Kinetics of CO_2 sequestration by *Anabaena variabilis* in a stirred tank batch type photobioreactor

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in
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Submitted

by

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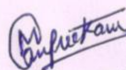
Declaration

I, the undersigned, hereby declare that the research work presented in the M. Tech. dissertation entitled "**Kinetics of CO_2 sequestration by *Anabaena variabilis* in a stirred tank batch type photobioreactor**" has been carried out by me under the supervision and guidance of Dr. N. Das, Professor, Department of Biotechnology and Dr. Haripada Bhunia, Professor, 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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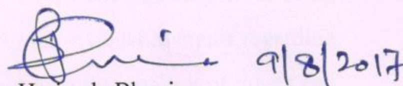
Certificate

This is to certify that dissertation entitled, "**Kinetics of CO_2 sequestration by *Anabaena variabilis* in a stirred tank batch type photobioreactor**" submitted by Ms. Manpreet Kaur 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.



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Abbreviations

AMOP	Aquatic Microbial Oxygenic Phototrophs
CCM	Carbon Capture Mechanisms
IEA	International Energy Agency
IGCC	Integrated Coal Gasification Combined Cycles
PBR	Photobioreactor
R & D	Research and Development
RuBisCO	Ribulose Bisphosphate Carboxylase Oxygenase

Abstract

Green house gases are strongly responsible for increase in the global warming since they absorb infrared radiations. Therefore, it is the need of the hour to reduce the production of green house gases up to a considerable extent. High emissions of CO_2 gas can be found at locations like thermal power plants, steel, cement, oil, automobile industries. Most of the CO_2 capture techniques are not environment friendly as they cause leakage of CO_2 back to the environment. Recent researches show that carbon dioxide sequestration by microalgae has become one of the alternatives to combat climate change due to the various limitations of the higher plants however, not much work has been done to meet the increasing demand for energy along with replacing the fossil fuels. Biological methods involve the utilization of photosynthesis for sequestering the CO_2 and thereby producing various valuable by-products under controlled conditions. This study involves the use of *Anabaena variabilis*- a cyanobacteria which is cultivated in BG (-N) 11 media under optimum conditions like 28ºC temperature, 7.2 pH, 120 rpm agitation rate, and around 5000 lx light intensity in a stirred tank photobioreactor for 7 days. The effect of varying the gas flow rate as 0.5 lpm, 1.0 lpm and 1.5 lpm on CO_2 sequesteration efficiency was studied along with the growth kinetics of *Anabaena* and protein content estimation. The kinetic parameters calculated like the productivity, CO_2 fixation rate, average specific growth rate were higher at 1.0 lpm as 0.052 g/l/day, 0.095 g/l/day, 0.558 l/day, respectively. However, maximum biomass was observed at 1.5 lpm as 0.416 g/l. The protein content was found to be similar in all the batches i.e. 0.14 g/l. Hence, the experimental data shows that 1.0 lpm is the most suitable gas flow rate for mass cultivation of microalgae as well as CO_2 sequestration in photobioreactor.

Keywords: CO_2 biosequestration, *Anabaena variabilis*, Growth kinetics, Photobioreactor, Aeration rate

CHAPTER 1

INTRODUCTION

1.1 Need for CO_2 sequestration

Green house gases are the main reason for increase in the global warming because of their tendency to absorb infrared radiations. They can cause several problems which include vast climate changes, cardiorespiratory disorders, heat wave–related disorders, psychological and social instability, etc. **[1].** Combustion of fossil fuel is the main reason for gradual increase in global CO_2 concentration. It represents approximately 75% of the total CO_2 emissions **[2].** Therefore, it is the need of the hour to reduce the production of green house gases up to a considerable extent. Thus, global warming can be prevented by reducing or managing the emissions of greenhouse gases like anthropogenic carbon dioxide, which is the most abundant green house gas. IEA reported that global CO_2 emissions in 2013 were found to be approximately 32.2 $GtCO_2$. This shows that there has been a successive increment of 2.2 % over the 2012 levels **[3].**

According to the report of Belbute and Pereira (2015), CO_2 emissions are assumed to increase from 36,131 Mt in 2013 to around 51,883 Mt in 2100 **[3].** India's share of global CO_2 emissions is found to be around 3%, but on per capita basis it is much lower than the world average. Therefore, to reduce the excess CO_2 levels, India also started research on CO_2 sequestration in 2004 through industrial and governmental support.

Various approaches commonly used for reducing the rise in CO_2 concentrations are given below:

i. To increase the efficiency in generating electricity and its optimum utilization.
ii. To expand the use of renewable energy sources like wind, biomass, geothermal, solar, nuclear power, etc.
iii. Judicious utilization of energy to change the present scenario.
iv. To capture carbon dioxide emissions from fossil-fueled electricity generating plants for permanent sequestration.

The top three approaches require public acceptability for changing their consumption needs and necessity to invest but it can lead to making the planet more inhabitable. However, the success of fourth approach requires more research.

1.2 Sources of CO_2

High emissions of CO_2 gas can be found at locations like steel, thermal power plants, oil, cement and automobile industries. Combustion of fossil fuel such as coal, oil, and gas leads to excessive CO_2 emissions. Flue gas emitted from these industries mostly contains CO_2, N_2, O_2, and water vapour along with the minute amounts of CO, SO_x, NO_x and particulate matters. A mutant strain of *Scenedesmus obliquus* was reported to fix high amount of CO_2 present in the flue gas. Up to 67% of CO_2 was captured in the pilot scale systems **[4].**

1.3 Microalgae as a biosequestration agent

Algae are also known to be one of the primitive life-forms. Depending on the type of species, algae can vary in size from 1 micron (µm) to a few hundred microns. Microalgae allow biomass generation and effective genetic and metabolic research in a much shorter time period as compared to the conventional plants **[5].** Photosynthetic efficiency of microalgae has been observed to be nearly 10 times higher than that of terrestrial plants **[5].** Around 1.6 to 2 grams of CO_2 is captured for every gram of the biomass produced. Hence, out of all the methods available, culturing of photosynthetic microalgae in photobioreactors is the most sustainable method for CO_2 biofixation since they can also produce various essential compounds like exoploysaccharides, biofuels, proteins, etc. Microalgae can be cultivated either in large open ponds or in closed photobioreactors. Airlift and bubble column photobioreactors are most commonly used for cultivating the microalgae on large scale. Maximum carbon sequestration can be achieved through proper choice of microalgal species and by optimizing certain parameters like temperature, nutrient availability, light intensity and pH **[6].** *Chlorella, Spirulina, Haematococcus pluvial, Scenedesmus, Dunaliella salina and Porphyridium cruentum* are some of the microalgae which have been successfully cultured on large scale **[7].** Photosynthetic efficiency of microalgae can vary from 3 % (*Spirulina* sp.) to 20 % (*Chlorella* sp.) **[3].**

Besides giving environmental and economic benefits, large scale cultivation of microalgae can also create a numerous job opportunities. CO_2 sequestration accompanied by other multi-functional systems like aquaculture farms and waste water treatment can be used to increase the economic feasibility of biological mitigation systems. The scientific community is currently researching for the potential approaches required to reduce the CO_2 emissions leading to global

warming. Use of several physico-chemical methods and proper conversion of CO_2 into valuable products by using microalgae are some of the sequestration options that are still in the R & D panorama **[8].** The extent of its utilization depends upon factors like capacity, cost, its impact on the environment, the rate at which that technology is introduced and several social factors like public acceptance.

1.4 Research objectives

The main objective of this research is CO_2 sequestration by using microalgae which is required to solve the various problems caused by global warming. The specific objective includes:

- To study the growth kinetics of *Anabaena* sp. in a stirred tank photobioreactor
- To study the impact of different aeration rates on CO_2 sequestration by microalgae

1.5 Dissertation overview

This dissertation includes five chapters. Chapter 1 involves a brief introduction to the above mentioned research topic. Chapter 2 includes the detailed literature review related to the research like basic information on CO_2 sequestration by microalgae, the microalgae used in this research i.e. *Anabaena*, ways to reduce CO_2 level the factors affecting the general CO_2 sequestration process, types of photobioreactors that can be used for proper microalgae cultivation, the process of photosynthesis. Chapter 3 contains briefly discussed materials and methods that are involved in the process. While chapter 4 contains the results obtained which are thoroughly analyzed and discussed. The last chapter includes the conclusions that can be drawn from analyzing the results obtained along with recommendations for the future work.

CHAPTER 2

LITERATURE REVIEW

2.1 CO_2 sequestration by microalgae

Recent researches show that carbon dioxide sequestration by microalgae has become one of the alternatives to combat climate change due to the various limitations of the higher plants however, not much work has been done to meet the increasing demand for energy along with replacing the fossil fuels. Microalgae are the source of attraction for various reasons like carbon dioxide sequestration, biofuel production, heavy-metal removal, pigment production, sunscreen formulation, etc. **[9].** *Nannochloropsis* sp. have been found to be an alternative source of EPA (eicosapentaenoic acid), a nutritionally important PUFA that is useful in the prevention and treatment of several human diseases **[10].** Microalgae promote formation of high value products like fatty acids, biogas, proteins, amino acids, pharmaceuticals, bioethanol, animal feed, biodiesel, biokerosene, biohydrogen, etc. They are efficient in the removal of pollutants and are useful in the process of bioremediation**,** waste water treatment, and act as N_2 fixing biofertilizers for soil**.** *Dunaliella* sp. has been used in the industrial production of b-carotene. *A. variabilis* was found to be the potential biocatalyst for biohydrogen production. Production of algal lipid by involving the photosynthesis–fermentation model with double CO_2 fixation has been reported. It enhances the carbon-conversion ratio of sugar to oil **[11].**

2.2 Ways to reduce CO_2 level

Most of CO_2 capture techniques are not environment friendly as they cause leakage of CO_2 back to the environment. Also, dissolution of CO_2 gas in water is 1.45 g/l at 25°C, which is very less. Hence, use of safe method for CO_2 disposal is important. Some of the ways to fix CO_2 are shown in **Fig. 1**:

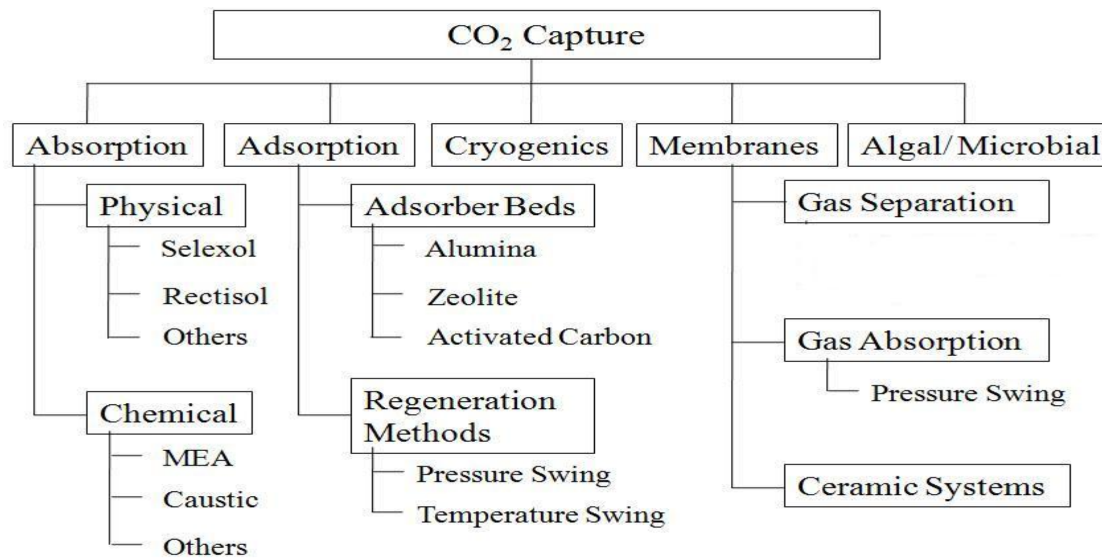

Fig. 1: Some of the carbon capture and CO_2 sequestration technologies **[12].**

2.2.1 Use of chemical/physical methods

To avoid the problems associated with low dissolution rate of CO_2 in the medium, an alkaline chemical scrubber (lime) can be used which can readily be utilized by microalgae for their growth **[13].** Another strategy is to chemically convert CO_2 into stable liquids or solids such as carbonate salts which can be buried or used as construction material.

2.2.2 Adsorption onto solids

Conventional catalysts such as activated alumina, magnesium oxide, zeolites, graphene, clays and zinc oxide can be used to reduce CO_2. However, their efficiency is not enough for reducing the large quantity of atmospheric CO_2.

This type of CO_2 capture system is generally used in the chemical as well as oil industries. Physical absorption is usually temperature and pressure dependent. Here the absorption occurs at high pressures and low temperatures. However, chemical absorption of CO_2 from gases like flue-gas depends upon the acid–base neutralization reactions involving the use of basic solvents. Amines are a perfect example of the preferred solvents. The emitted CO_2 is compressed while the regenerated absorbent solution would be recycled in the column **[14].**

2.2.3 Pressure swing adsorption (PSA)

This technique requires high pressure of the IGCC for enhancing the extent of CO_2 adsorbed. When CO_2 is passed to the adsorption tower, it is adsorbed on the adsorbent to a large extent than under normal conditions. Also, in PSA the thermal energy losses are much lower than the other methods.

2.2.4 Cryogenic/condensation systems

This type of system involves an air separation process in which various gases are separated by condensation. This method can be used for capturing CO_2 in aqueous form because of easy transportation and storage. CO_2 and H_2O are generally separated from the gases on the basis of their differences in the dew point and sublimation point **[14].** The aqueous form of CO_2 called bicarbonate (HCO_3^-), is the preferred source of carbon to be captured by microalgae as they can import it into the cells through the cell membranes. The major limitation of CO_2 trapping in the soluble form is the low CO_2 dissolution rate and retention time in aqueous state.

2.2.5 Electrochemical pumps for separating CO_2 from the gas

The molted carbonates and liquid alkaline fuel cells can be used for separating the carbon dioxide from air and gases. Operating the molted carbonate fuel cell in a closed circuit (along with an external emf) would result in transporting the carbonate ions through the membrane. The molted carbonate electrochemical separator needs oxidizing conditions for forming carbonates from the carbon dioxide, but this process is less applicable for directly separating the CO_2 from gases. Sulfur dioxide found in the flue gas is poisonous for the cell, hence forming the sulfates. There are several drawbacks in using this method like segregation of electrolyte, electrode degradation, etc are common in the severe high temperature conditions of flue gas [**14].**

2.2.6 Chemical looping

It is a novel method which involves segregation of carbon dioxide from flue gases. The oxygen required for combusting the fuel is provided by a metal oxide catalyst that can be regenerated. The lattice oxygen from the catalyst can be used as the oxidizing agent, in lieu of gaseous oxygen from the air, thereby resulting in the flue gas undiluted by the nitrogen and causing the

concentration of carbon dioxide in the flue gas. Since, air is not utilized in combusting the fuel, NO_x emissions are very less [**14].**

2.2.7 Membrane separation of CO_2

Developing the membrane separator for the purpose of making the highly economic technology requires selective removal of CO_2 when either CO, H_2, H_2S, H_2O from the flue gas or N_2, O_2, NO, H_2O, SO_2, HCl from the flue gas are present. It requires a membrane material which will either allow the selective transport (diffusion) of CO_2 or the selective exclusion of CO_2. Membranes can easily separate carbon dioxide from the gas by the process of size exclusion or the chemical affinity. Extensive research has been done on the properties of carbon dioxide selective membranes that are based upon inorganic materials like zeolites, alumina, carbon, etc. Hence, it has been found that membranes are required for successfully applying on the flue gases.

2.2.8 Geological sequestration and deep ocean sequestration

Injecting CO_2 directly into the geological formations is also a strategy for the mitigation of CO_2 from favorable point sources. However, this method is inappropriate for long term CO_2 sequestration. Hence, to overcome this limitation CO_2 can be converted into a more stable form known as biochar. Oceans also play a vital role in absorbing the atmospheric CO_2. Absorption of CO_2 by ocean is a very time consuming process and thus, it is difficult to cope with its excessive amounts that are emitted every day. 40% of the annual global CO_2 emission is fixed through photosynthesis by deep ocean organisms **[15].**

2.2.9 Biological methods

It involves the utilization of the process of photosynthesis for stabilizing the CO_2 and thereby producing various valuable by-products under controlled conditions. This method helps in the net removal of CO_2 from the environment as well as from anthropogenic sources. Their overall effect called 'negative net emissions' can also be observed sometimes.

The photosynthetic microalgae uptake CO_2 efficiently therefore they grow quickly. Their biomass production produces 5.4–10 fold more than most of the higher plants. Cyanobacteria and microalgae are found to be one of the suitable candidates for converting CO_2 into organic

matter. Not only can they help in the production of commercial by-products like bioethanol, carotenoids etc., but also, these photosynthetic microalgae are useful in bioremediation applications while some can also act as nitrogen fixing biofertilizers.

2.2.10 Immobilized microbial enzymes

Algae have active bicarbonate pumps and can concentrate bicarbonate molecules in the cell. Reports show that these bicarbonates are subsequently dehydrated, either spontaneously or by microbial enzymes such as carbonic anhydrase from *C.freundii*. However, CO_2 is captured through Calvin-cycle activity in the form of algal biomass **[16].** Carbonic anhydrase is a metallo-enzyme which contains zinc. This is one of the CCMs that can fix the CO_2 by catalyzing the reversible hydration of carbon dioxide into bicarbonate ions and a proton **[15].**

2.3 Cyanobacteria: an effective way of CO_2 sequestration

Cyanobacteria are photosynthetic microorganisms that can capture the sunlight by using Chlorophyll a and various other pigments. They can be found in various places like springs, lakes, ponds, wetlands, etc. They also play an important role in the N_2, C, and O_2 dynamics of many of the aquatic environments. They are free living organisms with the symbiotic associations with more complex biota like N_2-fixing cyanobacteria. *Anabaena azollae* has symbiotic association with the floating fern called *Azolla*, which is generally found in ponds and flooded soils.

Cyanobacteria are also classified as blue-green algae as they have algal-like appearance. They also possess chlorophyll pigment and are similar to higher plants in using photosynthesis process resulting in the production of oxygen. It utilizes a two-photosystem process. Structural studies also show that the microalgae are prokaryotic as they do not have nuclei and other cellular organelles. They have peptidoglycan cell wall which is also a characteristic of gram-negative eubacteria. Cyanobacteria include around 2000 species, 150 genera and 5 orders, which also involves wide variety of different shapes and sizes.

2.3.1 *Anabaena*

Anabaena sp. is a filamentous nitrogen-fixing marine cyanobacteria having a high growth rate (up to 0.1 h^{-1}). It is tolerant to high temperatures (up to 45°C) and wide pH levels (6-10). It

shows maximum absorption at the wavelength of 435 nm and 676 nm for chlorophyll-a, and 621 nm for phycocyanin. It has been reported that *Anabaena* sp. was partially photo-inhibited at high irradiances (> 1,300 $\mu Em^{-2}s^{-1}$) **[17].** *Anabaena* sp. has been reported to transform CO_2 into valuable products and it can metabolize CO_2 at rates of 1.2 gCO_2 l^{-1} day^{-1} outdoors **[18].** It grows fast in nitrate-free medium, has high productivity of exopolysaccharides. It can easily be harvested by sedimentation. However, it is sensitive to stress damage by aeration. **Fig. 2** shows the microscopic view of *Anabaena* sp.

Fig. 2: Microscopic view of *Anabaena* sp.

2.4 Cultivation of microalgae

Two of the most common microalgal culturing systems are open ponds and closed photobioreactors (PBRs). At moderate pH (~ pH 7) and temperatures (< 30°C), CO_2 is predominantly present as bicarbonate in water.

2.4.1 Open pond systems

They can be grouped into natural waters bodies such as lakes, lagoons, ponds and artificial ponds. The most commonly used systems are shallow big ponds, raceway ponds, tanks and circular ponds. Carbon dioxide or bicarbonate-capturing efficiencies as high as 90% have been reported in open ponds. The advantage of open ponds is that they are easier to construct, operate

and easy to clean after cultivation. However, major limitations include poor light utilization by the cells, high evaporation rate, diffusion of carbon dioxide to the atmosphere, requirement of large lands and are prone to contamination resulting in low biomass productivity. Sosa Texcoco Co. has developed the largest single algal production systems (900 ha) near Mexico City **[19].**

2.4.2 Closed pond systems

Limitations of open culture systems can be overcome by using the closed culture systems. Till now various types of closed system has been developed such as vertical column, flat–plate photo-bioreactor, tubular, etc. Closed systems have high biomass productivities and low contamination rate. They are good for the immobilization of algae. Closed PBRs have the advantage of the regulated and well-controlled microalgal cultivation, in addition to low contamination chances, greater CO_2 fixation efficiency, more metabolic flexibility, and well controlled hydrodynamics. These systems generally have larger S/V ratio or surface/volume ratio and more surface area is exposed to the light source thereby reducing the shadow effect, which can cause inhibition of microalgal growth in open systems. However, there are several drawbacks like improper light utilization, inadequate biomass circulation, limited mass transfer (CO_2/O_2), etc. Hence, it is not suitable for large-scale processes.

There are different types of closed photobioreactors that are being used for microalgae cultivation like (i) vertical tubular systems, (ii) plate-type systems, and (iii) column systems. Usually, column PBR systems are relatively less costly and tubular PBR systems are mostly used for large-scale microalgal cultivation **[20].** Scientists have found that air-lift PBRs or vertical tubular-type PBRs, can achieve the better CO_2 fixation efficiency since it has better circulation and mass transfer properties. Also, bubble column PBRs are more suitable because they have well-defined flow patterns as well as circulation times by using risers and down-comers. However, airlift PBRs take more time for circulation thereby reducing the general photo-inhibitory effects which can influence cell density along with CO_2 fixation ability **[21].** Also, it has been found that most of the light can directly be reflected by small bubbles when operating the air-lift PBRs, which thereby lowers the photosynthetic efficiency. Despite being more suitable, tubular PBR systems are known to be effective microalgal-CO_2 fixation systems. But the excessive O_2 removal limits the reactor sizes and length, causes CO_2 depletion, improper

parameter control and being highly costly. Hence, tubular PBR systems are inappropriate to scale up indefinitely, so multiplication of several reactor units can be done **[22].**

2.5 Factors affecting CO_2 sequestration process

The efficiency of CO_2 capture by algae usually varies according to the state of the algal physiology, temperature and pond chemistry. However, some of the most common factors influencing the sequestration process are:

2.5.1 Temperature

It is crucial to select microalgae that can grow in large temperature range. Certain thermophilic micro-organisms are known to survive till temperatures of 60°C. Ratio of solubility of O_2 to that of CO_2 increases with temperature which leads to O_2 fixation by RuBisCO enzyme. At 60°C, 100% CO_2 tolerance is observed for *Cyanidium caldarium* **[23].** High temperature may also lead to decrease in RuBisCO affinity for CO_2. It has been found that the cyanobacteria *Synechococcus* elongates when it is sparged with different CO_2 concentrations and temperatures. It proves that solubility of CO_2 is dependent on temperature. Hence, thermophilic microalgae can be useful to provide tolerance under high CO_2 concentration **[24].** Temperature plays an important role in regulating the cellular, physiological and morphological characteristics of cyanobacteria. The optimal temperature is variable among different cyanobacterial species; but they depend upon on other environmental parameters, like light intensity as well. Optimal growth temperature that has been observed are generally in range 15–30 °C for some of the microalgae species, while maximum cell densities were also observed at 27 °C **[25].**

2.5.2 pH

The pH of the culture medium 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, and with higher SO_x concentrations even down to pH 2.6 have been reported. Whereas the pH change due to the CO_2 had just minor influence on the algal growth, SO_x inhibited all growth. Microalgae usually prefer neutral pH for optimal growth but some can show tolerance to extreme pH conditions as well. *Spirulina platensis* and *Chlorococcum littorale* can show suitable growth at pH 9 and pH 4.

2.5.3 Light

At low light intensity, light becomes the limiting factor for the microalgal productivity. While exposing the cells to high light intensity for long duration causes photoinhibition which is caused due to free radical formation. Wavelength, cell concentration, penetration distance of light and the geometry of photobioreactor play important role in the attenuation of light intensity. According to Fernandes et al. (2010), circular geometry allows a better penetration but non-uniform distribution of light throughout the bioreactor, than the plane geometry. Proper light intensity is unavailable in high density cultures; hence, optical depth should be considered as an important issue in designing the bioreactor **[25].** The PAR required is generally in the range of 400-700 nm. It has been observed that biomass production declines with decrease in the duration of light **[26].** The ideal light intensity for optimum algal growth has been reported to be in the range of 4000-6000 lx **[27].**

2.5.4 Culture density

Below the optimum cell concentration cells can't capture all the light energy, while at high cell concentration the cells remain in the dark. However, highly dense culture also makes cells more tolerant to high CO_2 concentration.

2.5.5 Culture strain

By selecting the adequate species, microalgae can assimilate 0.04% to 100% (v/v) CO_2. Tolerance to high temperature and pH range, low risk of contamination, high growth rates and resistivity to shear stress play important role in deciding the best strain suitable for CO_2 sequestration **[23].** For example, *Dunaliella* is an extremely fragile species due to lack of cell wall, while *Spirulina* can tolerate stress up to large extent.

2.5.6 O_2 accumulation

The oxygen is produced due to the oxygenase activity of RuBisCO enzyme which is commonly found in photosynthetic organisms. Excess oxygen in the culture can cause toxic effects like photo-bleaching and may result in the reduction of photosynthetic efficiency of the microalgal cells. It may cause damage to the metabolism of the microalgal cells if allowed to accumulate.

However, an efficient degassing system such as sodium bisulphite, can be used to combat with it since it helps releasing the excess oxygen **[28].**

2.5.7 CO_2 Concentration

Early adaptation of cells with lower percentage of CO_2 concentration can make the cells tolerable to higher percentage of CO_2. For example, *Chlorococcum littorale* could grow under 60% CO_2 using the stepwise adaptation technique **[23].**

2.5.8 Mixing rates

Mixing is important to ensure suitable microbial growth in a photobioreactor. Low mixing rates interferes with the gaseous mass transfer and causes biomass to settle. Poor mixing causes formation of stagnant zones, where there is no sufficient availability of light and nutrients. This in turn, may lead to formation of anoxic/anaerobic conditions resulting in low productivity. Compromise in culture viability is also an issue because of the accumulation of toxic compounds in these stagnant zones. Conversely, high mixing rates result in shear damage to cells. The most common methods for mixing in bioreactors involve pumping, mechanical stirring and gas injection **[25].**

2.5.9 Volumetric mass transfer coefficient (k_La)

k_La is an important factor in determining and improving the aeration efficiency. k_La and cell growth rate generally varies in the various region of liquid flow. Liquid flow region in photobioreactor can be separated into laminar flow, turbulent flow and transition zone according to the parameters, gas velocity and rate of agitation. By using the feedback technique and varying the aeration rate and stirring speed, change in k_La can be achieved. High aeration rate causes shear stress thereby reducing the cell growth rate. Zhang et al. compared the k_La values in different photobioreactors at varying CO_2 concentration and determined that k_La value increases with decrease in the CO_2 concentration to supply sufficient CO_2 to microalgal cells **[29].**

2.5.10 Nutrient requirements

Microalgae require carbon and nitrogen as basic energy source for making the nucleic acids and proteins. However, phosphorus is also important for microalgal growth. Marine microalgae also utilize sea water supplemented with nitrates and phosphates. Nevertheless, vitamins and trace

metals like Cu, Mb, Mg, Ca, Mn, Zn, etc. also play important role for optimum microalgal growth **[25].** Lipids contain more energy than carbohydrates therefore they can also act as energy boosters. Decrease in nitrate, silicate or phosphate in the growth medium has been found to produce 2-3 fold increase in glycogen production in microalgae or lipids in many AMOPs. Many AMOPs are considered as rich sources of proteins when they are grown under nutrient replete conditions **[19].**

CHAPTER 3

EXPERIMENTAL SECTION

3.1 Materials

3.1.1 Microalgae and its culture medium

A culture of *Anabaena variabilis* ARM 441 which is a photoautotrophic microalgae, was obtained from the laboratory at Science and Technology Entrepreneur's Park (STEP), Thapar University, Patiala. BG (-N) 11 media (also known as $BG11_o$), was used for growing the microalgae as well as for the process of subculturing. The chemical composition of BG (-N) 11 media used in this project is given in **Table 1**.

Table 1: Composition of BG (-N) 11 media

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

The trace metal mix contains several metallic ions in minute concentrations which basically act as a cofactor for various enzymes. Deficiency in any of the metallic ions may cause the condition of impaired or no growth of microalgae at all. The chemical composition of the standard trace metal mix used in the project is given in **Table 2**.

Table 2: Composition of trace metal mix

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

All the chemicals used during the project were of standard AR grade and were obtained from Himedia, Loba Chemie and SD Fine Chemicals.

3.1.2 Instruments/ equipments

i. Weighing balance

The weighing balance used was of mettle Toledo company for weighing all the different chemicals.

ii. Temperature controlled orbital shaker

This instrument was used for maintaining the fresh stock of the microalgal culture through repeated subculturing. Suitable illumination was provided to the culture by using the cool white fluorescent light (85 Watt).

iii. Lux meter

The lux meter used was from Mastech, India, model MS 6610 for monitoring the instantaneous light intensity provided to the microalgal culture.

iv. Laminar air flow chamber

Laminar Air Flow used was from Thermodyne Pvt. Ltd., Faridabad to transfer the microalgal culture under aseptic conditions.

v. UV-Vis spectrophotometer

The absorbance at the wavelength of 683 nm was taken for the microalgal cell suspension extracted at regular intervals. It was obtained from Lambda 35 UV/ Visible Spectrophotometer (Perkin Elmer, USA).

vi. Autoclave

The vertical autoclave used was obtained from Equitron, India for sterilizing the media, glassware and the glass photobioreactor at standard conditions of 121°C temperature and 15 psi pressure.

vii. Whatmann no. 1 filter paper

It was used for filtering the microalgal suspension extracted from the photobioreactor.

viii. Microscope

Live microalgal cells were observed with the help of a Nikon eclipse 50 i microscope at 40X magnification.

ix. Digital pH meter

Digital pH meter (Thermo Scientific, Orion 5-Star model) was used for determining the pH of the media and then adjusting it by using 0.1 N HCl and 0.1N NaOH. The pH probes (of digital pH meter and the photobioreactor) were calibrated using the standard pH buffers of pH 7.0 and 9.2.

3.1.3 Set-up for subculturing the microalgae

For the process of subculturing the microalgae, a light source i.e. 85 Watts cool white CFL and an orbital shaker having controls for temperature and rotating speed was used. Flasks containing the required media inoculated with the microalgal culture were placed in the shaker. The cultures were maintained under optimum conditions i.e.28ºC temperature, 120 rpm agitation rate, and around 5000 lx light intensity. The cultures were allowed to be incubated till the stationary phase was reached.

3.1.4 Experimental setup for CO_2 sequestration

During the project, the stirred tank bioreactor i.e. Bioflo® / Celligen® 115 Bioreactor (New Brunswick) was used which had 7.5 liters capacity and 5.5 liters working volume. The compressed CO_2 gas cylinder used for providing CO_2 into the bioreactor was obtained from Lalit Gases, Patiala. An oil-free air compressor from Apcon, India was used for supplying the air. White CFL bulbs were used for providing the light having the intensity of approximately 5000 lux to the bioreactor. The Schematic diagram of the experimental setup for sequestering CO_2 is given in **Fig. 3** while the flow diagram of the general process involved is provided in **Fig. 4.**

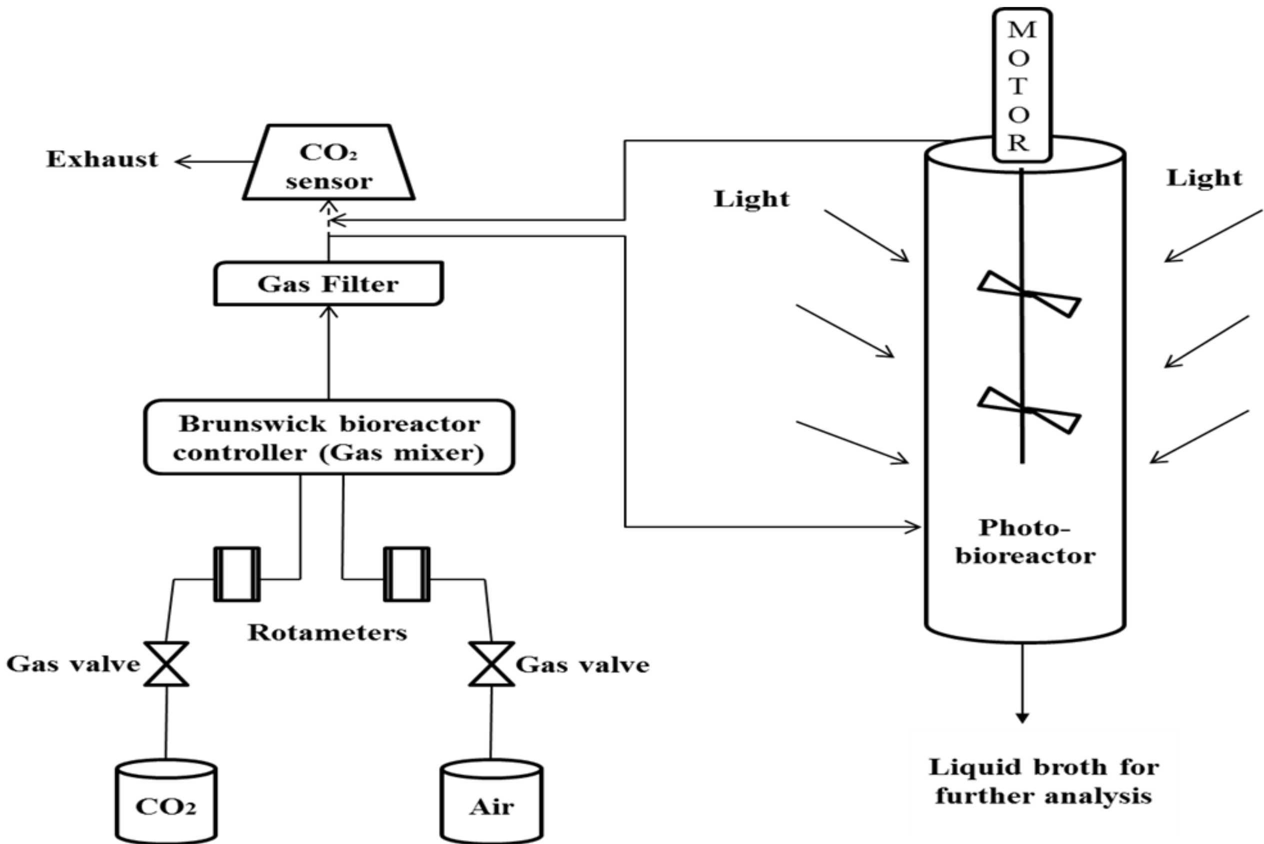

Fig. 3: Schematic diagram of the experimental set up for sequestering CO_2

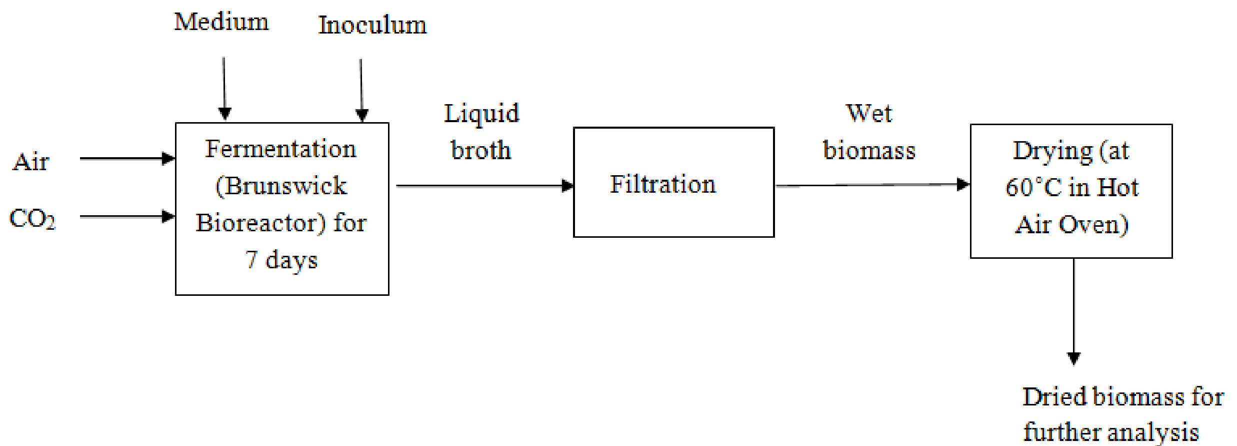

Fig. 4: Flow diagram of the general CO_2 fixation process

3.2 Procedure

3.2.1 Subculturing/maintenance

Subculturing of microalgae was performed with 6% inoculum (mid exponential phase) in 200 ml shake flasks filled with BG (-N) 11 media under optimum conditions of 28°C temperature and 120 revolutions per minute (rpm) agitation for 5 days. The flasks were plugged with the cotton balls to allow CO_2 from the atmosphere to dissolve in the media for proper growth of microalgae as well as to prevent contamination. The microalgal culture was provided with continuous illumination of 5000 lx through the CFL bulb. Before the sterilization and inoculation in the flasks, pH of the media was adjusted to 7.2±0.2 with the help of pH meter. The culture was incubated for 15 days and the process is repeated to maintain fresh stock.

3.2.2 Autoclaving

Sterilizing the photobioreactor is necessary before the initiation of a batch. Hence, autoclave was employed since moist heat can kill not only the microorganisms but can also destroy their spores. However, before autoclaving the unassembled photobioreactor, it was washed first with chromic acid and then it is rinsed with soap solution and then water. After that, the photobioreactor was air dried and reassembled. Probes for pH and D.O. were cleaned with the help of ethanol. The next step was to add the media into the photobioreactor and set pH at 7.2±0.2 before autoclaving. All the immersed pipes were tightened up with the help of stoppers to avoid any escape of media from the photobioreactor during autoclaving. Opening of each pipe extended outside the bioreactor, was further plugged with the cotton balls and covered with aluminum foil. DO and pH probes and impeller shaft were also covered with the caps. Afterward, photobioreactor was sterilized at set conditions of 121°C and 15 psi for half an hour. Finally the air is removed from the exhaust valve of the autoclave before opening the lid of the autoclave.

3.2.3 Inoculum preparation

6% inoculum was used and the microalgal culture was taken in mid exponential phase because more number of cells can be observed and rapid cell division takes place in this phase. Prior to inoculation, Bunsen flame was used to sterilize the head plate of photobioreactor.

3.2.4 Experimental set up for CO_2 fixation

Photobioreactor of 7.5 L as total capacity and working volume of 5.5 L can be used for mass culturing of microalgae. The temperature of the media inside the photobioreactor was set at 28±2˚C. It was measured with a temperature sensor i.e. Resistance Temperature Detector (RTD) and was maintained with chiller and heating jacket. The pH of the photobioreactor was set at 7.2±0.2 and was continuously measured by pH probe. Illumination of around 5000 lx was provided to the photobioreactor by 85 Watts CFL. The air and CO_2 mixture was sparged at 0.2 vvm (volume of gas/ volume of liquid/ min). The flow of gas and air was set by using the rotameter. Agitation of the media was carried out at 120 rpm to ensure proper nutrient distribution and breaking the air bubbles for improving mass transfer of CO_2 in the photobioreactor. CO_2 and air was passed through 0.2-μm filter before injecting in the photobioreactor. Similarly the outlets were also guarded by the micropore filters to prevent the escape of microalgal cells from the photobioreactor. The outlet was also installed with an exhaust condenser to prevent the evaporation of the media. Baffles present in the photobioreactor allow the proper mixing of gases and nutrients by creating turbulence while agitation.

3.2.5 Gas mixture

This project involves the use of pure CO_2 obtained from a cylinder along with a preheater (to avoid choking at low temperature) and the air obtained from an oil free compressor. CO_2 concentration was kept constant at 10% but the aeration rate was varied at 0.5 lpm, 1.0 lpm and 1.5 lpm during each batch with the help of rotameter.

3.2.6 Estimation of the dry weight of biomass

The samples were collected aseptically from the photobioreactor and were filtered through the filter paper. The filter paper were washed and dried in hot air oven at 60ºC for overnight. Then the dry filter paper was weighed to obtain the dry biomass weight **[30].** Absorbance at 683 nm was measured using UV/visible spectrophotometer for the corresponding day. This process is repeated for five days and the calibration curve between dry biomass and optical density was plotted. This plot was used to convert the optical density of the collected samples into dry biomass weight.

3.2.7 Determination of growth kinetic parameters

The values of biomass obtained were plotted against time for constructing the microalgal growth curve. From the log phase, the specific growth rate μ (1/d) was calculated through Equation (1)

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} ----(1)$$

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 calculated. The cell doubling time was calculated by Equation (2)

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

The maximum biomass obtained was represented 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 determined by using Equation (3)

$$P_{overall} = \frac{\Delta X}{\Delta t} ----(3)$$

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 represented as the maximum productivity.

3.2.8 Determination of CO_2 utilization efficiency

The initial biomass concentration of inoculum and maximum biomass concentration achieved in the photobioreactor 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 (4)

$$F_c = \frac{P_{overall} * 0.5 * 44}{12} ----(4)$$

where, 0.5 is taken as carbon content of dried biomass which is assumed, 12 (g/mol) and 44 (g/mol) represents the molecular weight of carbon and CO_2, respectively.

3.2.9 Determination of growth kinetics of microalgae using logistic equation

Using logistic equation to determine the growth kinetics is a good choice. It helps in explaining the growth curve since it does not use the term "substrate" to explain the whole growth profile of microalgae. 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 microalgal culture satisfactory **[31].** Equation (5) is the logistic equation.

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{X_{\max}}\right) \text{-------------------- (5)}$$

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 Equation (5), it can be written as Equation (6) where the initial biomass concentration X_0 is in (g/L).

$$X = \frac{X_{\max}}{1 + \left(\frac{X_{\max}}{X_0} - 1\right)e^{-kt}} \text{------------------------ (6)}$$

This may be further rewritten in the form of Equation (7)

$$y = \frac{a}{1 + be^{-kt}} \text{-------------------- (7)}$$

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 (7) in Origin Pro 8.0 using curve fitting tool. A confidence bound of 95% was taken into consideration to find the best fit.

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

3.2.10 Protein estimation

Protein estimation was carried out by Lowry method. [**30]** However, firstly the samples were homogenized with lysis buffer with the composition of 0.5 M Tris HCl, 8 M Urea, 5% (w/v) SDS, 20% (w/v) glycerol, 10% (v/v) ß mercepto-ethanol. Final pH of the buffer was adjusted to 6.8. The suspension was centrifuged at 4°C for 20 minutes at 10,000 rpm. After this, the lowry method was proceeded with the supernatant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Dry Weight Calibration Curve for *Anabaena variabilis*

Fig. 5 shows the graph plotted between absorbance of biomass at 683 nm and dry weight of biomass for measuring its growth. Calculating the biomass concentration is easier by measuring absorbance instead of cell dry weight. Hence, the relationship between the above two factors can be established by linear regression. The precision of the relationship between absorbance and dry cell weight was found to be as R^2value of 0.9915. Hence, these O.D. values were used for calculating the biomass.

The regression equation for this graph was found to be as follows:

$y=1.19923x+0.10939$; $R^2=0.9915$

The x axis represents biomass concentration (g/l) while the y axis represents optical density of biomass at 683 nm.

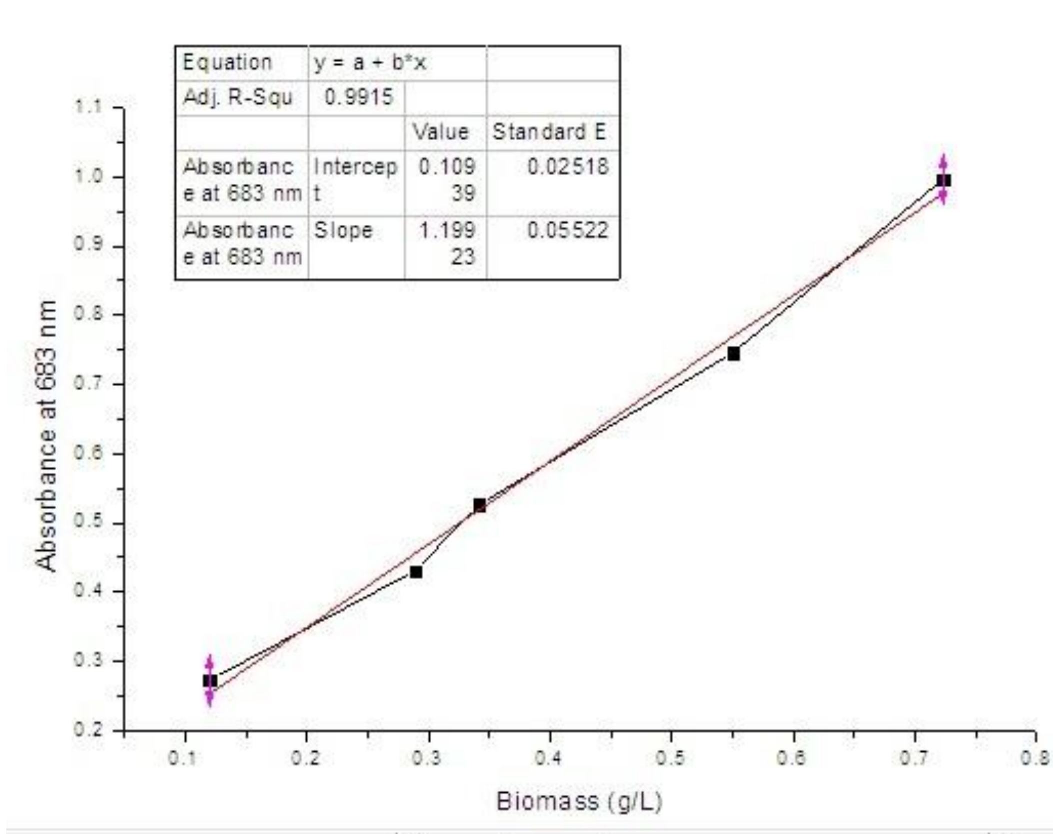

Fig. 5: Biomass dry weight as function of the absorbance

4.2 Growth of biomass at different aeration rate

Fig. 6 shows the growth of microalgal biomass with time at different aeration rates, i.e. 0.5 lpm, 1.0 lpm, and 1.5 lpm. Except aeration rate, all the other operating parameters like illumination, agitation, temperature, CO_2 concentration and pH were kept constant at 5000 lx, 120 rpm, 28 ± 2°C, 10% and 7.2 ± 0.2 respectively. *Anabaena variabilis* showed maximum growth i.e. 0.416 g/l at the input of 1.5 lpm. Initial biomass concentration in all the batches was found to be similar i.e. around 0.0594 g/l. It can be observed from **Fig. 6** that by comparing the values of 1.0 lpm and 1.5 lpm, there is not much difference in increase in biomass concentration with increase in gas flow rate. However, 0.5 lpm shows very less microalgal growth. Maximum growth of microalgae can be observed at the gas flow rate of 1.5 lpm but the specific growth rate is higher at 1.0 lpm. It can be concluded that *Anabaena* can fix CO_2 best at 1.0 lpm. Growth at both 1.0 lpm and 1.5 lpm was found to be almost same which means that minimum 1.0 lpm is required for effective microalgal growth and CO_2 sequestration. Variation in gas flow rate may affect the efficiency of CO_2 sequestration by microalgae since this process also depends upon the solubility of CO_2 into the media for easy uptake. Nutrient depletion, reduction in light availability, etc. are some of the reasons for decrease in CO_2 sequestration after a certain period. Gas flow rate can also affect the size of the bubbles as well as their residence time in the liquid phase. In the beginning, CO_2 sequestration will increase due to rapid microalgal growth but after some time it is adversely affected by the high shear stress and CO_2 levels. Hence, gas flow rate should not be kept too high i.e. less than 300 ml/min **[32].**

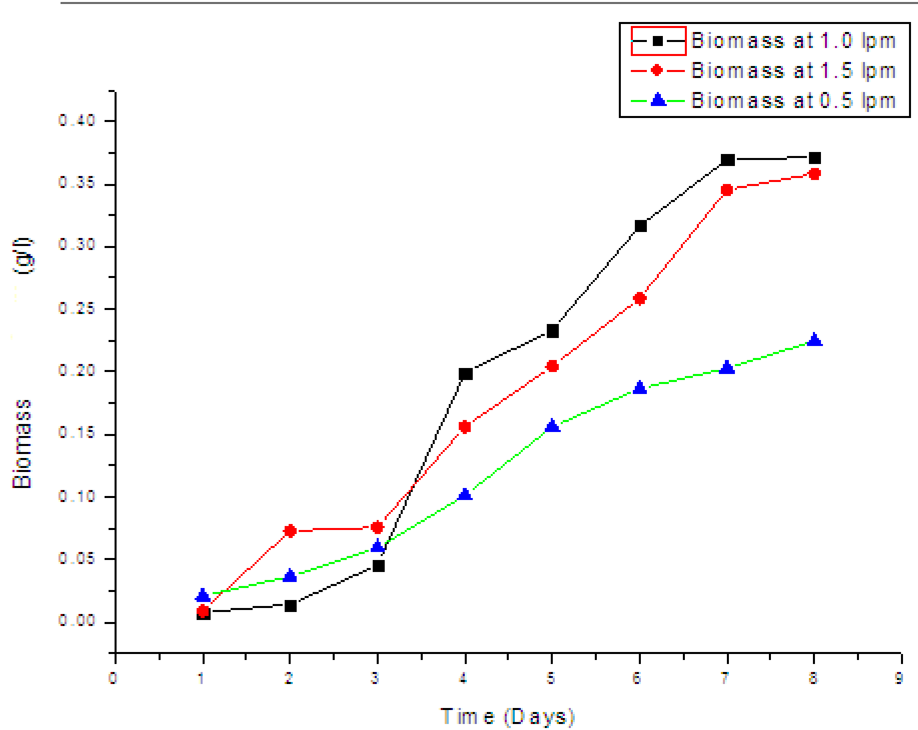

Fig. 6: Dry weight of biomass changes with time at different gas flow rates.

4.3 Total CO_2 Fixed by microalgae (assuming 50% carbon composition)

Fig. 7 shows the total CO_2 fixed at different gas flow rates by *Anabaena*. Continuous supply of CO_2 and air mixture was given for entire duration of each batch. It has been assumed that 1.83 grams of CO_2 can fix 1 gram of biomass. Therefore, the values of CO_2 fixed were determined by multiplying the biomass produced with 1.83 and then using **Equation (4)**. Here, dry carbon concentration of the microalgal sample was taken as 0.5. The maximum CO_2 fixed was found to be 0.761 grams at1.5 lpm.

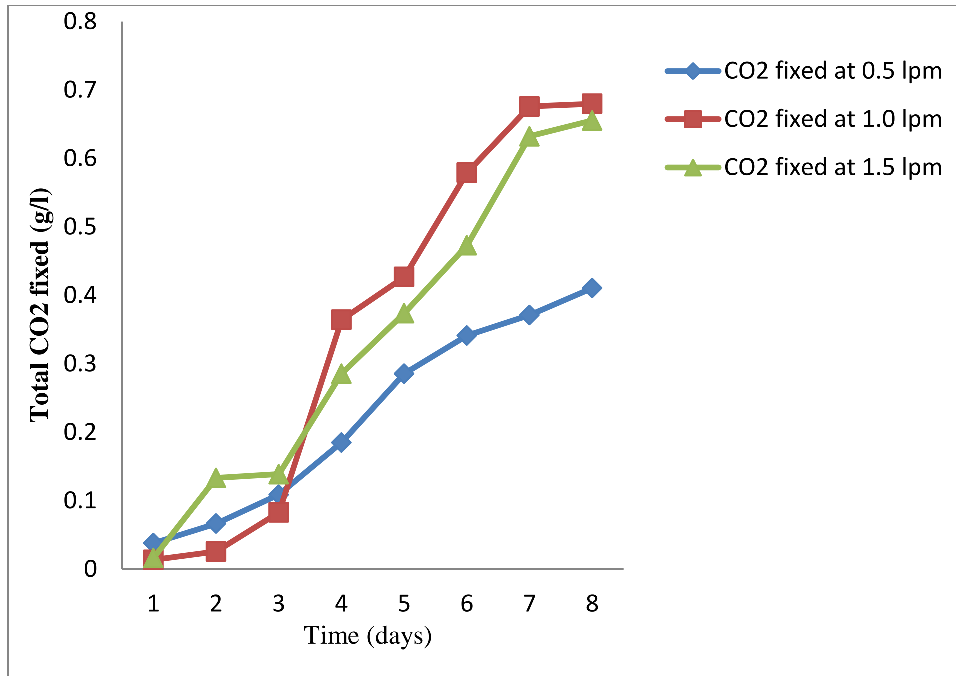

Fig. 7: Total CO_2 fixed at various gas flow rates (assuming the carbon composition as 50%)

4.4 Studies of kinetic parameters

Values of biomass (X) at different times were used along with exponential regression for calculating the maximum specific growth rate (μ_{max}, 1/day) during the exponential phase. μ_{max} of 2.100 (1/day) was found to be highest for 1.5 lpm. Similarly, the values of μ_{avg} and doubling time (t_d) were calculated to be 0.527 (1/day) and 0.33 days respectively. However, other kinetic parameters i.e. productivity, CO_2 fixation rates and total CO_2 fixed were highest at 1.0 lpm i.e. 0.052 g/l/day, 0.053 g/l/day and 0.686 g/l respectively. Although at 1.0 lpm and 1.5 lpm, all the kinetic parameters were almost same but total CO_2 fixed at 1.5 lpm was higher. All the kinetic parameters are listed in **Table 3**.

Table 3: Effect of different Gas Flow Rates on kinetic parameters of *Anabaena*

Gas flow rate (lpm)	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)
0.5	0.339	0.559	0.029	1.24	0.053	0.426
1.0	0.558	1.480	0.052	0.47	0.095	0.686
1.5	0.527	2.100	0.049	0.33	0.024	0.761

4.5 Growth kinetics using logistic equation

Logistic equation (8) was used to determine the growth kinetics by using OriginPro8 as a curve fitting tool.

$$X = \frac{X_{\max}}{1+\left(\frac{X_{\max}}{X_0}-1\right)e^{-kt}} \qquad \text{-------------------------- (8)}$$

Fig. 8 shows the predicted cell growth profile of microalgae *Anabaena variabilis* in stirred tank photobioreactor at different gas flow rates. Experimental data is fitted with the above mentioned logistic equation. X_{max} values obtained from this predicted model are listed in the **Table 4**. Here, the line represents the predicted data obtained from the actual experimental values.

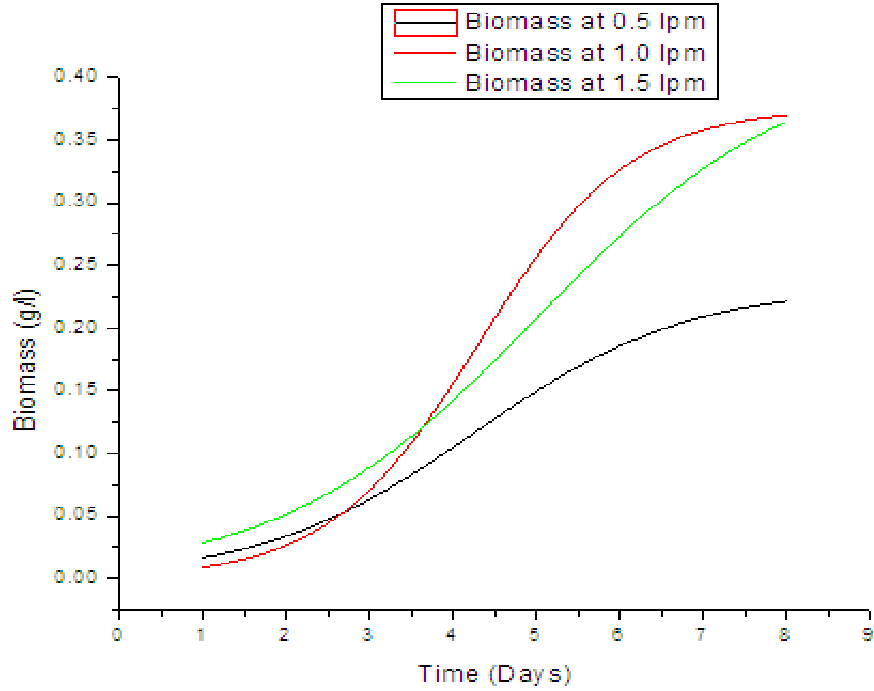

Fig. 8: Predicted cell growth profile of microalgae *Anabaena variabilis* in stirred tank photobioreactor at different gas flow rates.

The curve fitting was carried out in accordance with the actual experimental values since the R^2 values were equal to 0.99 (as shown in **Table 4**). From the predicted logistic curve, the values of X_{max} can be calculated. The best curve fitting for the experimental data is non-linear since it minimizes the sum of squared residuals.

Table 4: Some parameters used in the kinetic modeling of *Anabaena variabilis*

Gas flow rate (lpm)	$a=X_{max}$ (g/l)	c = k (1/d)	R^2 Value
0.5	0.233	0.783	0.99561
1.0	0.375	1.114	0.97113
1.5	0.416	0.651	0.97859

4.6 Protein estimation

Table 5 represents the amount of net protein present in *Anabaena* sp. The overall protein content did not appear to change much with varying gas flow rates. To estimate the protein content in microalgae, the calibration curve was plotted i.e. O.D. v/s protein concentration (µg/ml) which is shown in **Fig. 9**.

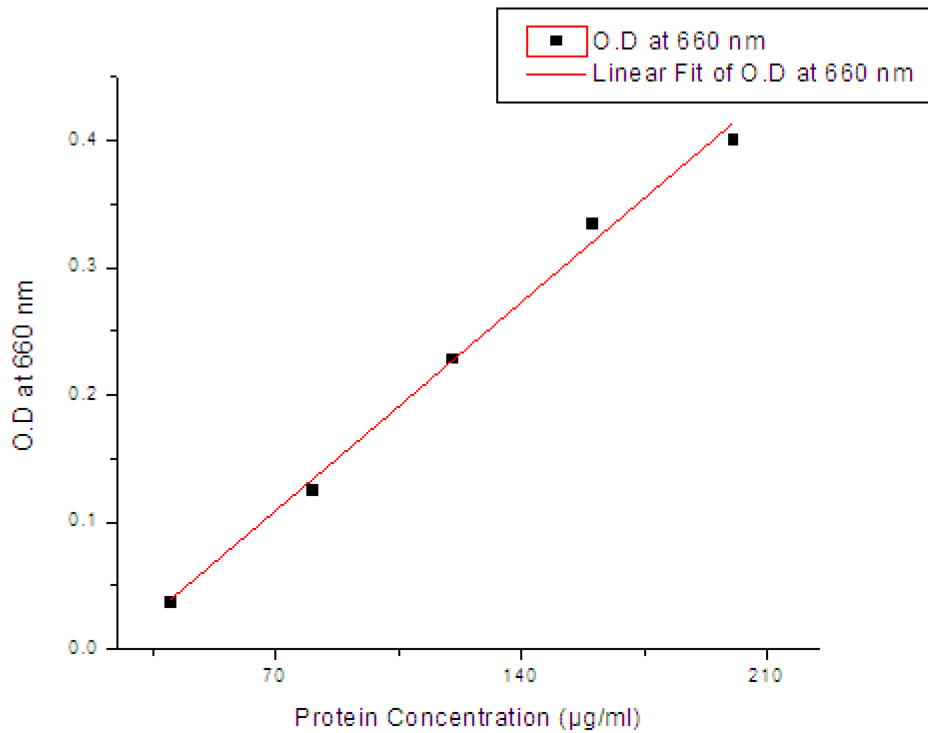

Fig. 9 Calibration curve of O.D. v/s protein concentration (µg/ml)

All the collected samples were diluted up to 1/20th fraction**. Fig. 9** follows the straight line equation $y= 0.0024x - 0.0558$

Where, y = O.D.at 660 nm and x = amount of protein obtained.

Table 5 Parameters calculated for protein estimation by Lowry's method

Gas flow rate (lpm)	**O.D. (at 660 nm)**	**Amount of protein (g/L)**
0.5	0.214	0.112
1.0	0.297	0.147
1.5	0.285	0.142

Table 5 shows that protein content was higher at 1.0 lpm. However, there is not much difference in the readings at different gas flow rates.

CHAPTER 5

CONCLUSIONS & RECOMMENDATION

5.1 Conclusions

i. The kinetic parameters have similar values for the data obtained in the batch with 1.0 lpm and 1.5 lpm as their gas flow rates. However, the values for the batch in which 0.5 lpm flow rate was provided are comparatively low.

ii. μ_{max} is highest at 1.5 lpm. However, productivity and CO_2 fixation rate are hjghest at 1.0 lpm which are 0.052 g/l/day and 0.095 g/l/day respectively.

iii. *Anabaena* can efficiently grow and sequester CO_2 even at the aeration rate of 1.0 lpm .

iv. Variation in gas flow rate does not affect the overall protein content of *Anabaena* since it remains approximately the same.

v. After few days, there is a decline in specific growth rate. It may be because of various reasons like high shear stress due to high gas flow rate, nutrient depletion, insufficient light, etc.

vi. The experimental data shows that 1.0 lpm is the most suitable gas flow rate for mass cultivation of microalgae as well as CO_2 sequestration in photobioreactor.

5.2 Recommendation

i. During the experiments gas flow rate was varied for each batch. The results may also vary if this parameter is kept constant.

ii. Photobioreactor with fixed S/V ratio was used during the experiment. Several changes in its dimensions may enable its performance to enhance considerably.

iii. In this research, effect of gas flow rate on microalgal growth and CO_2 sequestration were studied. However, other factors like illumination, media, temperature etc. can also be optimized for further refining the process.

iv. Instead of commercial CO_2 gas cylinder, other sources can also be tried like flue gas from the thermal power plants or exhaust gases from the industries, to make the process relatively inexpensive.

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