrients are insufficiently available and anoxic/anaerobic conditions will thus prevail, which results in a decrease of productivity. Culture viability might also be compromised by production and accumulation of toxic compounds in stagnant zones. Conversely, high mixing rates can cause shear damage to cells, besides requiring a large energy input. The most common methods of mixing in bioreactors are pumping, mechanical stirring and gas injection **[26]**.

vi. Temperature effects

Temperature is one of the major factors that regulate cellular, morphological and physiological responses of cyanobacteria: higher temperatures generally accelerate the metabolic rates of cyanobacteria, whereas low temperatures lead to inhibition of cyanobacterial growth. The optimal temperature varies among cyanobacterial species; however, optimal temperatures are also influenced by other environmental parameters, such as light intensity. Optimal growth temperatures of 15–30 °C have been reported for some species, with maximum cell densities obtained at 27 °C **[26]**.

vii. pH effects

Many cyanobacterial species are favored by neutral pH, however most species are tolerant to higher pH (e.g. *Spirulina platensis* at pH 9) and few to lower pH (e.g. *Chlorococcum littorale* at pH 4). There is a complex relationship between CO_2 concentration and pH in cyanobacterial bioreactor systems. Increasing CO_2 concentrations can lead to higher biomass productivity, but will also

decrease pH, which can have an adverse effect upon cyanobacterial physiology. By contrast, cyanobacteria have been shown to cause a rise in pH to 10–11 in open ponds because of CO_2 uptake. This increase in pH can be beneficial for inactivation of pathogens in cyanobacterial wastewater treatment, but can also inhibit cyanobacterial growth **[26]**.

viii. Nutrient requirements

In addition to the carbon, nitrogen is the most important element that is required for cyanobacterial nutrition and, as a constituent of both nucleic acids and proteins, nitrogen is directly associated with the primary metabolism of cyanobacteria. Phosphorus is the third most important nutrient for cyanobacterial 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 cyanobacteria, seawater supplemented with commercial nitrate and phosphate fertilizers is commonly used for production of cyanobacteria. Nevertheless, trace species, such as metals (Mg, Ca, Mn, Zn, Cu and Mo) and vitamins, are typically added for effective cultivation **[26]**. Lipids contain twice the energy stored per C atom than do carbohydrates, which translates directly into a two fold increase in fuel energy content. Depletion of nitrate (–N), silicate (–Si) or phosphate (–P) from the growth medium has been shown to produce twofold to threefold 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 **[13]**. **Table 2.2** shows the basic growth conditions and advantages of different species of cyanobacteria and microalgae. Based on this we can select a specie to serve a particular desired purpose.

Table 2.2: Different species of cyanobacteria and microalgae **[30]**

Sr. no.	*Species*	Growth conditions	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 hours, high growth ability, high temp. tolerance
3	*Viridiella*	Temp 15–42°C,pH: 2–6	Accumulates lipid granules inside the cell, high temp. and CO_2 Tolerance
4	*Anabaena*	Temp 25-35 °C, pH: 7-7.5	Temp resistant, N_2 fixation
5	*Enteromorpha clathrata*	Temp 24–33 °C, pH: 7.5 – 8.0	Very little agitation is needed

6	*Spirulina platensis*	Temp 35–37 °C, pH: 8.3-11	Very tolerable to pH change

2.7 Photobioreactors

Cyanobacteria are found growing within nearly every biotope because of their ecological diversity and their physiological adaptability. This diversity explains why almost everybody who is active in their cultivation has their own technical solutions in mind for mass cultivation. Within this multitude of technical solutions one can basically distinguish between open ponds or reactors, which are open to air, and closed systems. Although the term ‗photo-bioreactor' 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 photo-bioreactor 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 scaleup methodologies for photo-bioreactors 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 and (iv) sensible scalability of the photo-bioreactor technology **[31]**.

i. Tubular reactors

Most configurations of tubular reactors (TR) are one of the following three types:

(i) Horizontal tubular reactor, which is composed of horizontal transparent tubing, usually bearing gas transfer systems attached to the connections.

(ii) Simple airlift and bubble column, which is composed of vertical tubing (in the form of a vertical tubular reactor) that is transparent so as to allow for light penetration and where CO_2 is supplied via bubbling.

(iii) Helical tubular reactor, which is composed of a flexible plastic tube coiled in a circular framework.

Horizontal tubular reactors

Horizontal tubular reactors (HTR) have been on the rise; gas transfer takes place in the tube connection or via a dedicated gas-exchange unit, and the angle toward sunlight is particularly adequate for efficient light harvesting. Such systems can handle large working volumes, because they are not susceptible to contamination. On the other hand, they may generate considerable

amounts of heat, which may attain temperature amplitudes of 20 °C within a single day if (costly) temperature control systems are not provided; thus, it will likely pose a problem for regular operation **[30]**. A basic outline of horizontal tubular reactors is given in **Figure 2.4**. However, the major drawback of this system was probably its low gas transfer rate, arising from a large length and a small diameter.

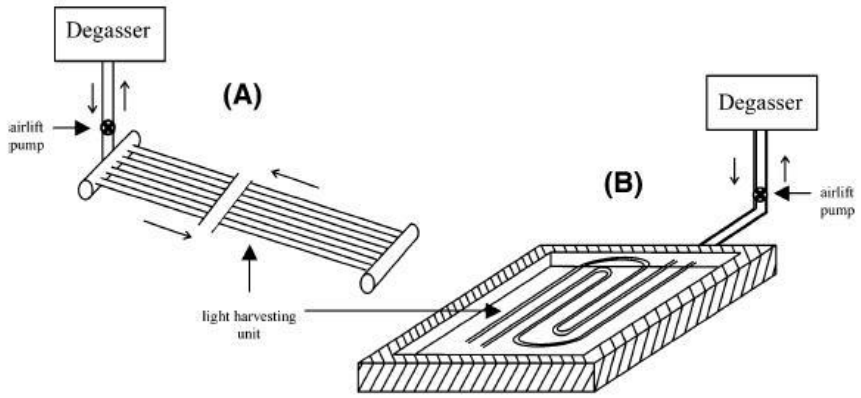

Figure 2.4: Schematic representation of horizontal tubular reactor with a degassing unit and a light harvesting unit, composed of parallel sets of tubes (A) or a loop tube (B) **[32]**

Vertical tubular reactors

The airlift and bubble column reactors depicted in **Figure 2.5** are examples of vertical tubular reactors (VTR), regularly composed of polyethylene or glass tubes, which are sufficiently transparent to allow good light penetration but are manufactured with sufficiently common materials so as to be non-expensive. Air is bubbled at the bottom- a strategy that provides good overall mixing, sufficient supply of CO_2, and efficient removal of O_2. Polyethylene bags have frequently been used, with advantage taken from their particularly low cost, high transparency and good sterility at startup-due to the high temperatures used during film extrusion. Scale-up is not straightforward; furthermore, in order to provide enough culture volume, as well as efficient gas transfer rate, the reactor diameter should be relatively high when compared to flat plate or tubular loop reactors, a requirement that in turn decreases the area-to-volume ratio and consequently constrains photosynthetic efficiency. Another major drawback is the large angle relative to the direction of sunlight, which causes a high fraction of incident.

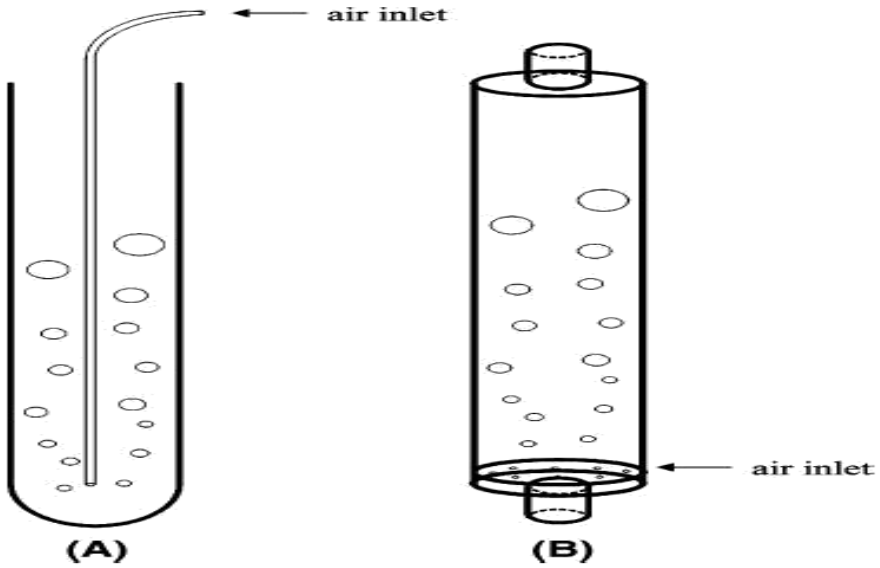

Figure 2.5: Schematic representation of airlift (A) and bubble column (B) reactors. **[32]**

ii. Flat plate reactors (FPR)

Flat plate reactors (FPR) are conceptually designed to make efficient use of sunlight; hence, narrow panels are usually built so as to attain high area-to volume ratios. The greatest advantage of this system is its provision of an open gas transfer unit, which has proven efficient in overcoming the problem of oxygen buildup; however, such an open zone restricts effectiveness of contamination control, as compared with completely closed reactors. A schematic representation of FPRs is given in **Figure 2.6**. In general; the main advantages of FPR are their high productivity and uniform distribution of light and, in the specific case of bubbled column FPR, the absence of a driving pump. Furthermore, these reactors can be oriented toward the sun, hence permitting a better efficiency in terms of energy absorbed from incident sunlight. The main disadvantage of alveolar panels is oxygen buildup, which arises from the high photosynthetic activity reached, coupled with the small diameter of the reactors used.

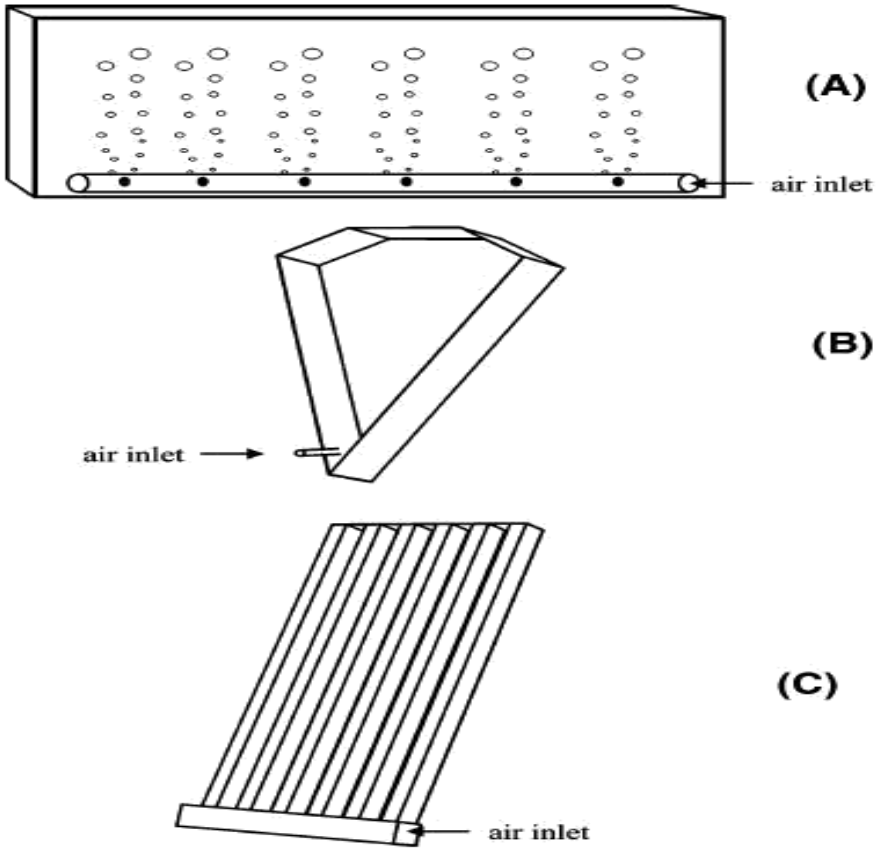

Figure 2.6: Schematic representation of flat panel reactors: flat panel bubbled in the bottom (A), V-shaped panel (B) and alveolar panel (C) **[32]**

iii. Fermenter-type Reactors

The least expanded systems for cyanobaterial cultivation are conventional fermenter-type reactors (FTR). These systems present indeed an intrinsic disadvantage: the area-to-volume ratio is quite low, so sunlight harvesting efficiency is poor. To overcome this nuclear drawback, sophisticated systems of internal illumination were developed, which are able to provide a more homogeneous distribution of light. When possible, cyanobacteria may be heterotrophically cultivated in FTR, using appropriate organic carbon sources. The main advantage of these systems is of course the accurate control of processing parameters, including light, coupled with the vast experience accumulated in food and pharmaceutical industries in terms of the scale-up thereof. Therefore, should productivity be enhanced, FTR would certainly become a competitive alternative for industrial manufacture of biochemical products brought about by cyanobacteria **[32]**. The advantages and disadvantages of different closed photo-bioreactor systems mentioned above are illustrated in **Table 2.3**.

Table 2.3: Advantages and disadvantages of different closed photobioreactor systems for cyanobacterial cultivation **[32]**

Photobioreactor System	**Advantages**	**Disadvantages**
Tubular PBRs	• Large illumination surface area • Fairly high biomass productivity • Relatively cheap • Suitable for outdoors	• Fouling • Large area of land needed • Poor mass transfer • High O_2 accumulation • Photo-inhibition risk • Hard to control temperature
Flat-plate PBRs	• Huge illumination surface area • Good light path • High biomass productivity • Easy to scale up • Suitable for outdoors • Relatively low O_2 accumulation • High photosynthetic efficiency	• High hydrodynamic stress • Hard to control temperature
Column PBRs	• Low shear stress • Low energy consumption • Cheap and easy operation	• Small illumination

CHAPTER 3

EXPERIMENTAL SECTION

3.1 Materials

i. Cyanobacteria and culture medium

Culture of *Anabaena Viriabilis* ARM 441, a photoautotroph and fast growing cyanobacteria, was obtained from Science and Technology Entrepreneur's Park (STEP), Thapar University, Patiala. BG (-N) 11 media was used for the growth of the culture as well as for subculturing. The chemical composition of BG (-N) 11 media is given in **Table 3.1**.

Table 3.1: Composition of BG (-N) 11 media

Sr. no.	Ingredients	Concentration (g/l)
1	Di-sodium hydrogen phosphate	0.04
2	Calcium chloride	0.036
3	Citric acid	0.006
4	Ferric ammonium citrate	0.006
5	EDTA	0.001
6	Sodium carbonate	0.02
7	Trace metal mix	1 ml/l

As the name suggests trace metal mix contains the metallic ions in small concentration which act as a cofactor for different enzymes. Any deficiency of a metal ion may lead to impaired or no growth at all. The chemical composition of trace metal mix is given in **Table 3.2**.

Table 3.2: Composition of trace metal mix

Sr. no	Ingredients	Concentration (g/l)
1	Boric acid	2.86
2	Manganese chloride	1.81
3	Zinc sulphate	0.222
4	Sodium molybdate	0.039
5	Copper sulphate	0.079
6	Cobalt nitrate	0.0492

All chemicals were of AR grade and obtained from Himedia, Loba Chemie and SD Fine Chemicals.

ii. Instruments/ Equipment

Weighing balance

Weighing balance from mettle Toledo was used to weigh different chemicals.

Temperature controlled orbital shaker

Subculturing was done to maintain the fresh stock of culture in a temperature controlled orbital shaker. Illumination was provided using 85 Watt cool white fluorescent light CFL.

Lux meter

Lux meter was obtained from Mastech, India, model MS 6610 to monitor the light intensity provided to the algal culture.

Laminar air flow chamber

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

UV-Vis spectrophotometer

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

Experimental setup

Bioflo® / Celligen® 115 Bioreactor (New Brunswick) of 7.5 liters capacity and working volume of 5.5 liters was used. Schematic representation of experimental setup is given in **Figure 3.1 (a)** and the total process is represented in the form of a block diagram **Figure 3.1 (b)**

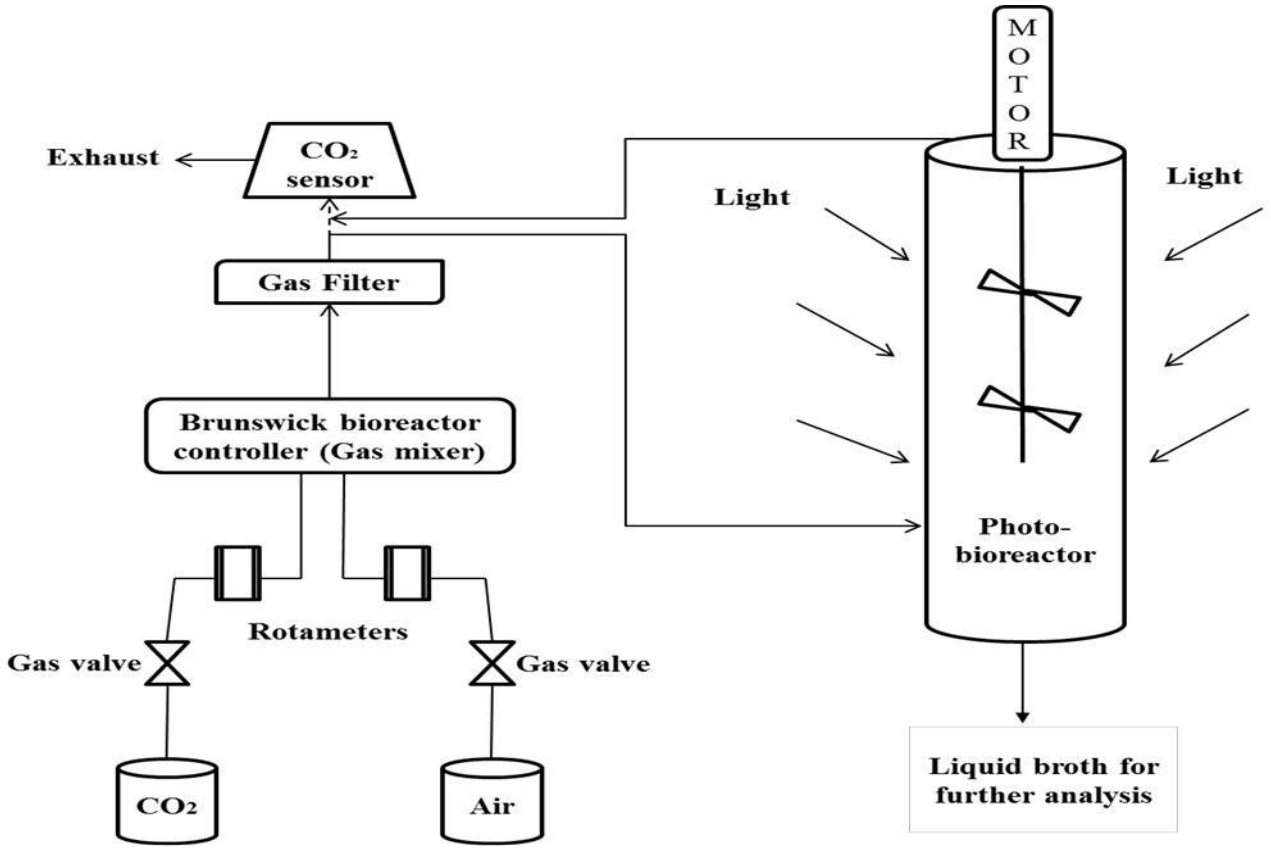

Figure 3.1(a): Schematic diagram of experimental set up for CO_2 fixation

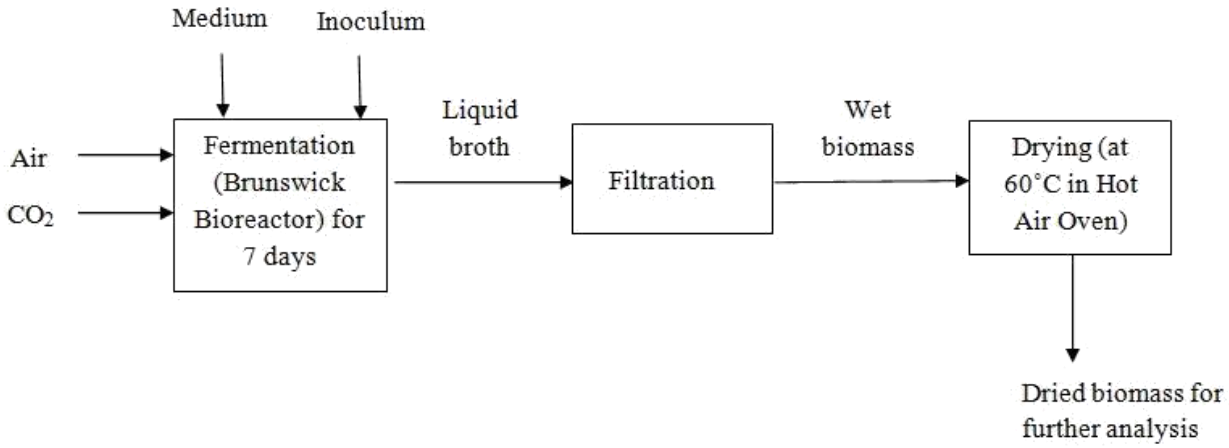

Figure 3.1(b): Block diagram of CO_2 fixation experiment

Autoclave

Autoclave obtained from Equitron, India was used for sterilization of media, glassware and photobioreactor at 121°C temperature and 15 pound per square inch (psi) pressure.

Centrifuge

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

Whatmann no. 1 filter paper

It was used to filter the cyanobacteria.

Organic elemental analyzer (CHNS-O analyzer)

CHNS-O analyzer (Thermo scientific (UK) Flash 2000) was used to determine the concentration of carbon, nitrogen, sulphur and hydrogen in the dry biomass sample of *Anabaena*.

BOD incubator and thermoreactor

BOD incubator was used to store oxitop respirometer systems (OxiTop IS 6, IS 12) for determining Biochemical Oxygen Demand. Thermo reactor (model 2025 D) was used for digestion of sample at 150° C for Chemical Oxygen Demand analysis.

3.2 Methods/ Experimental Procedure

i. Sub-culturing/ maintenance

Sub-culturing was carried out for 5 days (till mid exponential phase) with the 10% inoculum in shake flask containing 500 ml of BG (-N) 11 media in temperature controlled orbital shaker at 27°C and 125 revolutions per minute (rpm) stirring. The flask was plugged with cotton balls and was not aerated. Self-dissolved carbon dioxide from air was the only carbon source available, in order to avoid the growth of other heterotrophic bacteria. Culture was grown under continuous illumination of 5000 lx, which was provided with the help of 85 Watt CFL. Initial pH of the media was set at 7.2 ± 0.2. Subculturing was performed after every 15 days to maintain the fresh stock.

ii. Autoclaving

Sterilization of the photobioreactor is necessary before starting a batch. Autoclave is used for this purpose, as it kills not only microorganisms but also their spores. But before autoclaving, it was completely unassembled and washed with chromic acid and soap solution. Probes of photobioreactor were cleaned using ethanol. After that it was allowed to air dry for 1 hour and was reassembled again. Then media was added to the photobioreactor aseptically and pH was set at 7.2 ± 0.2 (pH is set before autoclaving). All the pipes attached to photobioreactor were plugged using stoppers to prevent any escape of media during autoclaving. Opening of every pipe was further plugged using cotton balls and aluminum foil. DO probe, pH probe and impeller shaft were covered with their respective caps. Water level was checked in the autoclave before switching the autoclave on. Photobioreactor was then kept in autoclave for half an hour at 121°C and 15 psi. Removal of air from the autoclave was ensured before autoclaving.

iii. Inoculum

10% concentration of inoculum in the media was always taken. Culture was always taken in their mid-exponential phase as the numbers of cells are more and cells divide actively during this period. Before inoculation, the headplate of the photobioreactor was sterilized using Bunsen flame along with the ethanol. Funnel used for this purpose was also autoclaved.

iv. Experimental setup

Bioflo® / Celligen® 115 Bioreactor (New Brunswick Sci., USA) made up of glass with 7.5 liters capacity and working volume of 5.5 liters was used. It has various components like agitation system, temperature control, aeration, pH control, exhaust system, DO measurement etc. Agitation of 200 rpm was provided through a Rushton-style impeller powered by removable agitation motor for proper distribution of nutrients as well as breaking of the air bubbles to improve the mass transfer of CO_2. Moreover, as *Anabaena* forms cluster and settles down very easily, agitation prevents the same from happening. The temperature of 27 ± 1 ˚C was measured with a sensor, (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 Watt CFLs. The mixture of air and CO_2 was sparged through a ring sparger in the form of small bubbles into the culture at 0.5 vvm (vol. gas/vol. liquid/min.) i.e. 2.75 l/min to ensure maximum mass transfer between liquid and gaseous phases. The mixture of air and CO_2 was passed through a micropore filter (0.2-µm pore size) prior to injection. Outlet of air was also guarded by a similar filter to ensure that no microorganism 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.

v. Gas mixture

Pure CO_2 from a cylinder with a preheater (to prevent choking at low temperature) and zero air through a compressor were mixed at different concentrations of 5%, 7.5%, 10%, 12.5% and 15%. Aeration rates were fixed at 0.5 vvm.

3.3 pH Optimization

The culture of *Anabaena viriabilis* was grown in BG (-N) 11 under different pH regimes i.e. 6, 7, 8 and 9. However, other conditions like 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 BG (-N) 11 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.

3.4 Estimation of Dry Weight of Biomass

Aseptically collected samples were filtered using Whatmann no. 1 filter paper, washed and dried at 60ºC overnight to obtain the dry weight of the biomass. Optical density of *Anabaena* for the corresponding day was measured using UV/visible spectrophotometer (PerkinElmer, USA) at 680 nm.

3.5 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/day) was calculated according to the Equation 3.1:

$$\mu \quad \frac{\quad}{\quad} \qquad (3.1)$$

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/day) was determined. The cell doubling time was estimated by Equation 3.2**:**

$$(\quad) \quad \frac{\quad}{\quad} \qquad \text{---------- (3.2)}$$

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/day) was calculated using Equation 3.3**:**

---------- (3.3)

where X_t is the biomass concentration at time t and X_o, the initial biomass concentration at inoculation time, t_o. P_{max} (g/l/day) was designated as the maximum productivity **[33]**.

3.6 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/day) was calculated according to Equation 3.4:

———————— ---------- (3.4)

Where 0.5 is taken as carbon content of dried biomass; 12 (g/mol) and 44 (g/mol) presents the molecular weight of carbon and CO_2, respectively **[33]**.

3.7 Growth Kinetics Using Logistic Equation

Logistic equation was used to determine the growth kinetics of cyanobacteria. 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 **[34]**. Equation 3.5 is the logistic equation.

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

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/day) for this strain. On integrating with the boundary conditions $X(0) = X_0$ and rearranging Equation 3.5, it can be written as Equation 3.6 where X_0, is initial biomass concentration (g/l).

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

This may be further rewritten in the form of Equation 3.7:

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

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 Equation 3.7 in OriginPro8 using curve fitting tool. A confidence bound of 95% was taken into consideration to find the fit.

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

3.8 CHNS Analysis

Elemental analysis of total nitrogen, carbon, hydrogen and sulfur was done to determine carbonate and organic carbon and to get 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 vicious reaction (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).

3.9 *Anabaena* Grown in Dairy Wastewater

Culture of *Anabaena viriabilis* was grown in dairy wastewater (obtained from Verka plant, Patiala) for 7 days at 27°C in orbital shaker in a shake flask to study the effect of waste water on its growth. Sample of dairy wastewater was first filtered and then autoclaved before it was used to grow *Anabaena*.

Biochemical oxygen demand (BOD)

After 7 days of experiment, sample of *Anabaena* 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.

Chemical oxygen demand (COD)

Supernatant obtained after centrifuging (at 10,000 rpm for 10 minutes) *Anabaena* 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 thermoreactor at 150°C for 2 hours. Finally, the glass tube was cooled and sample was taken in cuvette for its spectrophotometric analysis.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 pH Optimization

The pH of media is an important factor which significantly affects the growth of the cyanobacteria. 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. Culture of *Anabaena viriabilis* was grown on BG (-N) 11 media for 5 days. **Figure 4.1** shows the effect of initial pH on its growth. Its growth was observed under different pH i.e. 6, 7, 8 and 9. All other conditions i.e. light intensity; CO_2 concentration and agitation were kept constant. Growth is represented in the form of optical density.

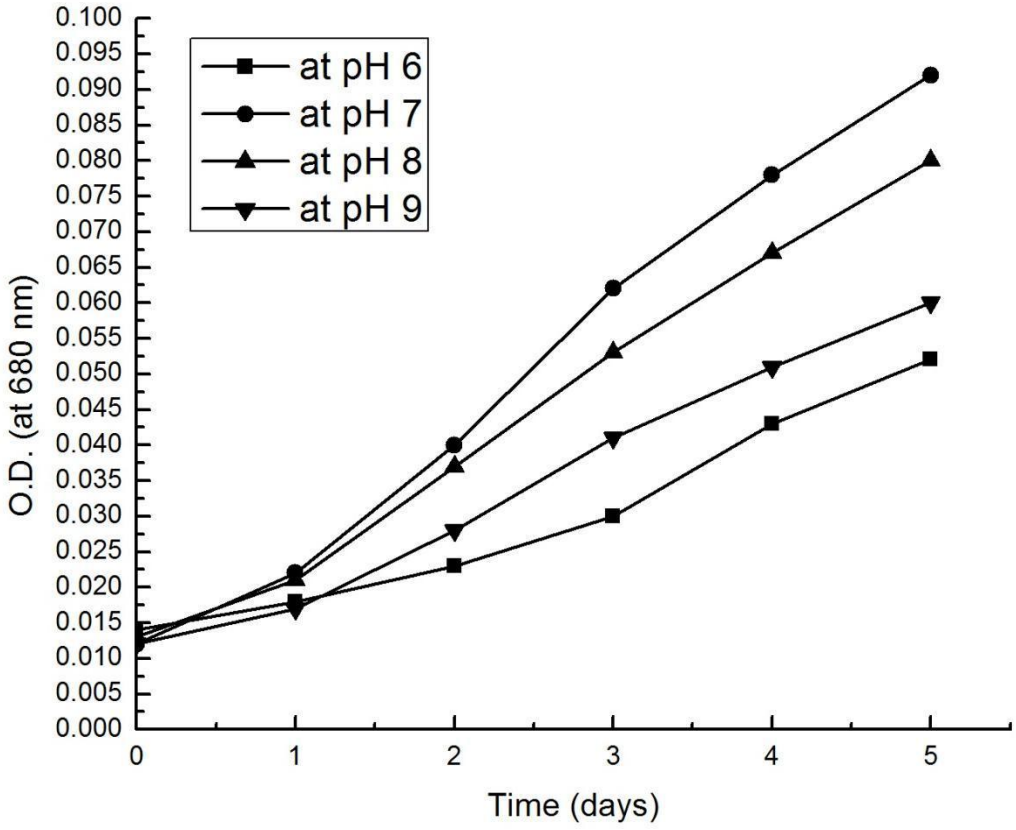

Figure 4.1: Effect of pH on growth of *Anabaena*

As shown in **Figure 4.1** maximum growth was observed at pH 7. Although difference in growth is not much between pH 7 and 8, however, cyanobacteria showed slightly higher growth at pH 7. At pH 6, elongated lag phase was observed which extended to 3rd day. In addition to that it can be observed that growth of *Anabaena* is more biased towards alkaline condition. Growth at pH 8 and 9 is higher than pH 6 which can be attributed to the fact that when CO_2 is introduced into the system, a dip in pH is observed in all cases. In case of alkaline conditions even after this dip the pH does not fall below 6.5 however, in case of batch with initial pH 6 the dip is quite significant exposing the batch to much acidic condition which may affect the growth of culture. So it is concluded that though *Anabaena* can grow at varied range of pH 6 to 9 but optimal pH is 7.

4.2 *Anabaena viriabilis* Dry Weight Calibration Curve

Figure 4.2 shows the absorbance of biomass as function of its concentration. Growth was measured in terms of biomass dry weight (g/l). Biomass can be more easily measured by using absorbance than by measuring cell dry eight directly. So, the relationship between optical density and cell dry weight for *Anabaena viriabilis* was established by linear regression. It was found that optical density precisely predicted the dry weight (R^2=0.9978). Hence, O.D. values were used to calculate the biomass.

Regression equations of the relationship between optical density and cell dry weight were established and shown as follows:

y=17.224x+0.0222; R^2=0.9978

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

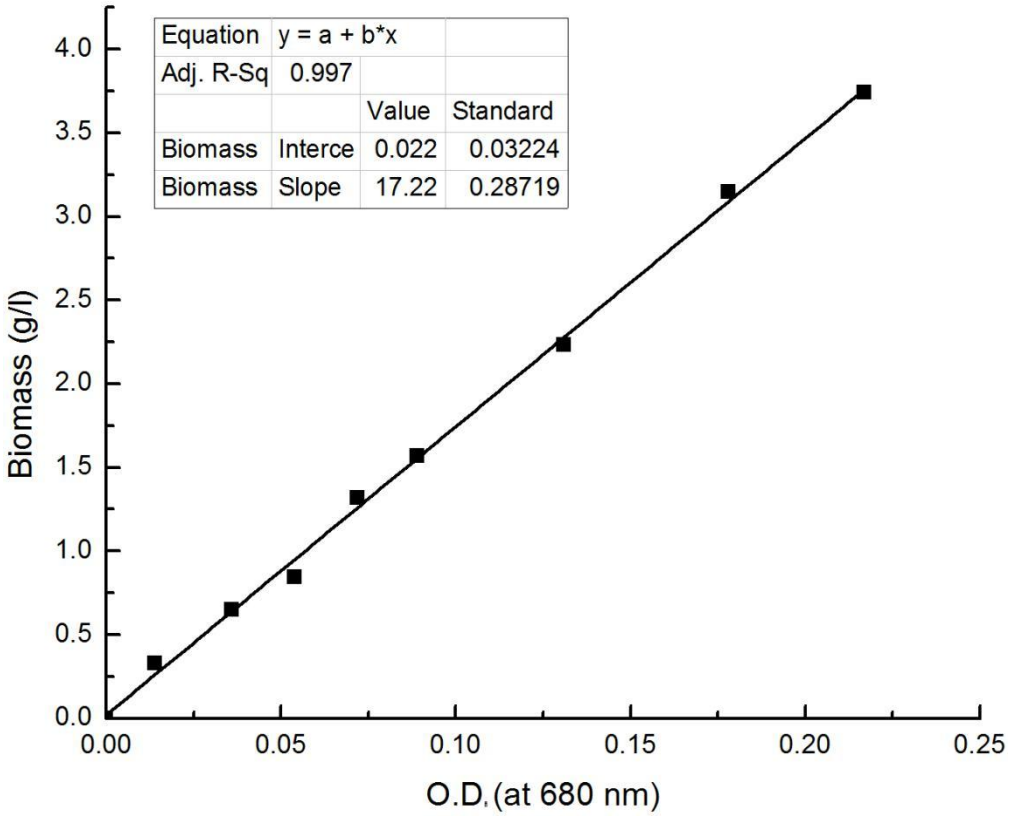

Figure 4.2: Concentration of biomass as function of its absorbance

4.3 Biomass Fixed by *Anabaena viriabilis*

Figure 4.3 shows the biomass growth with time for different CO_2 concentrations, i.e. 0.04%, 5%, 7.5%, 10%, 12.5% and 15%. Conditions like illumination, temperature, pH and agitation were kept constant at 5000 lx, 27 ± 1°C, 7.2 ± 0.2 and 200 rpm respectively. *Anabaena* showed maximum growth i.e. 4.9 g/l at input of 10% CO_2 concentration. Initial cyanobacterial concentration of all the batches was almost same i.e. 0.36 g/l. It is established from **Figure 4.3** that biomass concentration increases with increase in CO_2. It follows an upward trend up till 10% CO_2 concentration and then starts declining. It can be concluded clearly from the fact that biomass growth is higher at 7.5% and 5% than at 12.5%. *Anabaena* fixes CO_2 best at 10% CO_2 concentration. Growth at 0.04% and 15% was almost same which shows that at low CO_2 concentration, due to shortage of substrate, Rubisco enzyme shifts towards its oxygenase activity and at high CO_2 concentration *Anabaena* was not able to utilize the CO_2 due to non-representative biomass determination caused due to substrate inhibition. When CO_2 level increased more than 10%, *Anabaena* preferred aggregative growth form (like bio-floc or biofilm) than individual suspended form. 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 which we can observe from the curve of 10% CO_2 concentration. Although cyanobacteria grew very fast but it exhausted its vital nutrients resulting in bend in growth curve after 5^{th} day.

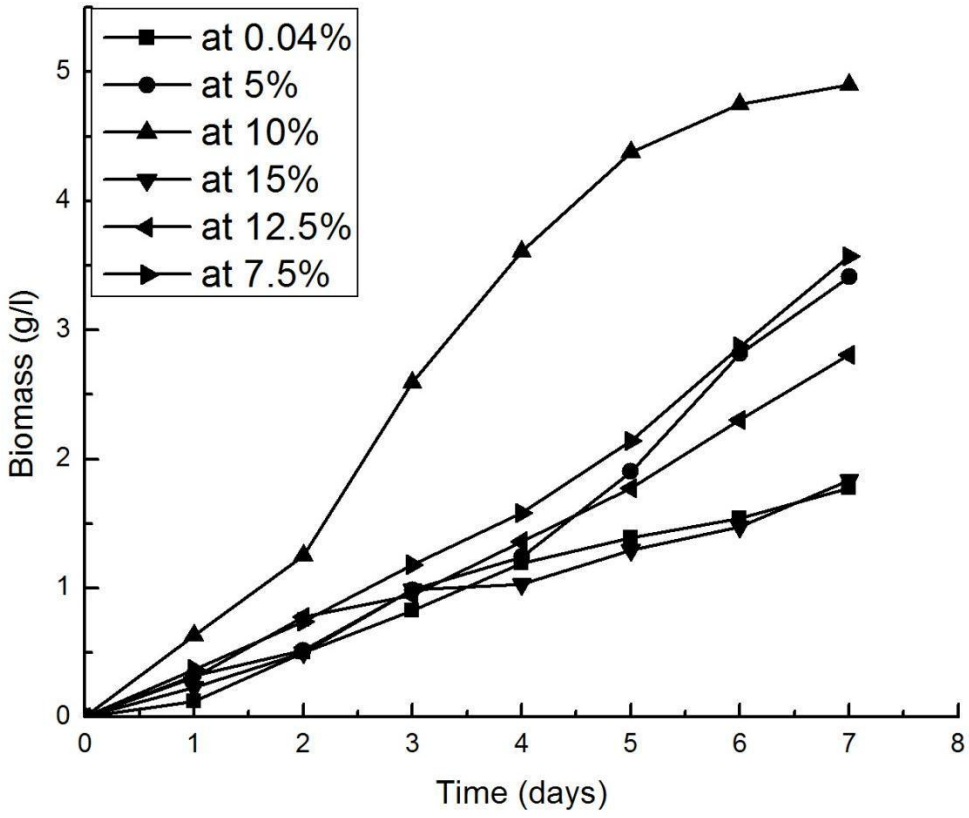

Figure 4.3: Biomass dry weight changes with time under autotrophic growth conditions.

4.4 Total CO_2 Fixed (assuming 50% carbon composition)

Figure 4.4 shows the total CO_2 fixed by *Anabaena* at different CO_2 concentrations. Continuous supply of CO_2 was given for entire duration of batch. It is assumed by other investigators that 1.83 grams of CO_2 is required to fix 1 gram of biomass. So the values of CO_2 fixed were calculated by multiplying biomass produced with 1.83 and using **Equation 3.4**. In this equation, dry carbon concentration in the sample was taken as 0.5. The maximum CO_2 fixed was 8.97 grams at 10% CO_2 supply.

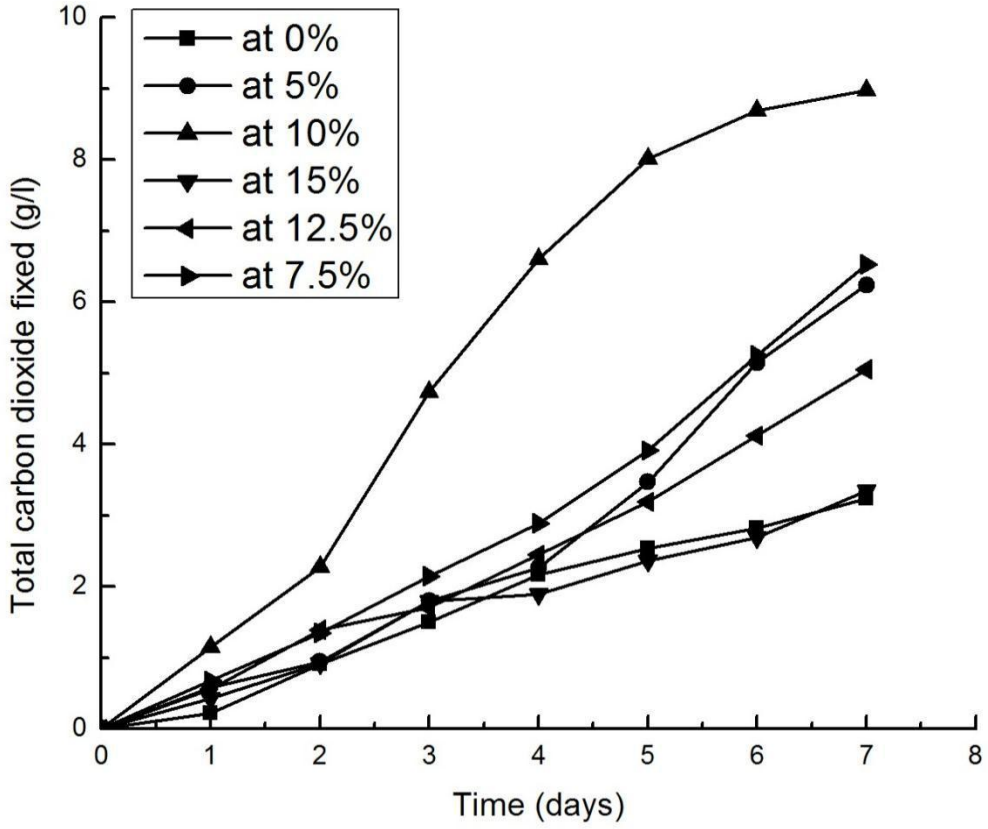

Figure 4.4: Total CO_2 fixed with time at different CO_2 concentrations (assuming 50% carbon composition)

4.5 Total CO_2 Fixed (at 40 % carbon composition)

Carbon dioxide fixation by *Anabaena* for all CO_2 concentration was calculated from the CHNS biomass carbon content values. From this data it can be concluded that the average carbon concentration in cyanobacterial biomass is 40% and not 50%. Thus, the multiplication factor to calculate CO_2 fixed is 1.46 and not 1.83 as assumed earlier, and can be calculated by using

Equation 3.4. **Figure 4.5** shows that actual maximum CO_2 fixed by *Anabaena* at 10% CO_2 supply is 7.15 g/l. However, the carbon content in the cyanobacteria began to decline after 10% CO_2 concentration thus CO_2 fixed also fell with increasing CO_2 as we can see it clearly from figure that biomass concentration of cyanobacteria was almost same at 0.04% and 15% however the CO_2 fixed is low for 15% CO_2 sconcentration.

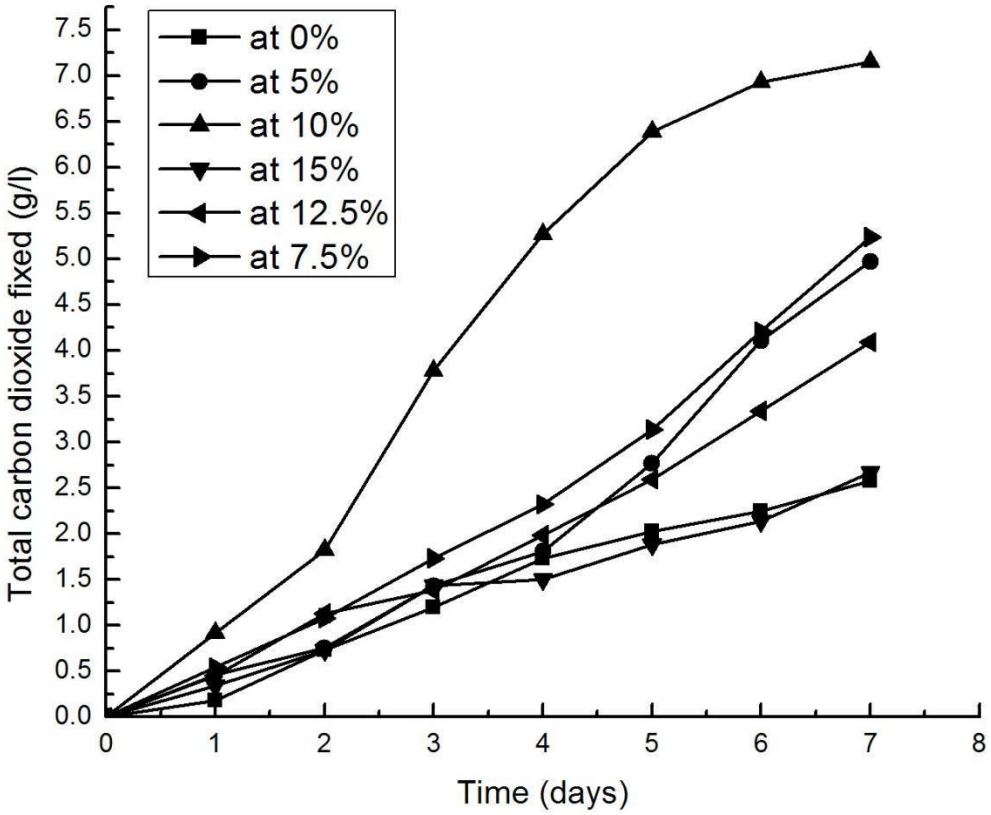

Figure 4.5: Total CO_2 fixed with time at different CO_2 concentrations (at 40 % carbon composition)

4.6 Studies of Kinetic Parameters

Biomass (X) values at different times and exponential regression were used to calculate the maximum specific growth rate (μ_{max}, 1/day) during the logarithmic phase. μ_{max} of 1.39 (1/day) was calculated for 12.5% CO_2 was highest among all. Similar was the case with μ_{avg} and doubling time (t_d) which were calculated to be 0.28 (1/day) and 0.447 days respectively. However, other kinetic parameters like productivity, CO_2 fixation rates and CO_2 captured were found to be highest at 10% CO_2 supply which are 0.7 g/l/day, 1.021 g/l/day and 7.15 g/l respectively. Although at 5% and 7.5% CO_2 concentration, all kinetic parameters were almost same but total CO_2 fixed at 7.5% supply was higher. *Anabaena* showed growth from 0-15% CO_2 supply however noticeable growth was observed only between 5-12.5%. All the kinetic parameters are given in **Table 4.1**.

Table 4.1: Effect of different CO_2 concentrations on kinetic parameters of the *Anabaena*

CO_2 concentration (%)	**μ_{avg} (1/day)**	**μ_{max} (1/day)**	**Productivity (g/l/day)**	**t_d (day)**	**CO_2 Fixation rate (g/l/day)**	**Total CO_2 fixed (g/l)**
0.04	0.178	0.60	0.253	1.15	0.368	2.58
5	0.225	0.95	0.487	0.729	0.711	4.98
7.5	0.22	1.01	0.51	0.68	0.74	5.24
10	0.26	1.29	0.7	0.537	1.021	7.15
12.5	0.28	1.39	0.401	.447	0.58	4.09
15	0.162	0.47	0.26	1.47	0.38	2.67

4.7 Growth Kinetics Using Logistic Equation

Logistic equation 4.1 was used to determine the growth kinetics which is predicted with the help of OriginPro8 using curve fitting tool.

$$X = \frac{A_{max}}{1+\left(\frac{X_{max}}{X_0}-1\right)e^{-kt}} \qquad (4.1)$$

Figure 4.6 shows predicted cell growth profile of *Anabaena* in stirred photobioreactor at different proportion of air–CO_2 (v/v) gas mixture. Experimental data is fitted with logistic equation. X_{max} obtained from predicted logistic model are in **Table 4.2**. Data points are average of experimental runs where line represents the predicted and symbols represent the experimental data.

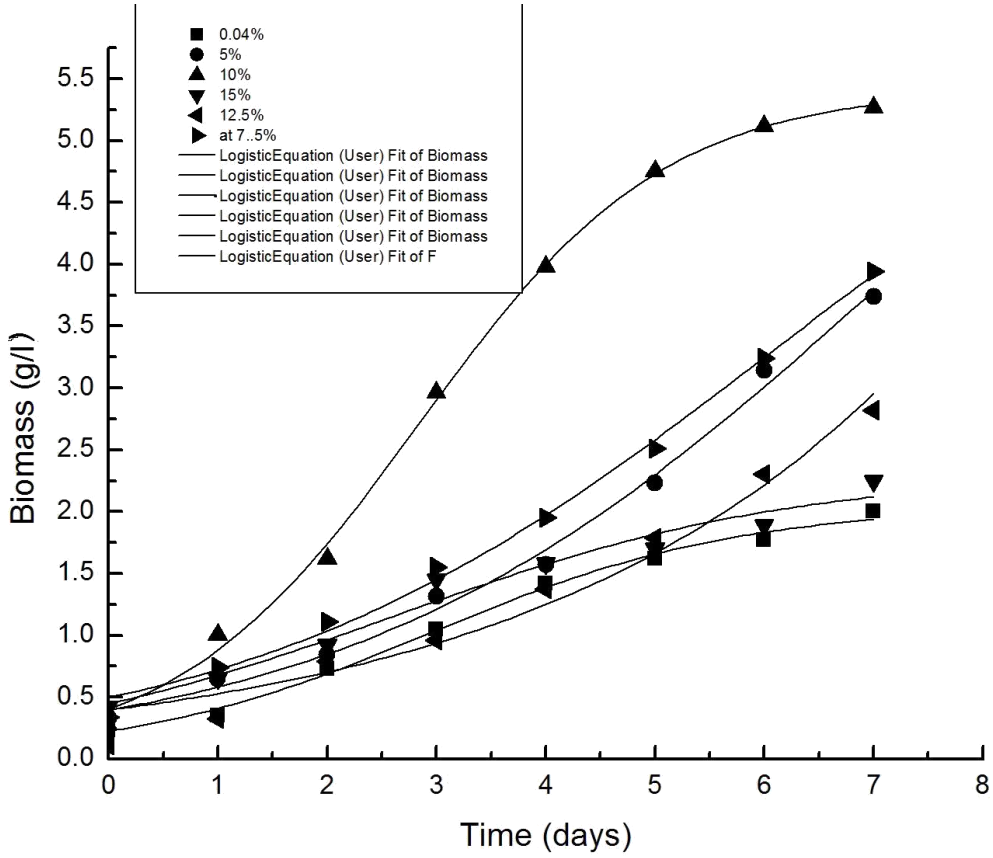

Figure 4.6: Predicted cell growth profile of *Anabaena* sp. in stirred photobioreactor at different proportion of air–CO_2 (v/v) gas mixture.

The curve fitting was found to be in good agreement with experimental values as the R^2 values were equal to 0.99 (**Table 4.2**). From the predicted curve, we can find the values of X_{max}. The pattern of X_{max} rise and fall was found to be quite similar to experimental values of X_{max}. The best curve fitting for our data is non-linear as it minimizes the sum of squared residuals.

Table 4.2: Summary of the parameters used in kinetic modeling of *Anabaena viriabilis*

CO_2 % (v/v)	**$a=X_{max}$ (g/l)**	**R^2**
0.04	2.04456	0.99279
5	4.62612	0.99193
7.5	4.98786	0.99389
10	5.41804	0.99815
12.5	3.92495	0.95857
15	2.31319	0.96234

4.8 CHNS Analysis

Table 4.3 shows that the concentration of carbon fixed decreased as we increased the concentration of CO_2. Although for first three CO_2 concentrations i.e. 5%, 7.5% and 10%, carbon fixed of the total concentration was around 40%, but with further increase in CO_2 concentration i.e. at 12.5% it came down to 38% and at 15% it became as low as 27%. The carbon fixed began to decline which clearly indicates that at high CO_2 concentrations *Anabaena* is not able to utilize carbon dioxide properly. The lower specific growth rate at 15% CO_2 level may be due to non-representative biomass determination. When CO_2 level increased, *Anabaena* preferred aggregative growth form (like bio-floc or biofilm) than individual suspended form. The phenomenon was less serious at 10% CO_2 level in the photobioreactor, but serious at 15% CO_2 level. This caused lower biomass measurement at 15% CO_2 level. However, concentration of nitrogen remained same which clearly indicates that high CO_2 concentration does not affect the nitrogen fixing mechanism of *Anabaena*.

Table 4.3: Elemental profile of *Anabaena* at different CO_2 concentrations

Sample	CO_2 concentration (%)	N (%)	C (%)	H (%)	S (%)
1	5	4.22	40.80	5.78	0.96
2	7.5	4.81	40.73	5.67	0.78
3	10	4.84	40.36	5.75	0.63
4	12.5	4.77	38.57	5.70	0.29
5	15	4.25	27.01	4.43	0.22

4.9 Cyanobacterial Growth in Dairy Wastewater

Culture of *Anabaena* was grown in dairy wastewater sample for 7 days at 27°C in orbital shaker in shake flask to study the effect of wastewater on *Anabaena*. Waste water without filtration is quite turbid, so it will hinder the light to penetrate thus affecting growth of *Anabaena*. Sample of dairy wastewater was first filtered and then autoclaved before it was used for culture growth. Decline in COD and BOD was observed after autoclaving. It can be seen from **Figure 4.7** that *Anabaena* grew at much higher rate in waste water than in synthetic medium. Growth is shown in terms of optical density.

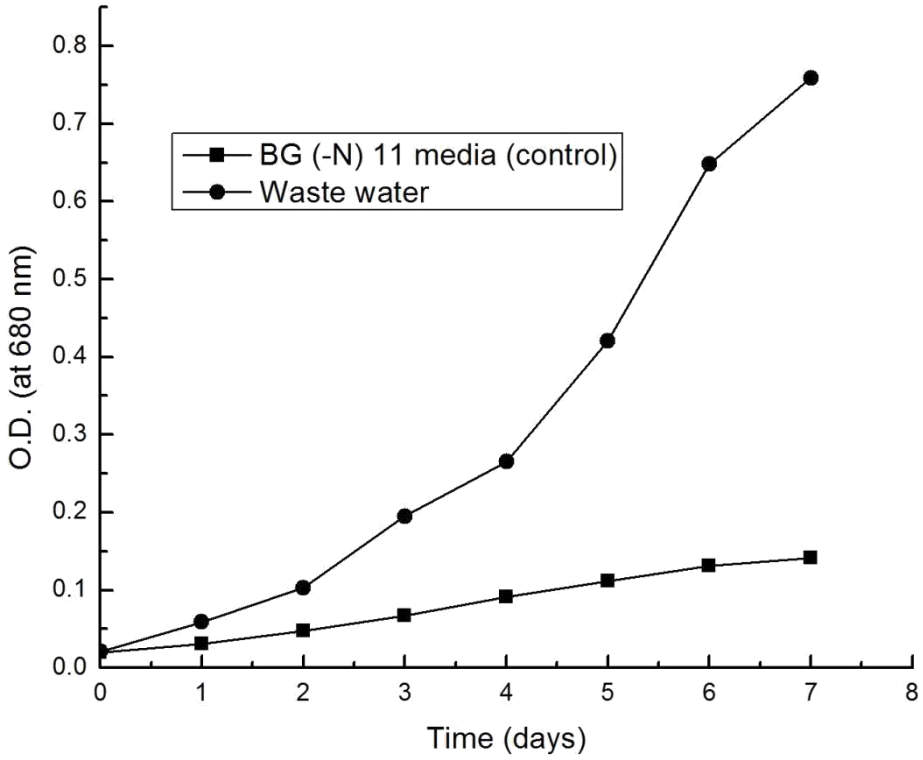

Figure 4.7: Growth comparison of *Anabaena* in BG (-N) 11 media and waste water

COD and BOD

Table 4.4 shows the decline in value of COD and BOD after inoculating the waste water with *Anabaena*. *Anabaena* utilized organic compounds present in sample utilizing atmospheric CO_2.

Table 4.4: COD and BOD values of waste water after inoculation with *Anabaena*

Sample (Waste water)	BOD (mg/l)	COD (mg/l)
Without filtration	2500	3100
After filtration and autoclaving	2300	2400
After 7 days of inoculation	80	100
Control (BG (-N) 11 media)	25	40

CHAPTER 5

CONCLUSIONS & RECOMMENDATIONS

5.1 Conclusions

i. *Anabaena* is not only active at high CO_2 concentration but can fix CO_2 very efficiently. However, it grows best at 10% CO_2 concentration.

ii. All the kinetic parameters show an upward trend till 10% CO_2 concentration. But they begin to decline after that.

iii. μ_{max} is highest at 12.5% CO_2. However CO_2 fixed, productivity and CO_2 fixation rate are maximum at 10% which are 7.15 g/l, 1.021 g/l/day and 0.7 g/l/day respectively.

iv. *Anabaena* grows best at pH 7.

v. Carbon composition of *Anabaena* is approximately 40% dry biomass and not 50% as assumed by other investigators.

vi. Concentration of CO_2 does not affect the nitrogen content of *Anabaena* and it remains almost 4.5% throughout.

vii. At high CO_2 concentration i.e. 15%, *Anabaena* shows lower specific growth rate, probably due to non-representative biomass determination. When CO_2 level increased, *Anabaena* preferred aggregative growth form (like bio-floc or biofilm) than individual suspended form.

viii. *Anabaena* shows higher growth rate in wastewater thus wastewater can substitute media for saving cost; moreover, it cleans wastewater making it fit to be released for future use.

5.2 Recommendations

i. During the course of experiment pH was allowed to vary however, growing *Anabaena* at constant pH may yield different results.

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

iii. 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.

iv. Instead of using commercial CO_2 gas cylinder, we can directly use mixture of air and flue gases (as a source of CO_2) to decrease the cost of CO_2 separation.

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3. Priyanka, **Sharma A.**, Bhunia H., Bajpai P.K. (2014). Kinetics of CO_2 sequestration by *Chlorella vulgaris* in CSTR batch type photobioreactor, *National Conference on Innovation and Development in Chemical Technology* (IDCT-2014), (28 Feb-1 March) (page no.-27) GGIPU, New Delhi.