CO_2 Sequestration using Microalga *Scenedesmus obliquus* SA1 isolated from Bio-diversity Hotspot Region of Assam

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CERTIFICATE

This is to certify that **Samarpita Basu** has been working under my supervision since December 2009. We hereby forward her thesis entitled **"CO_2 Sequestration using Microalga *Scenedesmus obliquus* SA1 isolated from Bio-diversity Hotspot Region of Assam"** to be submitted for the award of the degree of Doctor of Philosophy to IIT Guwahati. I certify that she has fulfilled all the requirements according to the rules of this institute and the investigations embodied in her thesis have not been submitted elsewhere for a degree or diploma.

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ABSTRACT

Flue gases emitted from coal-fired thermoelectric plants is responsible for up to 7% (v/v) of global CO_2 emissions, about 10-15% (v/v) of the flue gases emitted from the power plants being in the form of CO_2. Increased CO_2 concentration in the atmosphere is responsible for global warming and climate change. With heavy reliance on fossil fuels, worldwide CO_2 emissions still continue an upward trend. The thesis focuses on the isolation and characterization of high CO_2 and temperature tolerant microalga capable of sequestering CO_2 from flue gas and subsequent cultivation of the microalga in bench scale open system and lab scale photobioreactor for enhanced CO_2 sequestration. Microalga strain SA1 was isolated from a freshwater body of Assam and identified as *Scenedesmus obliquus* (KC733762). At 13.8 ± 1.5% inlet CO_2 concentration and 25 °C, maximum biomass of 4.975 ± 0.003 g L^{-1} and maximum CO_2 fixation rate of 252.883 ± 0.361 mg L^{-1} d^{-1} were obtained in the lab scale closed system studies. Also, at elevated temperature (40 °C) and 13.8 ± 1.5% CO_2 supply maximum biomass value of 0.883 ± 0.001 g L^{-1} and maximum specific growth rate of 0.54 ± 0.020 d^{-1} were obtained which were 21% and 53% higher respectively than the values (0.694 ± 0.002 g L^{-1} and 0.25 ± 0.017 d^{-1}) obtained for the control culture cultivated at 25 °C under ambient CO_2 levels. The carbohydrate, protein, lipid, and chlorophyll content of the CO_2 treated SA1 obtained in the lab scale closed system studies were 30.87 ± 0.64%, 9.48 ± 1.65%, 33.04 ± 0.46 and 6.03 ± 0.19% respectively. The inlet CO_2 concentration of 13.8 ± 1.5% was reduced to 0.5% during logarithmic growth phase of SA1. The maximum biomass productivity and CO_2 fixation rate of SA1 at CO_2 concentration of 13.8 ± 1.5% was higher than most

of the relevant studies. Since the power plant flue gas contains high concentration of CO_2 (around 12-15%) and is released from the power plant at high temperature (around 40-50 ºC after the desulfurization process), tolerance of high CO_2 concentration and high temperature of 40 ºC temperature by *S. obliquus* SA1 makes it a potential strain for CO_2 sequestration from flue gases.

SA1 strain was subsequently cultivated in bench scale open system at varying CO_2 levels ranging from 0.03-35% (v⁄v) and subsequently the carbonic anhydrase activity (CA) and the biochemical properties were monitored. Open culture system was our preferred choice since it is the most suitable system for handling the large quantity of flue gas generated from thermoelectric power plants. Maximum biomass concentration (1.39 ± 0.023 g L^{-1}), CO_2 fixation rate (97.65 ± 1.03 mg L^{-1} d^{-1}) and total Carbonic anhydrase (CA) activity (166.86 ± 3.30 E.U. /mg chla) were obtained at 35% CO_2 at a culture depth of 0.17 m. The culture depth was varied at 15% CO_2 concentration from 0.0425 m to 0.17 m. Overall biomass productivity (54.33 ± 0.19 mg L^{-1} d^{-1}), CO_2 fixation rate (102.13 ± 0.36 mg L^{-1} d^{-1}) and maximum biomass productivity (156.8 ± 4.37 mg L^{-1} d^{-1}) were the highest at a culture depth of 0.085m. These values decreased with further increase in depth of the culture. This may be accounted to increased light penetration at lesser culture depth resulting in better light utilization by the algal cells leading to increased photosynthetic efficiency. As evident from literature reports, CA activity is strongly induced when algae are grown in a low-CO_2 environment. This fact was evident from our experimental finding, as CA activity of control culture (grown at ambient CO_2 concentration) > CA activity of 15% CO_2 treated culture > CA activity of 35% CO_2 treated culture for most of the experimental period. CA inhibitors: acetazolamide and

ethoxyzolamide inhibited the external and internal enzyme activity respectively in SA1, thereby confirming the presence of periplasmic (external) and intracellular CA in the SA1 strain. High CO_2 levels were favorable for the accumulation of lipids and chlorophyll in the SA1 strain the values of which were 41.17 ± 0.77% and 8.47 ± 0.15% respectively. The increased lipid content could make the SA1 strain useful in biodiesel production. Also, chlorophyll is a useful commercial pigment and is regarded as an economically valuable co-product of the CO_2 sequestration process.

Finally, the operational parameters were varied to maximize the CO_2 utilization efficiency by the SA1 strain. In these optimization studies, SA1 strain was cultivated in a lab scale cylindrical glass photobioreactor (open system) under 15% CO_2 concentration at varied operational conditions (light intensity, CO_2 sparging duration and CO_2 flow rates). At light intensity of 4351 lux, CO_2 sparging duration of 12 h per day and flow rate of 0.43 liter per hour, maximum biomass concentration of 3.32 ± 0.022 g L^{-1}, maximum specific growth rate of 1.24 ± 0.028 d^{-1}, maximum CO_2 fixation rate of 1035.25 ± 52.98 $mgL^{-1}d^{-1}$ and maximum CO_2 utilization efficiency of 10.23% were obtained which were higher than most of the relevant literature reports. These parameters were thus inferred to be the optimum condition for maximum CO_2 utilization by the microalga in lab scale photobioreactor. SA1 has high biomass productivity, fast growth rates, an attractive biochemical profile, high CO_2 fixation rates and utilization efficiency when cultivated in presence of 15% CO_2 (typical flue gas concentration). It can thus prove to be a potential candidate for CO_2 sequestration from flue gas as well as find commercial utility.

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ABBREVIATIONS AND SYMBOLS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AZ	Acetazolamide
BG 11	Blue green medium
BLAST	Basic local alignment search tool
CA	Carbonic anhydrase
CCM	CO_2 concentrating mechanism
CHN	Carbon hydrogen nitrogen
DCW	Dry cell weight
DIC	Dissolved inorganic carbon
EDTA	Ethylenediaminetetraacetic acid
EU	Enzyme Unit
EZ	Ethoxyzolamide
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GCV	Gross calorific value
GF/C	Glass microfiber filter
GHG	Greenhouse gas
IPCC	Intergovernmental panel on climate change
LPH	Liter per hour
MEA	Monoethanolamine
$NADP^+$	Nicotinamide adenine dinucleotide phosphate

NCBI	National center for biotechnology information
OD	Optical density
PBR	Photobioreactor
PS I	Photosystem I
PS II	Photosystem II
RuBisCo	Ribulose-1, 5-bisphosphate carboxylase oxygenase
SEM	Scanning electron microscope
TIC	Total inorganic carbon
TOC	Total organic carbon
μ_{max}	Maximum specific growth rate (d^{-1})
Car_{tot}	Carotenoid total (mgL^{-1})
Chl_a	Chlorophyll a **(**mgL^{-1})
Chl_b	Chlorophyll b (mgL^{-1})
Chl_{tot}	Chlorophyll total (mgL^{-1})
C_i	Inorganic carbon
K_m	Michaelis-Menten constant
P_{max}	Maximum Biomass Productivity ($mgL^{-1}d^{-1}$)
$P_{overall}$	Overall biomass productivity ($mgL^{-1}d^{-1}$)
V_{max}	Maximum rate of enzyme catalyzed reaction
X_{max}	Maximum biomass concentration (gL^{-1})

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THESIS OUTLINE

The thesis is presented in the following five chapters

Chapter I: Introduction

Chapter II: Literature Review and Objectives

Chapter III: Materials and Experimental Methods

Chapter IV: Results and Discussions

Chapter V: Conclusion and Future Scope

A brief description of the contents of each chapter is furnished below:

Chapter I: Introduction

This chapter begins with the threats imposed by global warming and climate change due to increase in CO_2 emissions from fossil fuel combustion. Various available technologies for CO_2 capture from flue gas generated from thermoelectric power plants along with their limitations have been briefly discussed. Thereafter, microalgae as agents of CO_2 sequestration and their advantages over other means of CO_2 capture have been discussed. Microalgal photosynthesis and CO_2 concentrating mechanism (CCM) along with various cultivation systems and downstream biomass applications have also been discussed.

Chapter II: Literature Review and Objectives

In this chapter the effects of various physicochemical parameters like CO_2concentration, temperature, nutrients, light and pH of the medium on microalgal biomass production have been discussed. The microalgal species employed, photobioreactors used, biomass yield and CO_2 fixation rates obtained in previous literature reports have also been discussed. The importance of mixing and aeration of CO_2 into the culture and various biomass recovery options like flocculation,

sedimentation, filtration, and centrifugation for the downstream applications have also been discussed in detail. Based on the literature survey the major objectives of the present research project were formulated.

Chapter III: Materials and Experimental Methods

Initially, freshwater sample was collected from Guwahati, Assam (26°11'15"N 91°45'4"E) and isolation of microalgae was carried out in BG-11 medium as discussed in this chapter. A constant CO_2 supply of 13.8 ± 1.5% was used during isolation. The culture conditions employed during isolation and cultivation have been discussed in detail. 28s rRNA gene sequence of the isolated microalgae was obtained from M/s Qubebioscience private Limited, Hyderabad, India. The sequencing result was submitted to GenBank database and accession number was obtained.

The detailed procedure followed for the characterization of the microalgal isolate by scanning electron microscope and FTIR have been discussed. Procedures used for determination of microalgal biomass concentration, pH and CO_2 concentration have also been covered. The experimental section covers the details of all the studies carried out in lab scale closed system, bench scale open system and during operational parameter optimization in lab scale photobioreactor (open system). Growth kinetic parameters and CO_2 fixation rates were determined in all the above studies. The protocol used for assay of carbonic anhydrase (CA) and use of inhibitors of CA activity have been discussed in detail. Detailed procedures followed for biochemical composition, CHN, calorific value, total organic carbon and total inorganic carbon analysis have also been discussed.

Chapter IV: Results and Discussions

The microalgal isolate SA1 was identified as *Scenedesmus obliquus* based on partial 28S rRNA gene sequence. SEM and light microscopic analysis revealed the unicellular, globular cell morphology of the SA1 strain. FTIR spectra of 13.8 ± 1.5% CO_2 treated SA1 showed fourteen distinct peaks over the wavenumber range 4000-900 $cm^{-1}$and control (receiving atmospheric CO_2 for growth) SA1 showed five distinct peaks in the same range. Peaks representing nucleic acids, polysaccharides, and proteins were

obtained in case of both control and CO_2 treated SA1. However, peak at 1741cm^{-1} obtained in case of the CO_2 treated SA1 was attributed to C=O stretching of lipids (esters of fatty acids) and carbonyl groups of chlorophyll pigments. This peak was not observed in case of control which indicated that lipid and pigment accumulation in the present microalgal strain was being facilitated by the supply of CO_2. SA1 strain was found to completely fix the supplied CO_2 to organic carbon in the lab scale closed system studies since the inlet CO_2 concentration of 13.8 ± 1.5% was reduced to 0.5% after 14 days of incubation as evident from Gas Chromatography analysis of inlet and outlet gas streams. SA1 tolerated 40 °C temperature and CO_2 concentration of 13.8 ± 1.5% as evident from the lab scale closed system studies. At 13.8 ± 1.5% CO_2 with nitrate and phosphate concentration of 1.5 and 0.04 gL^{-1} respectively, photoperiod regime of 14:10 h (light: dark) and 25 °C, maximum biomass (4.975 ± 0.003 gL^{-1}), maximum biomass productivity (586 ± 7.21 $mgL^{-1}d^{-1}$) and highest CO_2 fixation rate (252.883 ± 0.361$mgL^{-1}d^{-1}$) were obtained which were higher than most of the relevant studies. The optimized conditions obtained in lab scale closed system were employed in the subsequent studies. The carbohydrate, protein, lipid, and chlorophyll content of the CO_2 treated SA1 were 30.87 ± 0.64%, 9.48 ± 1.65%, 33.04 ± 0.46 and 6.03 ±0.19% respectively in the lab scale closed system studies.

SA1 strain was cultivated in bench scale open system at varying CO_2 levels ranging from 0.03-35% (v/v) and subsequently the carbonic anhydrase activity (CA) and the biochemical properties were monitored. Highest value of maximum biomass concentration (1.39 ± 0.023g L^{-1}), and maximum specific growth rate (0.64 ± 0.040 d^{-1}) were obtained at 35% CO_2concentration at a culture depth of 0.17 m. Culture depth was varied from 0.0425m to 0.17 m at CO_2 concentration of 15%. Overall biomass productivity ($P_{overall}$) (54.33 ± 0.19 $mgL^{-1}d^{-1}$), CO_2 fixation rate (102.13 ± 0.36$mgL^{-1}d^{-1}$) and maximum biomass productivity (P_{max}) (156.8 ± 4.37$mgL^{-1}d^{-1}$) were the highest at a culture depth of 0.085m.

The total carbonic anhydrase (CA) activity obtained with the cell homogenate on 15th day of cultivation for the 35% CO_2 treated SA1 was 166.86 ± 3.30 E.U./mg chla while the external (periplasmic) CA activity as determined using intact cells on the same day was found to be 101.01 ± 2.62 E.U./mg chla. Thus, intracellular CA activity was

found to be 65.85 E.U./mg chla. Since extracellular (periplasmic) CA functions to accelerate the equilibration of CO_2 and bicarbonate in alkaline medium so that CO_2 is formed, the SA1 strain possessing an extracellular CA activity of 101.01 ± 2.62 E.U./mg chla appears to utilize CO_2 as the preferred species of inorganic carbon from the medium.

SA1 strain was cultivated in a lab scale cylindrical glass photobioreactor under 15% CO_2 concentration at varied operational conditions (light intensity, CO_2 sparging duration and CO_2 flow rates). At light intensity of 4351 lux, CO_2 sparging duration of 12 h per day and flow rate of 0.43 liter per hour, maximum biomass concentration of 3.32 ± 0.022 g L^{-1}, maximum specific growth rate of 1.24 ± 0.028 d^{-1}, maximum CO_2 fixation rate of 1035.25 ± 52.98 $mgL^{-1}d^{-1}$ and maximum CO_2 utilization efficiency of 10.23% were obtained which were higher than most of the relevant literature reports. These parameters were thus inferred to be the optimum condition for maximum CO_2 utilization by the microalga in lab scale photobioreactor. 15% CO_2 treated SA1 strain in the bench scale open cultivation system exhibited an attractive biochemical profile (total carbohydrate content of 32.60 ± 1.80%, total lipid content of 31.59 ± 1.32% and total chlorophyll content of 6.32 ± 0.45%) thus bringing out the commercial potential of this strain apart from its CO_2 sequestration ability.

Chapter V: Conclusion and Future Scope

This chapter summarizes the inferences drawn from the present work and recommendations for future work. The study concluded that microalga *S. obliquus* SA1 has high biomass productivity, fast growth rates, an attractive biochemical profile, high CO_2 fixation rates and utilization efficiency when cultivated in presence of 15% CO_2 (typical flue gas concentration). It can thus prove to be ideal for CO_2 sequestration from flue gas. Further research is needed for the performance evaluation of microalgae under harsh process parameters such as high temperature and toxic pollutants in flue gas. Also, cultivation systems and photobioreactor design need to be further studied and optimized prior to using microalgae-based CO_2 fixation and biomass production in the industrial fields.

CHAPTER I

INTRODUCTION

This chapter outlines the threats imposed by increased concentration of CO_2 in the atmosphere as a result of fossil fuel combustion for energy generation and the possible ways of CO_2 mitigation. Microalgal mitigation of CO_2 has been discussed along with its advantages over other available options. Further, microalgal cultivation systems and the commercial utility of microalgal biomass after CO_2 sequestration have been briefly discussed.

1.1. Introduction

Fossil fuel combustion releases large amounts of CO_2 into the atmosphere. CO_2 is one of the main greenhouse gas (GHG), increased concentration of which can cause global warming and climate change which are matters of great concern. GHG contributes not only to global warming but also has other impacts on the environment and human life. Oceans absorb approximately one-third of the CO_2 emitted each year by human activities and as its levels increase in the atmosphere, the amount dissolved in oceans will also increase turning the water pH gradually to more acidic. This pH decrease may cause quick loss of coral reefs and marine ecosystem biodiversity with huge implications in ocean life and consequently in life on earth (Mata et al., 2010). Flue gases from power plant are responsible for more than 7% of the total world CO_2 emissions. As estimated by the IPCC criteria, the CO_2 concentration of flue gas is up to 15% (Wang et al., 2008). Thus, CO_2 capture and utilization of flue gases are

important strategies for the sustainable operation of a power plant. The United Nations promoted the Kyoto Protocol (1997) with the objective of reducing GHG by 5.2% on the basis of emissions in 1990, and more than 170 countries have ratified the protocol (Ho et al., 2011).

The available technologies for CO_2 capture either consume huge amount of energy or are not cost effective when up-scaled to commercial stage. The most common method to separate CO_2 from industrial flue gas is through chemical absorption-desorption process, in which monoethanolamine (MEA) solution is usually used to dissolve the CO_2. After the solvent reaches its maximum absorption capacity, it is heated to 100-150 ℃ to release almost pure CO_2 for storage. However, the main concern in this process is the requirement of intensive energy during regeneration of MEA solution, which is about 3.7 GJ/tonne CO_2. Another issue is that the MEA solution can easily react with SO_2 (which co-exists with CO_2 in a typical coal-fired power plant), resulting in irreversible degeneration of MEA with precipitation of stable salt. Furthermore, MEA also can react with oxygen in flue gas, producing corrosive degradation products and subsequently causing corrosion to equipments. Industrial scale CO_2 capture using solid adsorbent appears to be another promising technology due to low energy input. Examples of CO_2 adsorbents are activated carbon, zeolite 5A, Na_2CO_3, SBA-15, CaO, etc. The main disadvantage of using solid adsorbent to capture CO_2 is the need to pre-treat the flue gas before channeling to adsorber. This is because the high moisture content in the flue gas together with the presence of contaminants (e.g. SO_x and NO_x) can poison the adsorbents and thus frequent adsorbent regeneration is required. Other problems of this process include low adsorption capacity of adsorbents, requirement to create high pressure or high temperature depending on adsorber configuration and life span of adsorbents (Lam et

al., 2012). Due to such limitations of the existing CO_2 capture processes, biological CO_2 mitigation using microalgae, wherein CO_2 is biologically converted to organic matter has attracted a lot of research attention all over the world. Microalgae are fast growing microorganisms: 100 times faster than terrestrial plants and they can double their biomass in less than one day (Tredici, 2010). Due to their simple cellular structure and large surface to volume ratio, they can uptake large amount of nutrients from water sources which promotes their growth rate (Khan et al., 2009). In addition, phototrophic microalgae can convert solar energy to chemical energy with efficiency of 10-50 times greater than terrestrial plants primarily due to more chlorophyll per unit area (Raeesossadati et al., 2014). Plants are expected to contribute to only a 3-6% reduction in global CO_2 emissions (Skjånes et al., 2007). CO_2 is utilized as carbon source by microalgae during photosynthesis and is converted to different organic cell components including carbohydrates, lipids, proteins, and nucleic acids (Spolaore et al., 2006). One kilogram of algal dry cell weight utilizes around 1.83 kg of CO_2. Annually around 54.9-67.7 tonnes of CO_2 can be sequestered in raceway ponds corresponding to annual dry weight biomass production rate of 30-37 tonnes per hectares (Brennan & Owende, 2010). Algal biomass can be used for the production of biofuels (e.g. biodiesel, bioethanol, biohydrogen, which are alternative renewable fuels to existing fossil diesel and gasoline) and other commercially and scientifically important products such as medications, cosmetics and nutrious foods, representing additional benefits from the microalgal CO_2 reduction process (De Morais & Costa, 2007) (Fig. 1.1.). Microalgae can utilize CO_2 from different sources, such as atmospheric CO_2, industrial exhaust gases, or CO_2 in the form of soluble carbonates (e.g. $NaHCO_3$ and Na_2CO_3) (Kumar et al., 2010). Hence, microalgal capture of CO_2 is

thought to be more technologically feasible and microalgae can act as a strong backbone in CO_2 mitigation and production of renewable fuels.

1.2. Microalgae as Agents of CO_2 Sequestration

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure (Mata et al., 2010). Prokaryotic cells (cyanobacteria) lack membrane-bound organelles (plastids, mitochondria, nuclei, golgi bodies and flagella) and are more akin to bacteria rather than algae. Eukaryotic cells, which comprise of many different types of common algae, do have these organelles that control cellular functions, allowing it to survive and reproduce. Eukaryotic algae are categorized into a variety of classes mainly defined by their pigmentation, life cycle and basic cellular structure. The most important classes are: green algae (Chlorophyta), red algae (Rhodophyta) and diatoms (Bacillariophyta) (Brennan & Owende, 2010). Green algae and cyanobacteria (formally blue-green algae) are ubiquitously distributed throughout the biosphere and grow under the widest possible variety of conditions from aquatic (freshwater to extreme salinity) to terrestrial places. They are unique to other microorganisms due to the presence of chlorophyll and have photosynthetic ability in a single algal cell, which allows easy operation for biomass generation and effective genetic and metabolic research in a much shorter time period than conventional plants. Well defined nucleus, cell wall, chloroplast containing chlorophyll and other pigments, pyrenoid, a dense region containing starch granules on its surface, stigma, and flagella are the major components of green algae (Kumar et al., 2011). Microalgae though lacking roots, stems and leaves possess chlorophyll as their photosynthetic pigment.

As shown in Fig. 1.2., photosynthesis occurs in two main stages: light dependent reactions and light independent reactions. Light reactions occur only when the cells are illuminated, and carbon-fixation reactions, also known as dark reactions occur both in the presence and absence of light. Frequently, the following empirical equation is used to describe the overall reaction of photosynthesis:

$$6\,CO_2 + 6\,H_2O \xrightarrow{\text{Light energy}} C_6H_{12}O_6 + 6\,O_2 \qquad (1)$$

The light dependent reactions capture the light energy and convert ADP and $NADP^+$ into the energy carriers ATP and NADPH via the electron transport chain and produce oxygen. In this process, antenna complexes formed by chlorophyll and other carotenoids transfer light energy to P700 (part of photosystem I) or P680 (part of photosystem II), which are the different photochemical reaction centers located on the thylakoid membrane of the chloroplast. Excited electrons are transferred to electron acceptors, leaving the reaction center in an oxidized state. Then, the light independent reactions capture carbon dioxide and produce the precursors of carbohydrates using previously formed ATP and NADPH by the Calvin-Benson cycle (Zhao & Su, 2014).

Microalgae, however, face several challenges in acquiring CO_2 from the environment. The first challenge is presented by the properties of the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) which fixes CO_2 in the Calvin cycle, converting it into organic carbon. From Michaelis-Menten enzyme kinetics it is known that there is a hyperbolic relationship between the rate of an enzyme-catalyzed reaction and the concentration of the substrate. The rate of reaction when the enzyme is saturated with substrate is the maximum rate (V_{max}) and K_m (Michaelis-Menten constant) is the concentration of substrate which permits the

enzyme to achieve half of the maximum reaction rate (V_{max}). Thus, in photosynthesis, K_m, implies the concentration of CO_2 at which the rate of photosynthesis attains one-half of its maximum value and V_{max} implies the maximum rate of photosynthesis (Shelp & Canvin, 1980). When the rate of photosynthesis is highest, RuBisCo is fully saturated with CO_2 and has maximum catalytic capacity. However, at ambient CO_2 levels, RuBisCo can function at only about 25% of its catalytic capacity because the concentration of the substrate (CO_2) is lower than the concentration of CO_2 at which the rate of photosynthesis attains K_m. Also, ribulose-1, 5-bisphosphate and CO_2 are the substrates for RuBisCo in the Calvin cycle but the oxygenation of ribulose-1, 5-bisphosphate is competitive with the carboxylation reaction. Hence, the relatively high concentration of O_2 competes with CO_2 as a substrate for RuBisCo (Moroney & Ynalvez, 2007; Fang et al., 2012).

Also, algae often experience significant fluctuations in dissolved inorganic carbon ($C_i = CO_2 + HCO_3^-$) levels and pH, which change the availability of dissolved CO_2 and HCO_3^- for photosynthesis. At an acidic pH, the vast majority of C_i is in the form of dissolved CO_2, while at alkaline pH, C_i is mostly in the form of HCO_3^-, with dissolved CO_2 making up only a small fraction of the available C_i. Another challenge these organisms face is that the diffusion of CO_2 in an aqueous solution is 10,000 times slower than the diffusion of CO_2 in air (Moroney & Ynalvez, 2007). Algae have adapted to these challenges through the development of a CO_2 concentrating mechanism (CCM). It permits the maintenance of the photosynthetic activity at low concentration of CO_2 in the environment. Carbonic anhydrase (CA) (EC 4.2.1.1), an important component of CCM, is a zinc-containing metalloenzyme which catalyzes the inter-conversion of HCO_3^- and CO_2, i.e. $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$, a reaction which occurs spontaneously, but too slowly to meet the physiological needs of a cell

(Jaiswal et al., 2005). CAs are characterized by a diversity of structures and physiological functions, and by different locations in cells (Dudoladova et al., 2007).

1.3. Microalgae Cultivation Systems

Well-designed cultivation system may lead to significant increase of CO_2 fixation efficiency. The design of large-scale culture systems have to consider many factors, including light intensity, temperature, biology of the algae, nature of the product, mixing, aeration, source of CO_2 and sterilization (Wang et al., 2008). Mainly microalgae are cultivated either in open ponds or in closed photobioreactors. A brief comparison of these two types of cultivation system is shown in Table 1.1.

Open ponds system of microalgae cultivation, which is closest to the natural environment is quite common and is mostly applied for large scale microalgae cultivation due to its low cost and ease in operation and maintenance. It is commonly used for industrial application, to produce significant amount of products for commercial purposes at relatively low cost (Cheah et al., 2015). The types of ponds that are currently used in research and industry include raceway ponds, shallow big ponds, circular ponds, tanks and closed ponds. The location in which the pond is situated is a critical factor in determining the type of pond selected (Harun et al., 2010). The open-pond is commonly designed as 0.25 m in width, with area of 0.2-0.5 ha for commercial purpose microalgal productions. Open pond system possesses high surface area per volume ratio allowing for high CO_2 mitigation. Additionally, if the nutrient sources used is wastewater, incorporated with CO_2 supplied from flue gas, the open pond system is usually applied. However, there are certain drawbacks of open pond system such as requirement of significant land area, risk of contamination or predators and evaporative water loss. Thus, some ponds are covered with transparent

material to promote growing period of microalgae, prevent evaporation loss and facilitate CO_2 distribution. Raceway pond, an open pond system is usually shallow with 15-25 cm in depth. It is equipped with paddle wheel agitation, so as to ensure good circulation and nutrients homogenization. In addition, the flow of culture is controlled and guided with baffles placed in the flow channel. Raceway ponds are presently the most commonly used large scale cultivation system for commercial scale, used for commercial culture of *Chlorella* sp., *S. platensis*, *Haematococcus* sp., and *Dunaliella salina* (Cheah et al., 2015). Several studies have demonstrated that using microalgae for CO_2 fixation in photobioreactors (PBRs) is an effective method. In comparison with open systems, closed PBRs are characterized via regulated and well controlled cultivation, with additional benefits of low contamination risk, high CO_2 fixation efficiency, high metabolic flexibility, and controllable hydrodynamics. Such systems usually have a larger surface area (high surface/volume ratio) exposed to the light source to reduce the shadow effect, which is one of the main causes of the inhibition of microalgae in open systems. However, scaling up of most closed PBRs with a high tube length faces some serious limitations related to light utilization, biomass circulation, mass transfer (CO_2/O_2), and the control of growth parameters. Consequently, the widespread view that PBRs are more effective than open ponds has not yet been proven with large-scale system (Ho et al., 2011). The criteria for a good microalgal-CO_2 fixation PBR system are proper mixing, gas transfer, and light distribution (Chiu et al., 2009). Various closed photobioreactors are currently used for microalgae cultivation, including (1) vertical tubular systems (e.g. air-lift PBRs) (2) plate-type systems, and column systems (Ho et al., 2011). The variation in the properties of some of the PBRs and raceway pond has been briefly discussed in table 1.2. The main problem associated with the use of photobioreactors is the intensive

capital cost, which is expected to be overcome by technology development (Wang et al., 2008).

1.4. Downstream Biomass Applications

The diminishment of natural resources, such as coal and petroleum is soon to be occurred. Microalgae are known as valuable feedstock for renewable energy production due to their high growth rates and high lipid productivities (Cheah et al., 2015). Microalgal CO_2 mitigation needs to be coupled with generation of value added products for the economic feasibility and sustainability of the sequestration process. Microalgae can produce lipids, proteins and carbohydrates in large amounts over short periods of time which make them an attractive option for conversion to biofuels for energy (Farrelly et al., 2013). Firstly, the microalgal biomass needs to be harvested from broth and the methods available for the same include centrifugation, filtration, flocculation and gravity sedimentation. The method chosen depends greatly on the final product and the processes subsequently used; some processes require the algae to be completely dewatered and others do not (Ho et al., 2011).

Bioenergy from microalgal products include: biogas produced via anaerobic digestion or co-digestion of microalgal biomass, biodiesel after oil extraction and re-esterification with small-chain monoalcohols, ethanol through fermentation and liquid fuels via thermo-chemical conversions, such as pyrolysis, gasification or liquefaction. Most bioenergy studies that pertain to microalgae have focused on biodiesel production (Kumar et al., 2010). It is much cleaner than petroleum diesel with emissions of hydrocarbons, carbon monoxide and particulates greatly reduced. It is virtually free of sulphur, thereby eliminating the production of sulphur oxides. One of the biggest advantages of biodiesel is that it can be used in

existing diesel engines without modification and is suitable for blending at any ratio with petroleum diesel (Farrelly et al., 2013). The production of bioethanol from algae by fermentation involves firstly the conversion of starch to sugars using enzymes, and then the conversion of sugars to ethanol by yeast (Demirbas, 2010). However, CO_2 is released as a by-product of fermentation which must be recycled to microalgae cultivation to reduce emissions (Singh & Gu, 2010). Biogas (biomethane) production by anaerobic digestion of biomass consists mainly of methane (55-75%) and CO_2 (25-45%). It offers a solution to the problems of waste after lipid extraction while producing a gas high in energy for combustion. The spent material after anaerobic digestion can then be used as an agricultural fertilizer due to its high nutrient content (Farrelly et al., 2013).

Industrial chemicals extracted from microalgae include: glycerol, which is widely employed in food and personal care products; astaxanthin and other carotenoids, used as antioxidants and coloring agents in food, cosmetics and aquaculture; fatty acids used in cosmetics; ploy-β-hydroxybutyrate, used in plastics; and polysaccharides, such as agar, alginates and carrageenans which are employed as thickening agents for foods (Kumar et al., 2010). To date, only a few species have been successfully used for commercial applications including *Chlorella*, Dunaliella salina, *B. braunii*, *Spirulina platensis*, *Nannochloropsis*, *Arthrospira*, and *Haematococcus pluvialis* (Ho et al., 2011). At present, thousand tonnes of biomass are produced yearly for commercial application by using open pond cultivation system (Cheah et al., 2015).

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Table 1.1. A brief comparison of open and closed systems for microalgae cultivation (Harun etal., 2010; Ho et al., 2011)

Factor	Open system	Closed System
Space required	High	Low
Water Loss	High	Low
CO_2-loss	High, depending on pond depth	Low
Produced O_2 accumulation	Low due to continuous spontaneous outgassing	Buid-up requires gas exchange device
Temperature	Highly variable	Required cooling
Shear	Low	High
Cleaning	None	Required due to wall growth and dirt
Sterility	Low	High
Species control	None	Achievable
Biomass quality	Variable	Reproducible
Air pump	Built in	Built in
Automatic cooling system	None	Built in
Automatic heating system	None	Built in
Operation cost	Low	High
Scale-up	Easy	Difficult

Table 1.2. The variation in properties of different cultivation systems (Cheah et al., 2015)

Cultivation system	Cost	Scale-up	Energy Consumption	Space	Gas transfer	Light Efficiency	Growth rate
Airlift	High	Difficult	Moderate	Low	Good	High	High
Tubular	High	Difficult	High	Low	Good	High	High
Flate plate	High	Moderate	Moderate	Low	Good	High	High
Raceway pond	Low	Easy	Low	High	Fair	Low	Low

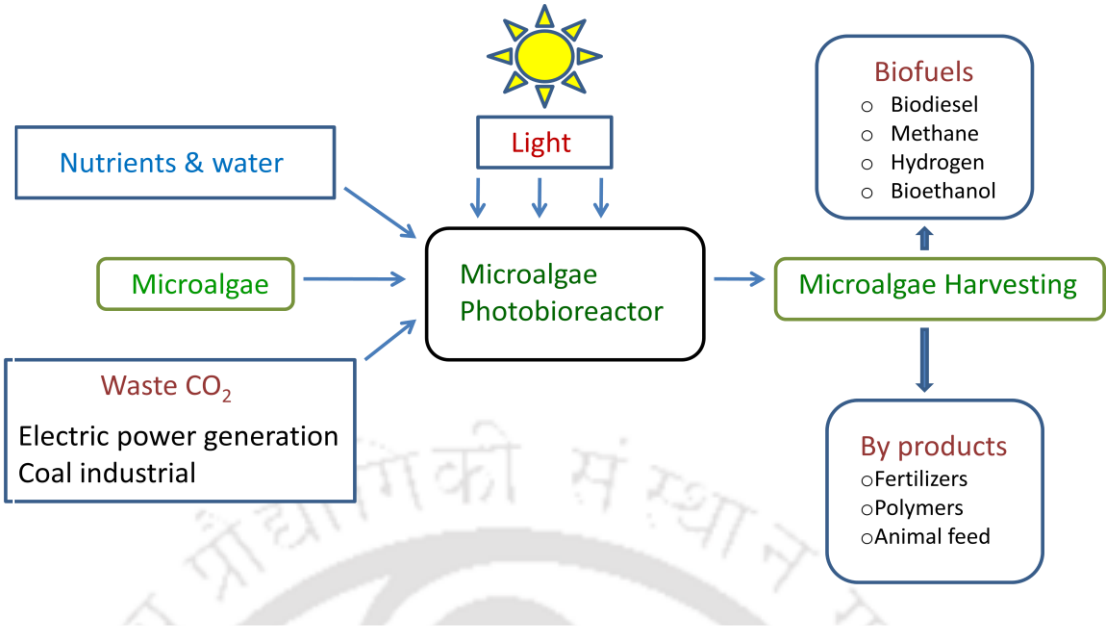

Fig. 1.1. Schematic of CO_2 mitigation in photo-bioreactor with generation of value-added products

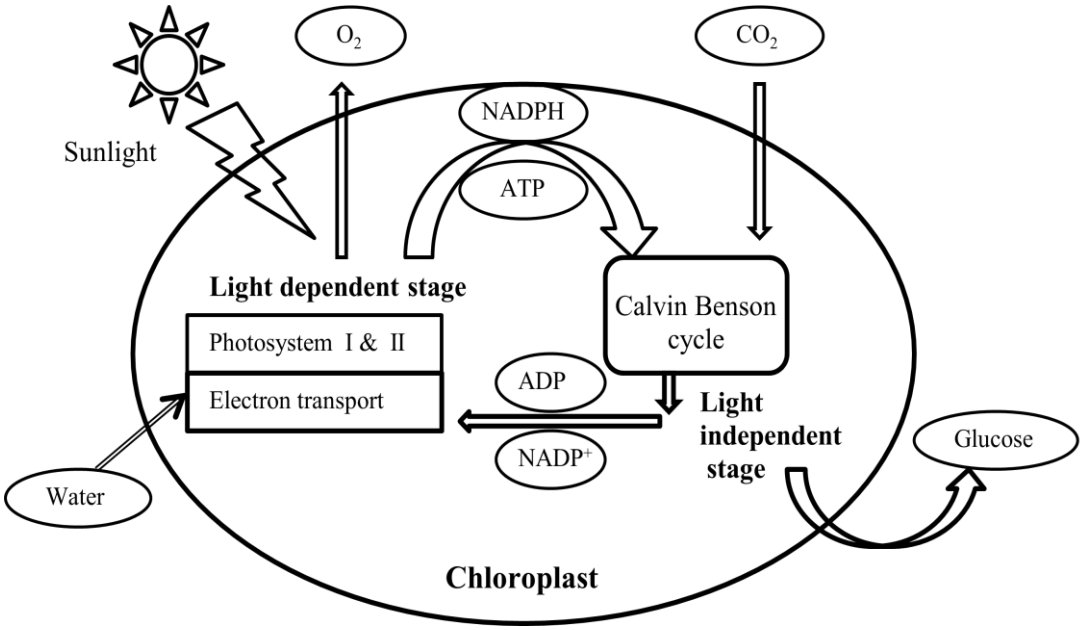

Fig. 1.2. An overview of photosynthesis

CHAPTER II

LITERATURE REVIEW AND OBJECTIVES

In this chapter, the effects of various physic-chemical parameters such as CO_2 concentration, culture temperature, light, nutrients and pH on microalgal CO_2 capture and biomass production are discussed. Also, the characteristics and kinetics of microalgal strains, and the cultivation systems employed in relevant studies have been discussed. Cultivation strategies like mixing and aeration of CO_2 into the cultivation medium and subsequent recovery of biomass for other applications have been briefly discussed. Based on the literature survey, the major objectives of the current research project have been formulated and discussed in this chapter.

2. Literature Review

2.1. Effects of Physico-Chemical Parameters on Microalgal Biomass Production

2.1.1. Effects of CO_2 concentration

Microalgal biomass production is greatly affected by the concentration of input CO_2 in the airstream. High concentration of CO_2 promotes photosynthetic efficiency of microalgae to reproduce within a shorter time and thus more quantity of microalgae biomass could be attained (Lam et al., 2012; Zhao & Su, 2014). However, it was demonstrated that the growth performance of some microalgae can be negatively affected when supplementing CO_2 higher than 1% (v/v) concentration (Cheng et al., 2006). *Chlorella* sp. could only tolerate CO_2 concentration up to 2% and further increment inhibited growth (Chiu et al., 2008). Some strains such as *Nannochloropsis oculata* grow much more effectively in 2% CO_2 than in air but above 5% CO_2 growth is suppressed (Hsueh et al., 2009). This is because high

concentration of CO_2 induced low pH in the culture medium and reduced the activity of extracellular carbonic anhydrase of microalgae which is responsible for carbon concentrating mechanism (CCM) (Tang et al., 2011). High density of cell inoculum promotes microalgae to achieve higher tolerance towards CO_2 and faster growth rate. This is because the high density of inoculums could minimize the initial lag phase resulting in immediate exponential growth of microalgae in the presence of high CO_2 concentration (Chiu et al., 2008). Most microalgae grow only at low CO_2 concentration level, and would be inhibited when CO_2 concentration level increases beyond 5% (v/v). Some microalgae can grow under higher flue gas-CO_2 concentration level (10-15%), but the carbon fixation and biomass production rate are less than that at lower CO_2 concentration (Zhao & Su, 2014). The CO_2 removal efficiency of a particular system depends on the cultivation technique, microalgal strain, cell density, light intensity, temperature, and the concentration of CO_2 in the airstream (Farrelly et al., 2013). In addition, CCM strongly regulates microalgal growth and CO_2 fixation. On increasing the concentration of CO_2, the hydrolysis of CO_2 moves to the direction of the positive reaction ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$), resulting in the increase in HCO_3^- and H^+ concentration which decrease the pH value in the medium. Low pH value may inhibit the activity of carbonic anhydrase (CA), which plays an important catalytic role in interchange between CO_2 and HCO_3^- and is regarded as an important component of the CCM. As a result, the microalgal carbon biofixation is weakened. However, some microalgae are able to adapt to the coercion of high concentration CO_2 through different methods, e.g., gene regulation and increasing the energy allocation proportion PSI/PSII. These methods can temporarily reduce the synthesis of organic carbon and simultaneously provide more ATP to maintain the pH stability inside the cell (Zhao & Su, 2014).

The carbon may be fixed in the form of protein, sugars, lipids and pigments in microalgae. Increasing the CO_2 concentration to cultivate microalgae tends to increase the accumulation of polyunsaturated fatty acid in the microalgae cells. For e.g., by supplying 50% CO_2 to cultivate *Scenedesmus obliquus*, the lipid extracted from the microalgae consisted of 76% unsaturated fatty acid, mainly linoleic acid (C18:2) and linolenic acid (C18:3). This could be because the high concentration of CO_2 supplemented resulted in relatively low concentration of O_2 in water that might affect enzymatic desaturation metabolism of microalgae and therefore, increasing the polyunsaturated content in microalgae cells. This is an added advantage because lipid containing higher concentration of unsaturated fatty acid reduces the pour point of the resulting biodiesel, making it feasible to be used in temperate countries (Lam et al., 2012).

2.1.2. Effects of temperature

Temperature is a major factor in microalgal growth, particularly cell morphology and physiology, with metabolic rates generally rising and falling with change in temperatures (Raeesossadati et al., 2014). The optimal temperature varies among microalgal species; however, optimal temperatures are also influenced by other environmental parameters such as light intensity and culture medium composition (Kumar et al., 2010) (Zhao & Su, 2014). Low temperature of culture medium is unfavorable for the enzyme activity of ribulose bisphosphate carboxylase oxygenase (RuBisCo), leading to reduction in the rate of photosynthesis. In contrast, high temperature inhibits microalgal metabolic rate and reduces CO_2 solubility. Low CO_2 solubility causes photorespiration whereby RuBisCo binds to O_2 rather than CO_2, thereby reducing carbon bioconversion rate by 20-30% (Cheah et al., 2015).

Since flue gases from point sources such as power plants have high temperatures, the use of algal strains tolerant to high temperatures would achieve significant reduction in cooling costs of the gases (Farrelly et al., 2013). According to previous studies, most of the microalgal species can tolerate temperatures between 15 °C and 26 °C. Usually, temperatures lower than 16 °C slow down microalgal growth, whereas those higher than 35 °C are usually lethal for a number of microalgal species. However, the tolerance and adaptability of some microalgae to high temperature can be improved by induced acclimation technology (Zhao & Su, 2014). The use of thermo-tolerant microalgae for CO_2 removal from hot flue gases has a major advantage in reducing production system cooling demands (Raeesossadati et al., 2014). *Chlorogleopsis* sp., a thermophilic cyanobacterial species showed maximum carbon uptake and cell concentration of 0.204 g L^{-1} d^{-1} and 1.24 g L^{-1} respectively at 5% CO_2 level and 50°C (Ono & Cuello, 2007). *Thermosynechococcus elongates* (a unicellular thermophilic cyanobacterium) grows in hot springs at temperatures of 48 °C - 55 °C. Cultures of *Thermosynechococcus elongates* were able to grow in up to 20% CO_2 and the maximum productivity and CO_2 fixation rates were 0.09 ± 0.01 and 0.17 ± 0.01 mg ml^{-1} d^{-1} respectively (Eberly & Ely, 2012). In general, at higher temperatures the available CO_2 decreases because of lower solubility in the microalgal culture. At 50 °C Henry's law constant of CO_2 in water is 1.817×10^{-2} mol/atm, while on lowering the temperature to 30°C, Henry's constant increases to 2.965×10^{-2} mol/atm, leading to higher CO_2 availability and generally higher microalgal CO_2 uptake. This is balanced with increasing metabolic rates at increasing temperatures (Raeesossadati et al., 2014). The flue gas from fossil-fired power plants is not hotter than 40 °C after desulfurization and nitrogen oxide removal (Sakai et al., 1995). Consequently, if a microalgal strain can flourish rapidly under 15% CO_2

concentration at a temperature of 40 °C, it can prove to be appropriate for CO_2 mitigation from flue gas.

2.1.3. Effects of nutrients

Cultivation medium must provide sufficient nutrients for microalgal growth and it is important to develop balanced media for optimal microalga cultivation and CO_2 fixation (Wang et al., 2008). Carbon, nitrogen and phosphorus are the three essential nutrients for biomass growth which are required for various metabolic activities (Cheah et al., 2015). Usually, in the culture medium for microalgae, nitrogen sources exist in the forms of nitrate, nitrite and ammonia salt such as urea while phosphorus exists in the forms of hydrogen phosphate and dihydric phosphate (Zhao & Su, 2014). Nitrogen is required for microalgal nutrition and, as a constituent of both nucleic acids and proteins, nitrogen is directly associated with primary metabolism of microalgae (Kumar et al., 2010). Phosphorus is the element required for photosynthesis, metabolisms, formation of DNA, ATP and cell membrane (Cheah et al., 2015). Phosphorus as a constituent element of ATP is essential to the cellular processes related to energy transfer (e.g. photophosphorylation). Photosynthesis requires large amounts of proteins (notably RuBisCo), and the proteins are synthesized by phosphorus rich ribosomes. Also, phosphorus-containing ATP/ADP are essential for photophosphorylation. As a result, phosphate starvation may have a severe impact on various aspects of microalgal metabolism, including photosynthesis and lipid accumulation (Wang et al., 2008). Appropriate concentrations of nitrogen and phosphorus are able to promote microalgal growth. Extremely low nitrogen and phosphorus concentrations cause microalgal growth inhibition while high concentration of these nutrients present toxic effects on microalgae decreasing their growth rate (Zhao & Su, 2014). Under partial nitrogen deprivation (stress condition),

microalgal growth rate diminishes but more lipids are produced (Kumar et al., 2010). An oil-rich microalgal species, *Neochloris oleoabundans* accumulated 35-54% lipids of its cell dry weight (of which 80% was comprised of triglycerides) under nitrogen deficient conditions (Kawata et al., 1998). It is important to find the balance between producing high lipid yielding cells and maintaining high biomass productivity by optimizing nitrogen in the cultivation medium (Wang et al., 2008). High N/P ratio is favorable for biomass production under conditions of atmospheric CO_2 as the carbon source. Moreover, the nitrogen source has an interactive influence with other process factors particularly with the carbon source (Zhao & Su, 2014). Other inorganic salts, sulfur and trace element like metals (Mg, Ca, Mn, Cu and Mb) and vitamins are usually added to the medium for effective photosynthetic activity (Cheah et al., 2015). Sulfur is an essential component of cysteine and methionine. In its absence, protein biosynthesis is impeded, and the photosynthetic system PS II repair cycle is blocked. Magnesium is required for nitrogenase activity. Iron is involved in electron flow from H_2O to nicotinamide adenine dinucleotide phosphate. Some trace metals play key roles in (non-cyclic) photosynthetic electron transport. For example, manganese is essential for O_2 evolution, and calcium has an important role in the thylakoid lumen in facilitating H_2O dehydrogenation and O_2 evolution (Wang et al., 2008).

Microalgae have the ability to assimilate nutrients from wastewaters for their growth thus serving as important agents of bioremediation. Algal pond could remove pollutants especially N and P, more efficiently compared to conventional activated sewage treatment ponds. However, a suitable nutrient profile of wastewater is required for successful microalgae cultivation for CO_2 sequestration (Cheah et al., 2015). Microalgae *Chlorella* sp. and *Scenedesmus* sp. are most notable in assimilating

nutrients in wastewaters, subsequently producing high biomass yield (Bhatt et al., 2014).

2.1.4. Effects of light

Light is the most influencing factor to microalgal CO_2 sequestration and biomass production due to the photoautotrophic nature of microalgae. For microalgal biofixation of CO_2 from flue gas, the light source can be divided into natural sunlight, which can be applied to both open and closed cultivation systems, and artificial cold light which is mainly applied in closed systems (Zhao & Su, 2014). The intensity and characteristics of light are important for microalgal growth. Sufficient light intensity needs to be delivered to all cells within the culture. High light intensity permits light penetration to a high-density culture (Cheah et al., 2015). The auto-shading effect of microalgal cells affects the light delivery efficiency. Thus, some microalgal cells inhibit the growth of others by reducing light availability. However, this limitation could be overcome by air delivery systems such as sparging air bubbles which increase Brownian motion uniformity of light delivery to the cells (Raeesossadati et al., 2014). With continuous increase of light intensity, microalgal photosynthesis has an increasing rate. Light intensity at maximum photosynthesis rate is called the saturated light intensity, after which the light saturated area is reached. After light-saturated area, photosynthesis can be weakened and inhibited with further increase in light intensity (photo inhibition). Similarly, the minimum limit of light-limited area is called compensated light intensity (Zhao & Su, 2014). Studies on *S. obliquus* WUST4 showed that with increasing light intensity from 6000 lux to 15000 lux, CO_2 removal rates increased, with the highest CO_2 removal ratio (67%) obtained at 12,000-13,000 lux. However, beyond 13,000 lux the CO_2 removal ratio decreased due to inhibition of the microalgal photosynthesis (Li et al., 2011). Hirata et al. (1996)

investigated the effects of sunlight, xenon lamps and fluorescent lamps on CO_2 fixation rates of *Chlorella* sp. and found that cultivation under white fluorescent lamps achieved the highest CO_2 fixation rate (0.865 g L^{-1} d^{-1}), biomass concentration (0.842 g L^{-1}) and biomass productivity (0.437 g L^{-1} d^{-1}). Thus, the characteristics of light are important for microalgal growth and CO_2 biofixation in addition to its intensity. Algae have developed several mechanisms like plants, to adjust to changes in quality and intensity of light. However, the adjustment capacities vary from one species to another. Algae with phycobilisomes may prefer low light intensities, while other algal strains (e.g., most dinoflagellates) often need higher light intensities (Razzak et al., 2013).

Apart from light intensity, proper light-dark photoperiods are required for the maintenance and growth of microalgal cultures. For a particular microalgal species, there are different growth characteristics under different light-dark periods (Zhao & Su, 2014). Some algal species (e.g. many tropical open-ocean *coccolithophorids*) may be destroyed by continuous illumination. In most algal cultures, light/dark periods of 12/12 to 16/8 hours of light to hours of darkness are required. Inappropriate hours of light to hours of darkness may lead to unwanted photoperiodic effects (Razzak et al., 2013). Thus, it is important to optimize the light intensity and photoperiod for a particular microalgal strain of interest.

2.1.5. Effects of pH

The pH of the culture media is an important factor influencing the growth of microalgae. The effect of pH on growth varies with different strains of microalgae. In most cases, freshwater eukaryotic algae prefer acidic environments (pH 5-7), whereas cyanobacteria prefer alkaline environments (pH 7-9) (Razzak et al., 2013). For

atmospheric CO_2, there is no significant change in pH of the culture medium. The pH ranges are about 7.9-8.3 for marine water and about 6.0-8.0 for freshwater. However, for post-combustion flue gas (CO_2 concentration 10-20%), pH sharply reduces to about 5.5. Usually, low pH inhibits the activity of enzyme RuBisCo and thereby leads to inhibition of microalgal growth. However, after acclimatization, some microalgal species may tolerate extremely low pH values (e.g. *Chlorella* sp. KR-1 can tolerate pH below 4, Zhao & Su, 2014). Microalgae have been shown to cause a rise in pH to 10-11 in open ponds because of CO_2 uptake (Kumar et al., 2010). Most microalgal species have their own optimal pH ranges. Kajiwara et al. (1997) obtained highest specific growth rate and final cell mass concentration at pH 6.8 using the cyanobacterium, *Synechococcus* PCC7942 sparged with 5% CO_2. Qiang et al. (1998) cultivated *Spirulina platensis* at pH 9.5 under 2% CO_2 enriched air at which the strain performed well. Kao et al. (2014) supplied flue gas to cultivate *Chlorella* sp. and observed only a slight decrease in the pH of the medium. They attributed the pH decrease to SO_2 and CO_2 present in the flue gas and obtained the lowest pH of 6.4. Thus, the minor change in pH resulting from aeration with flue gas indicated that the effect of SO_2 on microalgal growth was not significant.

2.2. CO_2 Sequestration from Flue Gas Using Different Microalgal Species

Besides the physico-chemical parameters described in section 2.1., the species of microalgae are of utmost importance in the CO_2 sequestration process. Optimal ranges and values of these parameters to achieve high CO_2 fixation rates and biomass productions are usually different for each microalgal species. Compared to atmospheric CO_2 culture process, microalgae are exposed to harsh conditions when grown in flue gas environment (such as high CO_2 concentration, high temperature and aeration ratio). These effects can be overcome by some microalgal species by

adaptation, making it possible for them to endure rigorous flue gas conditions and continue growth (Zhao & Su, 2014).

Some of the microalgae employed in CO_2 sequestration, CO_2 concentration supplemented, reactor types used for cultivation, maximum biomass yield and CO_2 fixation rates obtained have been presented in Table 2.1. *Scenedesmus* sp. and *Chlorella* sp. have been identified as promising microalgae strains for CO_2 sequestration. Depending on the microalga strain, supplement of 1-20% CO_2 is generally suitable for microalgal cultivation. Apart from high CO_2 concentration, flue gas may contain many different compounds (O_2, H_2O, CO, NO_x, SO_x, HCl, heavy metals, particulate matter etc.) depending on the source of flue gas. Among these compounds, NO_x and SO_x can cause significant negative impact towards microalgal growth due to their hazardous properties and high toxicity level. However, the effect of NO_x and SO_x towards microalgae growth is highly dependent on the type of strains. Based on most of the literature studies it can be concluded that by selecting suitable microalgae strains such as *Chlorella* sp., *Dunaliella tertiolecta*, and *Scenedesmus obliquus*, the presence of NO_x (less than 100 ppm) and SO_x (less than 50 ppm) have insignificant effects towards their growth. This is because the listed microalgae strains are well known for their high adaptability towards surrounding environment and are able to withstand extreme conditions (Lam et al., 2012). Moreover, it was found that microalgae can uptake the dissolved NO (major constituent of NO_x in flue gas) through diffusion which could become an alternative nitrogen source for microalgal growth (Nagase et al., 2001). Thus, the presence of NO_x in the flue gas may not cause any significant harmful effect towards microalgal growth. However, SO_2 (major constituent of SO_x in flue gas) concentration exceeding 60 ppm causes adverse effects towards microalgal growth such as cell membrane lipid peroxidation, bleaching of

chlorophyll and consequent damage to pigments and proteins and a drastic drop in pH of the cultivation medium. Therefore, a desulfurization unit is necessary to remove SO_2 prior to using flue gas for microalgal cultivation. It is worth mentioning that the high moisture content of flue gas is not expected to have any significant effect towards microalgae growth since water is part of the compounds used in photosynthesis (Lam et al., 2012).

2.3. Mixing and Aeration of CO_2 into the Cultivation Medium

CO_2 has low solubility in the medium, meaning the mass transfer from gaseous to liquid phases is low. This may result in lack of CO_2 distribution in the medium, which may limit microalgal growth. Optimal mixing is required to enhance CO_2 distribution and good biomass productivity (Cheah et al., 2015). Low mixing rates hamper gaseous mass transfer and might even permit biomass settling and emergence of stagnant zones, where light and nutrients are insufficiently available and anaerobic conditions will thus prevail, leading to a decrease in productivity. In addition to the physical transport of CO_2 required by microalgal photosynthesis, mixing also promotes chemical reactions between CO_2 and H^+, OH^-, H_2O and NH_3; all of these exert a great influence upon CO_2 uptake rates. These reactions are essentially reversible, so pH regulation is essential in microalgal bioreactors for control of CO_2 transfer (Kumar et al., 2010). In addition, it was also noted that mixing time/intensity is also an important factor not only for enhancing CO_2 mass transfer ability but also for improving the nutrients distribution in algal cell suspension; especially for high-cell concentration and high-viscosity culture systems (Ho et al., 2011). Mixing can be accomplished by pumping, mechanical stirring (e.g. rotation wheels, static mixer), and aeration or a combination of these means. Not all algal species can tolerate vigorous mixing. Mechanical agitation and bubble break-up often lead to

hydrodynamic stress, resulting in restriction of algal growth and metabolic activity (Wang et al., 2012). Fair mixing efficiency can be achieved by pumping; however, the associated hydrodynamic stress increases with rotation speed of pumps, or the number of passes of the microalgal suspension through the pump units. Gas injection (bubbling) produces lower hydrodynamic stress, while providing good gas transfer and reasonable mixing efficiency; however, cell damage in sparged cultures increases as the biomass concentration increases, because higher degrees of stirring are needed to maintain a high-density culture. One approach to minimize this problem is to maintain a low gas input per nozzle, so as to reduce shear stress and consequent cell damage (Kumar et al., 2010). Moreover, studies indicate that the aeration strategy has an influence, e.g. gradual increase of CO_2 supply could enhance the growth rate and CO_2 fixation rate compared to constant CO_2 supply. This is because microalgae could adapt to the new CO_2 concentration well and enhance their CO_2 tolerance with slow increase in CO_2 supply, especially under a relatively higher CO_2 concentration (Zhao & Su, 2014).

2.4. Biomass Recovery

Microalgae are typically cultured in highly diluted water suspensions and hence separating them from the culture media is needed for further analysis of the biomass. Separating algae from its culturing media is known as harvesting. Harvesting allows the drying, and further processing of microalgae to obtain the desired products (Razzak et al., 2013). The most common harvesting processes include flocculation, sedimentation, centrifugation, floatation and filtration (Kumar et al., 2010). Flocculation is used to aggregate the microalgal cells to increase the effective particle size and hence ease sedimentation, centrifugal recovery and filtration. Microalgae carry a negative charge which prevents them from self-

aggregation within suspension. The surface charge on algae can be countered by chemicals called flocculants. These cationic chemicals coagulate algae without affecting the composition and toxicity of the product. Some of the flocculants commonly in use include aluminum sulfate, ferric chloride and ferric sulfate (Harun et al., 2010). One main criterion for selecting a proper harvesting procedure is the desired product quality. Gravity sedimentation is useful for low value products, e.g. sedimentation tanks or settling ponds may be used to recover biomass from sewage based processes. On the other hand for high-value products such as recovery of algae for use as food or aquaculture applications, it is generally recommended to use continuously operating centrifuges that can process large volumes of biomass (Mata et al., 2010). Gravity sedimentation and centrifugation methods are based on Stoke's Law, i.e. settling characteristics of suspended solids is determined by density and radius of algal cells (Stoke's radius) as well as the sedimentation velocity. Centrifugation recovery process is rapid and energy intensive; biomass recovery depends on the settling characteristics of the cells, slurry residence time in the centrifuge, and settling depth. The disadvantage of the process includes high energy costs and higher maintenance requirements. However, harvesting efficiency of > 95% can be achieved. Flotation methods are based on trapping of algal cells using dispersed micro-air bubbles. Some strains naturally float at the water surface as microalgal lipid content increases. However, there is limited evidence of its technical or economic viability (Brennan & Owende, 2010). Filtration method of biomass recovery has proved to be one of the most competitive methods. Generally, filtration involves running the algal broth through filters on which algae accumulate and allow the medium to pass through the filter (Harun et al., 2010). A conventional filtration process is most appropriate for harvesting of relatively large (> 70 µm) microalgae

such as *Coelastrum* and *Spirulina*. It cannot be used to harvest algae species having dimensions < 30 µm like *Scenedesmus*, *Dunaliella* and *Chlorella*. Conventional filtration operates under pressure of suction. For recovery of smaller algae cells (< 30 µm), membrane microfiltration and ultra-filtration (a form of membrane filtration using hydrostatic pressure) are technically viable alternatives to conventional filtration. Owing to the cost for membrane replacement and pumping in larger scales of production, centrifugation may be a more economical method of harvesting algal biomass (Brennan & Owende, 2010).

2.5. Objectives of the Present Work

Thus, from the above discussion it is evident that the research studies have mainly focused on the abilities of the various algal strains to capture CO_2 under varying culture conditions namely CO_2 concentration, temperature, pH and the presence or absence of SOx and NOx. However, the information available so far is not adequate enough and thus, CO_2 reduction by use of microalgae is yet to be established in practice and made applicable for industrial use. Further investigation needs to be done to make the process of using microalgae for CO_2 reduction suitable for industrial use and also to find more suitable algal strains for the process of CO_2 sequestration from the flue gas environmental condition.

Thus, the parameters that need to be further studied include:

a. Suitability of microalgal strain(s) (growth rates and carbon-assimilation rates in flue gas environment)
b. Lab-scale and bench-scale microalgal studies in suitable photobioreactors (with respect to higher concentrations of CO_2 and high temperature)
c. Obtaining improved CO_2 utilization efficiencies by microalgae.
d. Studies of enzymes responsible for CO_2 uptake by microalgae

Moreover, it is important to isolate new microalgal strains for studying the CO_2 sequestration process. Microalgae thriving in the vicinity of power plants or in water bodies exposed to increased CO_2 levels could prove to be potential candidates for CO_2 mitigation process, since they would already possess the adaptation mechanisms and the cellular organization necessary to tolerate high CO_2 concentration, thereby facilitating CO_2 uptake.

To this end, the following objectives were established and accomplished throughout the research project:

- Isolation, identification and characterization of high CO_2 and temperature tolerant microalga
- Study of growth kinetics and carbon-assimilation rates of the isolated microalga in lab scale and bench scale
- Study of the performance of the microalga and carbonic anhydrase activity in bench scale open system under high concentration of CO_2
- Optimization of operational parameters in lab scale photobioreactor to maximize the CO_2 utilization efficiency of the microalga

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Table 2.1.

Comparison of maximum biomass yield and CO_2 fixation rate of different microalgae strains reported in literature

Microalgal species	CO_2 Concentration supplied (% v/v)	Reactor Type	Maximum Biomass yield (g L^{-1})	CO_2 fixation rate (g L^{-1} d^{-1})	Reference
Scenedesmus obliquus SJTU-3	10	Erlenmeyer flask	1.84	0.288	(Tang et al., 2011)
Chlorella pyrenoidosa SJTU-2	10	Erlenmeyer flask	1.55	0.260	(Tang et al., 2011)
Chlorella sorokiniana	10	Airlift	2.4	0.56	(Kumar & Das, 2012)
Chlorella sorokiniana	15	Airlift	2	0.54	(Kumar & Das, 2012)
Scenedesmus obliquus CNW-N	2.5	Glass made vessel	2.10	0.744	(Ho et al., 2012)
Scenedesmus obliquus CNW-N	10	Glass made vessel	2.03	0.324	(Ho et al., 2010)
Chlorella sp. NCTU-2	5	Bubble column	2.369	0.64	(Chiu et al., 2009)
Chlorella sp. NCTU-2	5	Centric tube airlift	2.534	0.73	(Chiu et al., 2009)
Botryococcus braunii	5	Fermentor	3.11	0.496	(Sydney et al., 2010)
Chlorella vulgaris LEB-104	5	Fermentor	1.94	0.251	(Sydney et al., 2010)
Dunaliella tertiolecta	5	Fermentor	2.15	0.272	(Sydney et al., 2010)
Scenedesmus sp. NIER-10060	15	Conical-ended cylindrical glass reactor	2.73	0.612	(Jin et al., 2006)
Chlorella vulgaris NIER-10003	15	Conical-ended cylindrical glass reactor	1.88	0.460	(Jin et al., 2006)

CHAPTER III

MATERIALS AND EXPERIMENTAL METHODS

This chapter contains the details of all the experimental conditions and analytical methods used. It introduces the culture medium and isolation procedure used for obtaining the CO_2 tolerant microalga from a freshwater body of Guwahati, Assam. The microalga was further characterized by SEM and FTIR as discussed here and subsequently used to capture CO_2 under varying conditions: in Erlenmeyer flasks (lab scale closed system) and in cylindrical photobioreactors (open system). Optimization of operational parameters such as light intensity, flow rates and CO_2 sparging duration were done as discussed in this chapter. Determination of microalgal growth kinetics, CO_2 fixation rate, CO_2 utilization efficiency and biochemical analysis were carried out, details of which have been discussed in this chapter. Also carbonic anhydrase activity assay, total inorganic carbon and total organic carbon estimations were performed, the detailed procedure of which have been covered in this chapter.

3.1. Chemicals and Culture Medium

The culture media used throughout the study was BG 11 medium (ATCC Medium 616) containing 1.5 g L^{-1} sodium nitrate ($NaNO_3$), 0.04 g L^{-1} dipotassium hydrogen phosphate (K_2HPO_4), 0.075 g L^{-1} magnesium sulfate ($MgSO_4.7H_2O$), 0.036 g L^{-1} calcium chloride ($CaCl_2.2H_2O$), 0.006 g L^{-1} citric acid ($C_6H_8O_7$), 0.006 g L^{-1} ferric ammonium citrate (C_6H_5+4yFexNyO_7), 0.001 g L^{-1} EDTA ($C_{10}H_{14}N_2Na_2O_8.2H_2O$), 0.02 g L^{-1} sodium carbonate (Na_2CO_3), 1 mL L^{-1} trace metal mix. The trace metal mix consisted of 2.86 g L^{-1} boric acid (H_3BO_3), 1.81 g L^{-1}

manganese chloride ($MnCl_2.4H_2O$), 0.222 g L^{-1} zinc sulfate ($ZnSO_4.7H_2O$), 0.39 g L^{-1} sodium molybdate ($Na_2MoO_4.2H_2O$), 0.079 g L^{-1} copper sulfate ($CuSO_4.5H_2O$) and 0.0494 g L^{-1} cobalt nitate ($Co(NO_3)_2.6H_2O$). Solid media was prepared by the addition of 15 g L^{-1} (1.5%) agar to the medium. Prior to use, the media was sterilized in an autoclave at 121 °C for 15 min. The pH of the media after autoclaving and cooling was 7.4. The carbon free BG 11 media used in this study was devoid of Na_2CO_3 and had a pH of 6.9. All inorganic chemicals were of analytical grade unless specified otherwise and were obtained from Merck India. The CO_2 and N_2 cylinders (purity 99.99%) were procured from Assam Air Products Pvt. Ltd., Guwahati, India.

3.2. Isolation and Identification of Microalgae

The microalgae strain was isolated from a freshwater body located in Guwahati, Assam (26°11'15"N 91°45'4"E). For microalgae isolation, 2 mL of freshwater sample collected in sterile sample bottle was inoculated into BG 11 medium in 250 mL Erlenmeyer flask and incubated at 25 °C. 14 hours of light and 10 hours of dark period was maintained during growth using daylight-type 20 W fluorescent tubes. Average light intensity maintained on the surface of the Erlenmeyer flask was 5496 lux using a Digital Light Meter (model LX-101A, Lutron, Taiwan). After 10 days of incubation, 2 mL of culture was transferred into fresh BG-11 medium without Na_2CO_3 and subjected to 13.8 ± 1.5% CO_2 continuously and incubated for two weeks. The gas flow rate into the culture medium was maintained at 8.0 LPH (1.2 LPH CO_2; 6.8 LPH N_2) using rotameters as shown in Fig. 3.1. The CO_2 treated culture was spread onto BG 11 agar plates and the single colonies obtained were sub-cultured into the same media (Fig. 3.2.). Purity of the isolates was checked using optical microscope (model CX41, Olympus, Japan). Pure cultures were sent to M/s Qubebioscience Private Limited, Hyderabad, India for 28S rRNA gene

sequencing. Homology study of the partial 28s rRNA gene sequence of SA1 was carried out using NCBI BLAST (http://www.ncbi.nlm.nih.gov). The sequencing result was submitted to GenBank database and accession number was obtained.

3.3. Characterization of the Isolate by SEM and FTIR

For scanning electron microscopic analysis, a drop of the algal sample was placed on a cover slip and air dried. It was then fixed for a period of 24 h using 4% (w/v) glutaraldehyde, rinsed in distilled water thrice and dehydrated in graded series of ethanol (30%, 50%, 75%, 85%, 95% and 100%) for 3 min followed by air drying under vacuum. The air dried film was gold coated and loaded for SEM analysis (model 1430VP, Leo, Germany) (Sadiq et al., 2011). For FTIR analysis, the algal biomass was dried and blended with potassium bromide powder and used for measurement (model IRAffinity-1, Shimadzu, Japan). A region of 4000-400 cm^{-1} was used for scanning (Phukan et al., 2011).

3.4. Determination of Microalgal Cell Concentration and pH

The cell concentration of the culture in the Erlenmeyer flask was determined regularly by measuring optical density at wavelength 685 nm (OD_{685}) using UV/Vis spectrophotometer (model Evolution 201, Thermo Scientific, USA). The dry cell weight (DCW) of microalgae biomass was obtained by filtering 5 mL aliquots of the culture through previously weighed glass microfiber (GF/C, 37 mm in diameter) filters (Whatman, England). Each loaded filter was dried at 70 °C until the weight was invariant. The dry weight of the blank filter was subtracted from that of the loaded filter to obtain the microalgae dry cell weight. The OD_{685} values were converted to biomass concentration via proper calibration between OD_{685} and dry cell weight and the conversion factor was determined (i.e., 1.0 OD_{685} approximately equals to 0.56 g

DCW L^{-1}). Aliquots were withdrawn from the CO_2 treated culture after every 72 h and the pH was determined in a digital pH meter (model pH Tutor, Eutech Instruments, Singapore) to study the variation of pH of the culture media with continuous CO_2 treatment.

3.5. CO_2 Concentration Analysis

Gas chromatography (GC) was used to determine the CO_2 concentrations in the air streams. The equipment used (model Ceres 800 Plus, Thermo Scientific, USA) was equipped with column Porapack Q and a thermal conductivity detector. The operational conditions were as follows: injector temperature of 56 °C, column temperature of 45 °C and detector temperature of 200 °C. The carrier gas, argon, was administered at a flow rate of 1.5 mL min^{-1}. The amount of CO_2 removed was determined from samples taken from the inlet and outlet gaseous phase of the system. The areas obtained using the integrator was compared with reference curves to determine the CO_2 concentrations. The flow rates of CO_2 and N_2 were measured using pre-calibrated rotameters.

3.6. Studies under Varying Culture Conditions

Microalga strain was cultivated in lab scale closed system and subsequently in bench scale open system. For the optimization of operational parameters, cultivation was carried out in a lab scale photobioreactor (open system). All the culture conditions used in these three different studies have been briefly discussed in this section.

3.6.1 Lab scale closed system studies

To determine the effect of varying nutrient concentrations on the growth kinetic parameters of the microalga, the complete BG 11 medium was modified with varying nitrate (0.5, 1, 1.5, 2, 2.5 and 3 g L^{-1}) and phosphate (0.02, 0.03, 0.04, 0.05, 0.06 and 0.07 g L^{-1}) concentrations. The light/dark-cycle of 14/10 h, temperature of 25 °C and light intensity of 5496 lux were employed. For the photoperiod variation studies, the effects of the following light/dark cycles were examined: 12/12 h, 14/10 h, 16/8 h and 24/0 h. Temperature of 25 °C and light intensity of 5496 lux were maintained. For the temperature variation studies, the effects of the following temperatures were examined: 25 °C, 30 °C, 35 °C and 40 °C. The light/dark cycle of 14/10 h and light intensity of 5496 lux were employed for the same. CO_2 was continuously supplied to these cultures, the average concentration of which was maintained at 13.8 ± 1.5% throughout the entire duration of the experiments. A control culture was maintained which received only atmospheric (0.03%) CO_2 level for growth. All the experiments were carried out till the stationary phase of growth was reached.

3.6.2. Bench scale open system studies

The microalga strain was cultivated in 5 L culture vessel (0.17 m diameter, 0.27 m length) with a culture depth of 0.17 m. Inoculum size was optimized in Erlenmeyer flasks prior to the bench scale studies. A constant inoculum size of 40 mg L^{-1} was used in each experiment (optimized value). The culture was subjected to varying concentrations of CO_2 supply, ranging from 15% to 35% and the growth of the organism was monitored periodically. A control culture was maintained which received only the ambient CO_2 (0.03%) for growth. At 15% inlet CO_2 concentration, the culture depth was varied from 0.0425 m to 0.17 m to assess the height of the

culture at which maximum CO_2 was sequestered. All the culture vessels were of open type mimicking the natural pond type algal cultivation used in practice (Fig. 3.3.a and 3.3.b). The cultures were incubated at 25 ± 1°C and illuminated with daylight-type 20 W fluorescent tubes with a light intensity of 6000 lux maintained on the surface of the culture vessels using a Digital Light Meter (model LX-101A, Lutron, Taiwan). Fourteen hours of light and 10 hours of dark period was maintained during each experiment. These growth conditions employed in our current study were optimized in our earlier study (Basu et al., 2013). The flow rates of CO_2 employed were 1.2, 2, and 2.8 liter per hour (LPH) respectively for CO_2 supply of 15%, 25%, and 35% respectively. CO_2 and N_2 from gas cylinders were mixed and the mixture gas was supplied to the culture at a flow rate of 8 LPH. The flow rates were maintained using pre-calibrated rotameters.

3.6.3. Operational parameter optimization studies

The photobioreactor (PBR) used for cultivation of the microalga strain consisted of an open cylindrical glass tube (0.05 m diameter, 0.6 m length and 0.05 m diameter, 0.8 m length). Length to diameter ratio of the working volume of the culture was varied (l/d ratio of 8, 10 and 12 were employed) (Fig. 3.4.a and 3.4.b). Since highest biomass concentration was achieved at l/d ratio 10 (0.05 m diameter, 0.5 m culture depth), all further studies were carried out using culture depth of 0.5m in the PBR having diameter 0.05m (working volume of culture media being 860 mL). The operational parameters that were studied were varying light intensities (2445, 2735, and 4351 lux), CO_2 sparging duration (6, 12, 18 and 24 h of CO_2 supply), and CO_2 flow rates (0.143, 0.43, 1.2 and 3.68 liter per hour CO_2). CO_2 and N_2 from gas cylinders were mixed and the mixture gas was supplied via bubbling from the bottom of the PBR to the culture at flow rates of 0.953, 2.86, 8 and 24.53 liter per hour (LPH)

with CO_2 flow rates of 0.143, 0.43, 1.2 and 3.68 LPH respectively to obtain a final CO_2 concentration of 15% in all the experiments. The flow rates were maintained using pre-calibrated rotameters. For the light intensity variation studies, continuous CO_2 supply (24 h CO_2) of 15% was provided at flow rate of 1.2 LPH. For the intermittent CO_2 supply studies, CO_2 was supplied at flow rate of 1.2 LPH and light intensity of 4351 lux was employed for the same. For the CO_2 flow rate variation studies, 18 h/day and 12 h/day CO_2 sparging was done and 4351 lux light intensity was employed. The cultures were incubated at 25 ± 1 °C and illuminated with daylight-type 20 W and 18W fluorescent tubes. The light intensity on the surface of the PBR was measured using a Digital Light Meter (model LX-101A, Lutron, Taiwan). Fourteen hours of light and ten hours of dark period was maintained during each experiment. Inoculum size of 40 mg L^{-1} was used in all the experiments.

3.7. Determination of Growth Kinetic Parameters and CO_2 utilization Efficiency

Time-course profile of the biomass concentration (X; g L^{-1}) was used to calculate the maximum specific growth rate (μ_{max}, d^{-1}). The maximum biomass concentration achieved was designated as X_{max} (g L^{-1}).

Overall biomass productivity ($P_{overall}$, mg L^{-1} d^{-1}) was calculated via following equation:

$$P_{overall} = \frac{\Delta X}{\Delta t} \qquad (1)$$

Where, ΔX is the variation of biomass concentration (mg L^{-1}) within a cultivation time of Δt (d)

Specific growth rate μ (d^{-1}) was calculated from the following equation:

$$\mu = \frac{\ln(X_1/X_0)}{t_1 - t_0} \qquad (2)$$

Where, X_1 and X_0 were the biomass concentration (g L^{-1}) on days t_1 and t_0 respectively.

The CO_2 fixation rate (mg L^{-1} d^{-1}) was determined from the following equation:

CO_2 fixation rate = 1.88 × Biomass productivity (mg L^{-1} d^{-1}) (3)

The CO_2 utilization efficiency (%) was estimated from the following equation (Ketheesan & Nirmalakhandan, 2012):

$$CO_2 \text{ utilization efficiency (\%)} = \frac{1.88 \times \text{Biomass productivity (g L}^{-1}\text{ d}^{-1})}{\text{Input CO}_2\text{ (g CO}_2\text{ L}^{-1}\text{ d}^{-1})} \times 100 \quad (4)$$

The typical molecular formula of microalgal biomass, $CO_{0.48}H_{1.83}N_{0.11}P_{0.01}$ (Chisti, 2007) was used. Thus the factor 1.88 is the mass of CO_2 fixed by unit biomass, considering 51.39% of carbon in the dry biomass and obtained as follows:

$$0.5139(44/12) = 1.88 \quad (5)$$

3.8. Determination of Biochemical Composition, CHN Analysis and Calorific Value of Microalga

Carbohydrate and protein estimation of the microalgal biomass were done by the Anthrone method and Lowry's method respectively (Sadasivam & Manickam, 2008). Total lipid was determined using modified protocol of Folch (Pruvost et al., 2009). Briefly, 10 mL of microalgae culture was harvested by centrifugation (model C-24 BL, Remi Instruments, India) at 4000 rpm for 10 min and the pellet was dried overnight at 65 °C. 4 mL methanol was then added to the pellet and incubated at 160 rpm for 1 h followed by addition of 8 mL chloroform and incubation at 160 rpm for 2 h. Centrifugation was done at 4000 rpm for 10 min and the supernatant containing lipids was transferred into another tube. The residue was extracted second time for 30

min with 3 mL of a mixture of methanol/chloroform (1/2) followed by centrifugation at 4000 rpm for 10 min. The supernatant was pooled with the first one and washed with 4.5 mL of a 0.88 % KCl solution. Separation in two phases was accelerated by centrifugation and the lower chloroform layer was transferred into another tube. A second washing was done with 1.5 mL of methanol. After centrifugation, the lower chloroform layer was again transferred into another tube and centrifuged at 4000 rpm for 10 mins for the complete elimination of water and any trace solids. Solvent was evaporated at 50°C, the extracted lipid was redissolved in 5 mL chloroform and transferred into a pre weighed tube and dried until constant weight. Thereafter, the weight of the crude lipid obtained was measured gravimetrically.

For chlorophyll and carotenoids extraction, two milliliters of culture were centrifuged (4400 rpm, 6 min) and 5 mL of pure methanol was added to the pellet. Samples were placed in an ultrasound bath for 5 min and incubated at 60 °C for 40 min. A temperature shock was then applied by transferring the samples to 0 °C for 15 min. After a new centrifugation step, the absorbance of the supernatant was measured in the UV/Vis spectrophotometer (Cuaresma et al., 2011). Modified Arnon's equations were used to calculate the chlorophyll and carotenoids concentrations in the extracts (Cuaresma et al., 2011):

$$\mathrm{Chl}_b = (16.72 \times A_{665} - 9.16 \times A_{652}) \times \text{dilution factor } [\mathrm{mg\,L^{-1}}] \quad (6)$$

$$\mathrm{Chl}_a = (34.9 \times A_{652} - 15.28 \times A_{665}) \times \text{dilution factor } [\mathrm{mg\,L^{-1}}] \quad (7)$$

$$\mathrm{Chl}_{tot} = \mathrm{Chl}_a + \mathrm{Chl}_b \ [\mathrm{mg\,L^{-1}}] \quad (8)$$

$$\mathrm{Car}_{tot} = \frac{\text{dilution factor} \times 1000 \times A_{470} - 1.63 \times \mathrm{Chl}_a - 104.96 \times \mathrm{Chl}_b}{221} \ [\mathrm{mg\,L^{-1}}] \quad (9)$$

The cellular content of carbohydrate, protein, lipid, chlorophyll and carotenoids were expressed as % of dry cell weight. C, H, N analysis of the dried algal pellet was carried out in an Elemental Analyzer (model EA3000, EuroVector, Italy), and the oxygen content was calculated by difference. Calorific value (CV) was determined in an oxygen bomb calorimeter (model RBC, Reico Equipments and Instruments Pvt. Ltd., India). The dried algal pellet was oxidized by combustion in presence of 300 psi oxygen under pressure.

3.9. Carbonic Anhydrase (CA) Activity Assay

CA activity was assayed electrometrically using a modified procedure of Wilbur and Anderson (1948). The sample was assayed at 3 °C by adding cells (equivalent to 200 µg Chlorophyll a) to 3 mL of HEPES buffer (pH 8.0) and sonicated for 10 min. The reaction was initiated by addition of 2 mL ice-cold CO_2 water. The time required for the pH to decrease from 8.0 to 7.0 was measured using digital pH meter (model Orion 3 Star pH Benchtop, Thermo Scientific, USA). The enzyme activity in the test sample was calculated using the equation:

$$EU = 10(T_0/T_1) \quad (10)$$

where EU is the enzyme unit, T_0 is the time required for pH change when sample is present, and T_1 is the time required for pH change when sterilized distilled water was used in place of the algal sample (Jaiswal et al., 2005). The periplasmic CA activity was measured with whole intact cells, and the total activity was determined on the cell homogenate. The intracellular CA activity was calculated by subtracting periplasmic CA activity from the total CA activity in the cell homogenate.

3.10. Inhibition of CA Activity

The specific inhibition of CA was studied in the presence of acetazolamide (AZ), a membrane-impermeant inhibitor of CA and ethoxyzolamide (EZ), a membrane-permeant CA inhibitor. The inhibitors were added to 10 mM HEPES buffer (pH 8.0) and kept in dark due to sensitivity of acetazolamide to light. Cells equivalent to 200 µg Chlorophyll *a* were harvested on day 15 of cultivation from culture receiving 35% CO_2. AZ and EZ were supplied at final concentrations from 10 to 50 µM to the cells and incubated for 30 min on ice. Subsequently, the CA activities of the samples were assayed electrometrically. Sonication was done only for the cells supplied with EZ prior to assay. The effect of the inhibitors were expressed as percent inhibition, calculated from (Weis et al., 1989):

$$100 - [(\text{CA activity in presence of inhibitor}/\text{CA activity}) \times 100] \quad (11)$$

3.11. Determination of Total Inorganic Carbon (TIC) and Total Organic Carbon (TOC)

The cell suspension obtained from the light intensity variation studies during optimization of operational parameters was harvested daily and centrifuged at 6000 rpm for 10 mins to pellet down the microalgal cells. The supernatant obtained was thereafter used for estimating the TIC and TOC. TIC was determined by the titration method as described by Sun et al. (2005). Briefly, the supernatant obtained was mixed with excess 0.1 N NaOH solution. Subsequently, 10 mL of 20% $BaCl_2$ solution was added. The solution was shaken well and $BaCO_3$ precipitate was formed. Then phenolphthalein indicator was added and the solution was titrated with 0.1N HCl till pink color disappeared. Next, methyl

orange indicator was added and again titration was done till pink color appeared. The amount of HCl consumed was noted and the TIC concentration was thereafter calculated.

TOC analysis of the supernatant was performed in TOC analyzer (model no. 1030, O-I- Analytical, Aurora, USA).

3.12. Statistical Analysis

All the experiments were carried out in triplicate, and values expressed as mean ± standard deviation (SD). Statistical analysis was carried out using OriginPro 8.0. Phylogenetic tree was inferred using Neighbor-joining algorithm in Mega 5.1.

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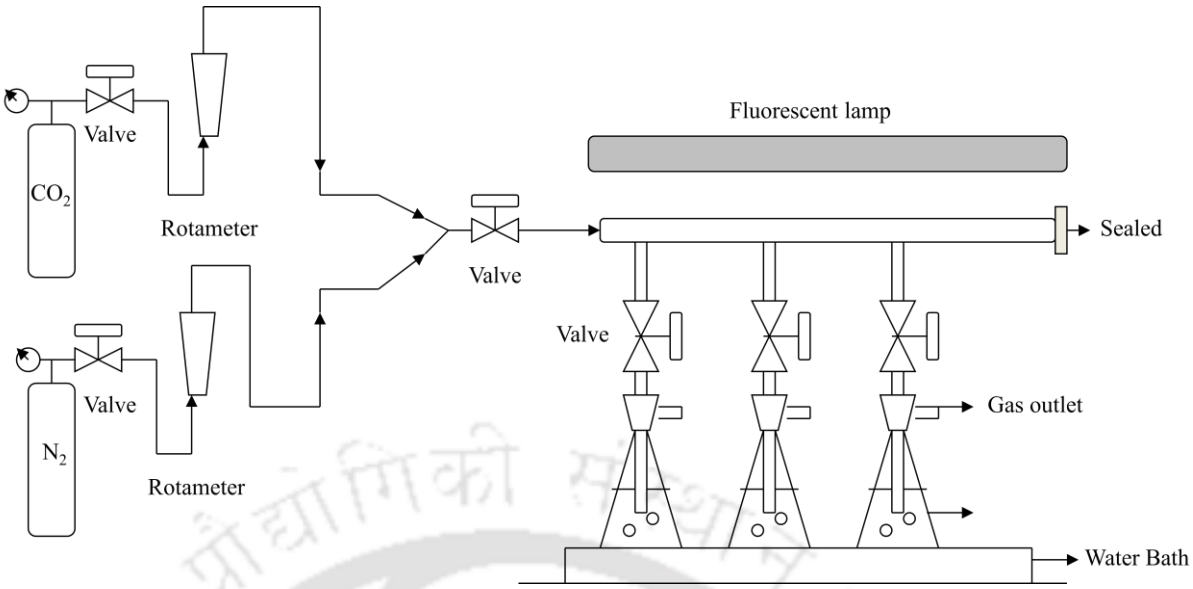


Fig. 3.1. Schematic of the experimental set up

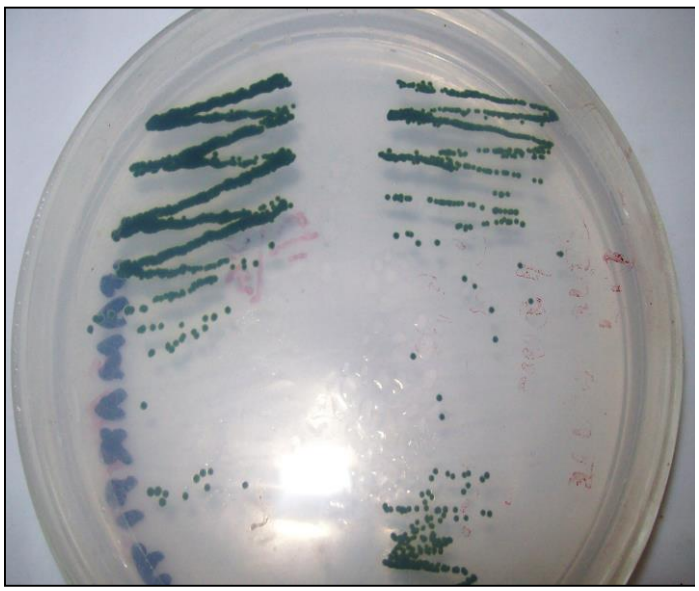
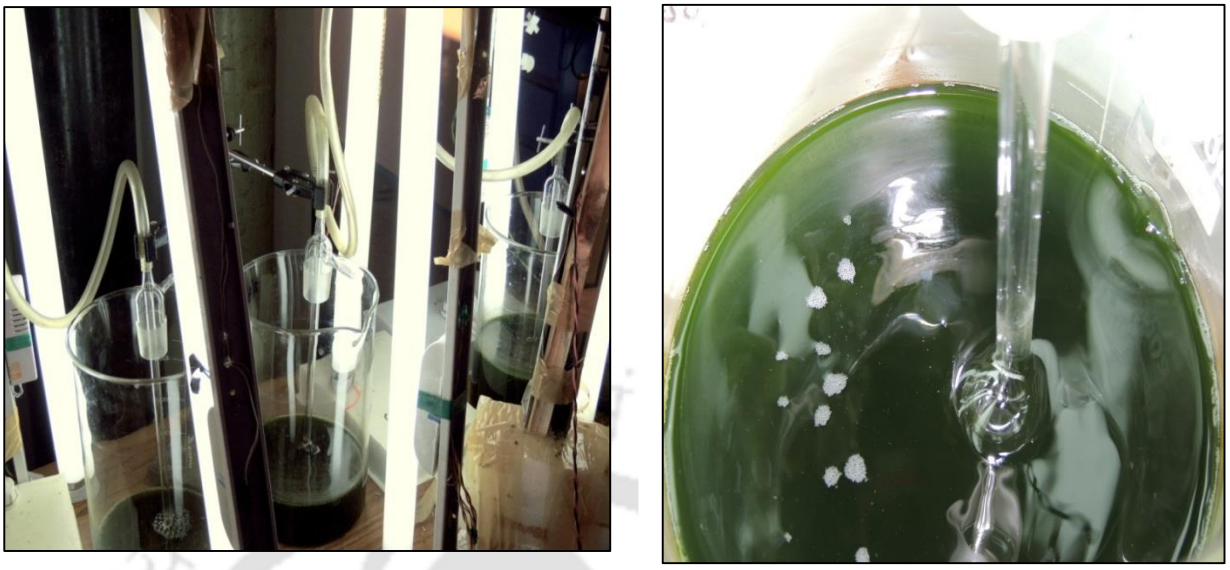
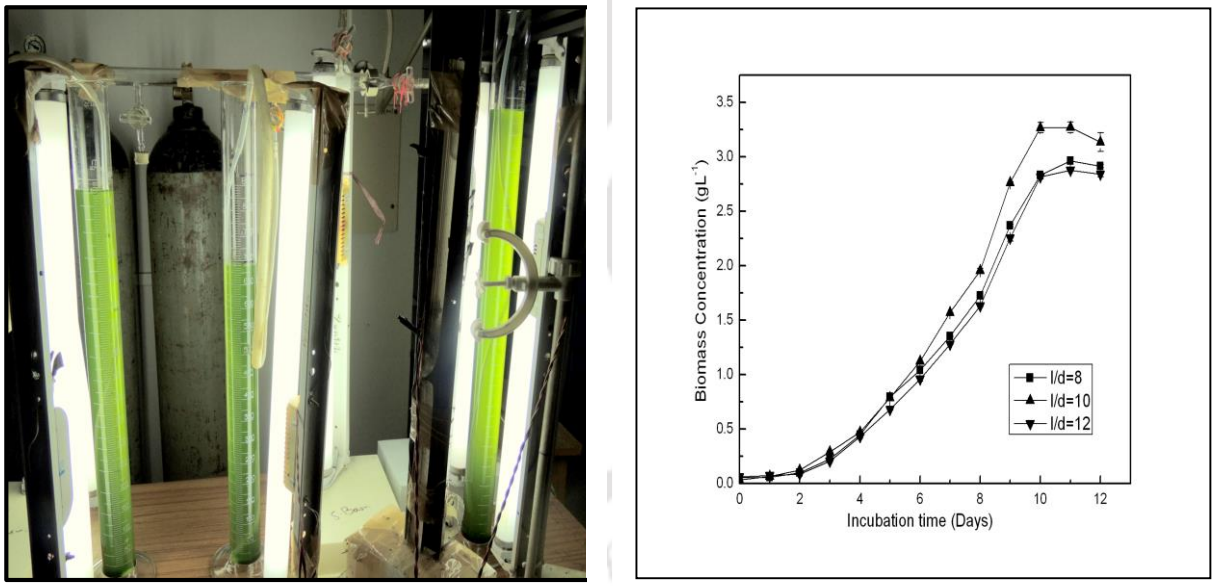


Fig. 3.2. Pure culture of microalgae



(a) (b)

Fig. 3.3. Photograph of (a) Set up used and (b) Biomass obtained in bench scale open system studies



(a) (b)

Fig. 3.4. (a) Photograph of l/d ratio variation study (b) biomass concentration obtained at varying l/d ratios

CHAPTER IV

RESULTS AND DISCUSSIONS

This chapter has been split into six divisions. Initially the isolation of a CO_2 tolerant freshwater microalga was carried out. The microalga SA1 was identified as S. obliquus by 28S rRNA gene sequencing and phylogenetic analysis. The SA1 strain was found to be tolerant to 13.8 ± 1.5 % CO_2 and 40 °C temperature and was further characterized by SEM and FTIR. The growth kinetic parameters and CO_2 fixation rate of the microalga were determined in lab scale closed system studies. Further, bench scale open system studies were carried out using microalga SA1 in presence of varying CO_2 concentrations ranging from 15% to 35%. The activity of enzyme carbonic anhydrase, the key enzyme involved in the CO_2 concentrating mechanism (CCM) in microalgae and growth kinetic parameters were studied under varying culture conditions in bench scale. The biochemical properties of the SA1 strain was studied both in closed as well as open system to bring out the commercial significance of the strain along with its CO_2 sequestration (from flue gas) potential. Finally, several operational parameters such as height of the culture, light intensity, CO_2 sparging duration and flow rate of CO_2 were optimized in open system for improving the CO_2 utilization efficiency by this SA1 strain.

4.1. Isolation of the Microalgae

The isolate SA1 was identified as *Scenedesmus obliquus* based on partial 28S rRNA gene sequence. Sequence results from BLAST searched into the GenBank databases confirmed that the obtained sequence was homologous to 28S rRNA gene of *Scenedesmus obliquus* with 96% similarity. The sequence obtained was deposited

to GenBank with the accession number KC733762. The genus *Scenedesmus* is placed in the member of the family *Scenedesmaceae*. The phylogenetic tree (Fig. 4.1.) indicated that strain SA1 fell within the evolutionary radiation occupied by the genus *Scenedesmus* and was closely related to *S. obliquus* strains YSR012 (96%), YSR007 (96%), YSR016 (96%), YSR019 (96%), YSR020 (96%), KGE 18 (96%), YSW013 (94%), and KGE 30 (95%).

4.2. SEM and FTIR Analysis

Scenedesmus obliquus SA1 is a unicellular green microalgae having globular cell morphology as evident from the SEM and light microscopic analysis (Fig. 4.2.a and 4.2.b). FTIR spectra of the SA1 strain subjected to 13.8 ± 1.5 % CO_2 treatment (lab scale closed system studies) showed fourteen distinct peaks over the wavenumber range 4000-900 cm^{-1} (Table 4.1.). A comparative study was done with the control culture of the SA1 strain which received only atmospheric CO_2 (0.03%) concentration for growth (Fig. 4.3.a and 4.3.b). For the control culture, five distinct peaks were obtained over the wavenumber range 4000-900 cm^{-1}. The peaks were assigned to specific molecular groups on the basis of published studies described previously (Dean et al., 2010; Mecozzi et al., 2011). Peaks at 3302cm^{-1} and 3344 cm^{-1} obtained in case of the CO_2 treated SA1 were attributed to OH stretching of carbohydrates, proteins, lipids (sterols and fatty acids) and nucleic acids. Peak at 1741 cm^{-1} obtained in case of the CO_2 treated SA1 was attributed to C=O stretching of lipids (esters of fatty acids) and carbonyl groups of chlorophyll pigments. This peak was not observed in case of control which indicated that lipid and pigment accumulation in the present microalgal strain was being facilitated by the supply of CO_2. Three peaks at ~ 1631 cm^{-1}, 1647 cm^{-1}, and 1654 cm^{-1} obtained for the CO_2 treated culture were associated with C=O stretching of proteins (amide I). Peak at 1654 cm^{-1} was also obtained in

case of the control. The peaks at ~1543 cm^{-1} and 1546 cm^{-1} were obtained in case of CO_2 treated SA1 and control respectively which were attributed to C-N stretching of proteins (amide II). A peak of particular interest at 1458 cm^{-1} corresponding to C=O stretching of carbonate ion was observed in case of the CO_2 treated culture only indicating that HCO_3^- might be accumulated and stored in chloroplast stroma as a result of high CO_2 supply (Chi et al., 2011). Peaks at 1381 cm^{-1}, 1384 cm^{-1} and 1404 cm^{-1} were attributed to CH bending of aliphatic groups. Peak at 1153 cm^{-1} obtained for both the CO_2 treated as well as control culture was associated with C-O-C stretching of polysaccharides. Peaks lying between 1020-1085 cm^{-1} for both the cultures were attributed to P=O of phosphodiester groups of nucleic acids and phospholipids.

4.3. Lab Scale Closed System Studies

4.3.1. Evaluation of cell biomass v/s pH

The cell concentration and pH of the culture medium being supplied with 13.8 ± 1.5 % CO_2 were monitored simultaneously over a period of 37 days as plotted in Fig. 4.4. The initial pH of the culture medium was 6.94 which gradually became alkaline with increase in biomass concentration. The biomass concentration showed a constant increase up to day 25 of cultivation reaching a value of 3.75 g L^{-1} indicating logarithmic growth phase of the microalga, and remained constant till day 28. The biomass concentration again increased till day 34 reaching the maximum value of 4.86 g L^{-1} after which it decreased to 4.56 g L^{-1} indicating decay phase of growth. The maximum biomass value of 4.86 g L^{-1} corresponded with a medium pH of 8.49 on day 34 of cultivation. It is an established fact that microalgae can uptake both CO_2 and HCO_3^- through the cell membrane leaving OH^- in the culture medium which in

turn increases the pH of the medium (Hansen, 1998). This fact was evident from our experimental finding.

Eukaryotic algae possess inorganic carbon (Ci) transporters in the chloroplast in addition to cell membrane Ci transporters, as photosynthesis occurs in the chloroplast. Once imported into the cell, CO_2 or HCO_3^- accumulates mainly as HCO_3^- in the chloroplast stroma and is transported into the thylakoid lumen and converted to CO_2 by enzyme carbonic anhydrase. The elevated concentration of CO_2 in the thylakoid lumen then diffuses through pyrenoid tubules in the thylakoid membrane to the pyrenoid matrix, where it is fixed by ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) in the Calvin cycle, converting it into organic carbon (Chi et al., 2011).

4.3.2. Analysis of CO_2 concentration

The average CO_2 concentration in the inlet gas stream was 13.8 ± 1.5% during the experimental period as determined by GC (Fig. 4.5.a). The flow rate of CO_2 was maintained at 1.2 LPH throughout the experiments. The CO_2 concentration in the outlet stream was found to be 0.5% after 14 days of incubation (Fig. 4.5.b) and the corresponding biomass value was 1.83 g L^{-1}. Thus SA1 strain was found to be completely fixing the supplied CO_2 to organic carbon subsequently leading to a very good CO_2 removal. *S. obliquus* SA1 strain can hence prove to be a potential strain for CO_2 mitigation from flue gases in small-scale (closed system).

4.3.3. Effect of varying culture conditions on the growth kinetic parameters of SA1

Biomass concentration as a function of incubation time at 13.8 ± 1.5% CO_2 concentration under different concentrations of nitrate and phosphate in the culture medium was presented in Fig. 4.6.a and 4.6.b. Nitrate and phosphate play a central

role in microalgal cell physiology and growth, the optimum concentration of which enhances the growth rate of microalgal cultures (Bhola et al., 2011). At low nitrate and phosphate concentration of 0.5 g L^{-1} and 0.02 g L^{-1} respectively maximum biomass values (X_{max}) obtained were 3.118 ± 0.003 and 3.07 ± 0.015 g L^{-1} respectively (Table 4.2.). However, these values were much higher than the control culture (containing 1.5 and 0.04 g L^{-1} of nitrate and phosphate respectively) with maximum biomass (X_{max}) of 0.694 ± 0.002 g L^{-1}. Thus, even at nutrient depleted conditions, 13.8 ± 1.5% CO_2 supply greatly enhanced cell division. With increase in nitrate and phosphate concentration, maximum biomass value increased and the maximum of 4.975 ± 0.003 g L^{-1} was obtained at a nitrate and phosphate concentration of 1.5 and 0.04 g L^{-1} respectively. The growth curve representing 0.04 g L^{-1} phosphate concentration in Fig. 4.6.b (truncated for better clarity) is the same as that obtained at 1.5 g L^{-1} nitrate concentration as shown in Fig. 4.6.a. de Morais and Costa (2007b) reported a maximum biomass concentration of 1.14 ± 0.01 g L^{-1} in *S. obliquus* receiving 12 % CO_2 after a cultivation time of 20 days. This value was 2.7 times lower than the maximum biomass (3.18 ± 0.002 g L^{-1}) attained in the present case after 20 days of cultivation at a nitrate and phosphate concentration of 1.5 and 0.04 g L^{-1} respectively. Tang et al. (2011) reported a maximum biomass value of 1.84 ± 0.01 g L^{-1} with *S. obliquus* SJTU-3 receiving 10 % CO_2 after 14 days of cultivation. The maximum biomass concentration of SA1 after 14 days of cultivation (1.907 ± 0.002 g L^{-1}) in presence of 1.5 g L^{-1} nitrate and 0.04 g L^{-1} phosphate was also 3.5% higher than the reports of Tang et al. (2011). SA1 showed an increase in all the growth kinetic parameters in terms of overall biomass productivity ($P_{overall}$), CO_2 fixation rate, maximum specific growth rate (μ_{max}) and maximum biomass productivity (P_{max}) when supplied with 13.8 ± 1.5% CO_2 (Table 4.2.). The values for

the above parameters in case of the control culture were 16.82 ± 0.05 mg L^{-1} d^{-1}, 31.62 ± 0.096 mg L^{-1} d^{-1}, 0.25 ± 0.017 d^{-1}, and 93.666 ± 0.577 mg L^{-1} d^{-1} respectively. The maximum specific growth rate (μ_{max}) of SA1 was highest (0.67 ± 0.01 d^{-1}) at 1.5 and 0.04 g L^{-1} nitrate and phosphate concentrations respectively. With increase in the nutrient concentrations, maximum specific growth rate value decreased. However, nitrate and phosphate concentrations of 3 g L^{-1} and 0.07 g L^{-1} were not inhibitory for the growth of SA1 which might prove to be beneficial if the microalga were cultivated in wastewater discharged through agricultural or industrial effluents which contain much lower amount of nitrate (~2 mg L^{-1}) and phosphate (~ 1 mg L^{-1}) salts (Kim et al., 2010). The highest maximum specific growth rate (μ_{max}) of SA1 obtained in the nutrient studies (0.67 ± 0.01 d^{-1}) was 64% higher than the findings of de Morais and Costa (2007b) who reported a maximum specific growth rate of 0.249 ± 0.021 d^{-1} in the microalga *S. obliquus* being supplied with 12% CO_2. Maximum CO_2 fixation rate of 252.883 ± 0.361 mg L^{-1} d^{-1} as well as maximum overall biomass productivity ($P_{overall}$) of 134.513 ± 0.191 mg L^{-1} d^{-1} was achieved at 2 g L^{-1} nitrate. The CO_2 fixation rate (315.71 ± 0.435 mg L^{-1} d^{-1}) obtained with SA1 strain at 2 g L^{-1} nitrate concentration after 15 days of cultivation was 20% higher than the reports by Sydney et al. (2010) who obtained a CO_2 fixation rate of 251.64 mg L^{-1} d^{-1} in *Chlorella vulgaris* LEB-104 being supplemented with 5% CO_2 after 15 days of cultivation and 13% higher than *Dunaliella tertiolecta* SAD-13.86 which showed a CO_2 fixation rate of 272.4 mg L^{-1} d^{-1} after 15 days of cultivation under 5% CO_2. Tang et al. (2011) reported CO_2 fixation rate of 246 ± 0.002 mg L^{-1} d^{-1} in *S. obliquus* SJTU-3 and 223 ± 0.002 mg L^{-1} d^{-1} in *Chlorella pyrenoidosa* SJTU-2 being supplemented with 20% CO_2 after 14 day cultivation period. The CO_2 fixation rate (293.05 ± 0.208 mg L^{-1} d^{-1}) obtained with SA1 strain at 2 g L^{-1} nitrate concentration

after 14 days of cultivation was 16% higher than that of *S. obliquus* SJTU-3 and 23% higher than that of *Chlorella pyrenoidosa* SJTU-2. Maximum biomass productivity (P_{max}) of 586 ± 7.21 mg L^{-1} d^{-1} was achieved at 1.5 g L^{-1} nitrate and 0.04 g L^{-1} phosphate concentration which was 1.5 times higher than that reported by Ho et al. (2010) who reported a maximum biomass productivity (P_{max}) of 382.7 mg L^{-1} d^{-1} using *S. obliquus* AS-6-1 in presence of 20 % CO_2 and 3.7 times higher than reports by Tang et al. (2011) who reported a P_{max} of 155 ± 0.004 mg L^{-1} d^{-1} in *S. obliquus* SJTU-3 cultivated with 10% CO_2. Higher biomass production could benefit downstream applications such as bio-energy production in addition to CO_2 fixation. Biomass concentration as a function of incubation time at 13.8 ± 1.5% CO_2 concentration, under different photoperiods and temperatures was presented in Fig. 4.6.c and 4.6.d. The light regimes to which the cultures are subjected are considered to be an important factor in the productivity and yield of photosynthetic reactions (Jacob-Lopes et al., 2009). Photosynthesis is a process comprising two steps, light reactions that only occur when the cells are illuminated, and carbon-fixation reactions, also known as dark reactions, that occur both in the presence and absence of light (Iverson, 2006). In this study, a comparison of different photoperiods on the growth kinetic parameters of SA1 was done in order to determine the most efficient light regime. As indicated in Table 4.2., 14 h light: 10 h dark regime was found to be optimum for maximum biomass production and CO_2 fixation. The maximum biomass (X_{max}) value (4.975 ± 0.003 mg L^{-1} d^{-1}) was 2.4 times higher for 14 h light: 10 h dark regime as compared to 12 h light: 12 h dark regime (2.013 ± 0.002 mg L^{-1} d^{-1}). Stationary phase was reached earliest when a light regime 24 h light was used for microalgal cultivation (Fig. 4.6. c). This might be because of the phenomenon of photo inhibition. Photo inhibition results from generally reversible damage to the

photosynthetic apparatus, as a result of excessive light (Chisti, 2007). The light period of 12 h was insufficient for biomass production and the overall biomass productivity (56.636 ± 0.124 mg L^{-1} d^{-1}) achieved was 2 times lower than that obtained using a photoperiod of 14 h light (119.863 ± 0.075 mg L^{-1} d^{-1}). The SA1 strain was gradually subjected to higher temperatures in presence of 13.8 ± 1.5% CO_2 (Fig. 4.6.d) to study the effect of increasing temperatures on the growth kinetic parameters. The maximum biomass values (X_{max}) were of the order 4.975 ± 0.003 g L^{-1} > 3.658 ± 0.001 g L^{-1} > 3.038 ± 0.001 g L^{-1} > 0.883 ± 0.001 g L^{-1} for the temperature values of 25, 30, 35 and 40 °C respectively. The SA1 strain tolerated temperatures up to 40 °C under which it showed a maximum specific growth rate (μ_{max}) of 0.54 ± 0.020 d^{-1} which was 2.16 times higher than the control culture cultivated at 25 °C. Also, the CO_2 fixation rates and overall biomass productivities ($P_{overall}$) at 40 °C were 56.966 ± 0.028 mg L^{-1} d^{-1} and 30.3 ± 0.017 mg L^{-1} d^{-1} respectively which showed 44% increase as compared to the control culture cultivated at 25 °C. The tolerance of 13.8 ± 1.5% CO_2 and 40 °C temperature by *S. obliquus* SA1 makes it a potential strain for CO_2 uptake from flue gases. Not much literature data is available on the high temperature tolerance of microalgae *Scenedesmus obliquus.*

4.3.4. Biochemical composition and CHN analysis

CO_2 treated SA1 strain was found to contain 30.87 ± 0.64% of carbohydrate, 9.48 ± 1.65% of protein, 33.04 ± 0.46% of lipid, 6.03 ± 0.19 % of total chlorophyll, and 0.02 ± 0.005 % of carotenoids. The corresponding values of the biomolecules for the control were 39.77 ± 0.67%, 12.92 ± 0.78%, 23.44 ± 1.08%, 4.92 ± 0.37% and 0.06 ± 0.02% respectively (Table 4.3.). Although the carbohydrate and protein content in the CO_2 treated SA1 strain were lower by 23% and 26% as compared to the control, lipid content and total chlorophyll content values showed 30% and 18% increase in

the CO_2 treated SA1 strain. This data corresponded well with the results obtained in FTIR analysis (Table 4.1.). The carbohydrate content obtained in case of the CO_2 treated SA1 was 47-59% higher than the reported values in other *S. obliquus* strains supplied with 2.5% CO_2 with 90% nitrogen source in the medium (Ho et al., 2012). Miranda et al. (2012) reported a carbohydrate content of 31.8 ± 0.5% in *S. obliquus* biomass and established *S. obliquus* as a good source of bioethanol production due to the high biomass sugar content. The carbohydrate content obtained for SA1 strain after CO_2 sequestration (30.87 ± 0.64%) was also high enough to make the CO_2 sequestering SA1 strain a good candidate for bioethanol production. The CO_2 treated SA1 strain was found to contain a 47-59% higher lipids as compared to reports by Ho et al (2010) in *S. obliquus* CNW-N cultivated under 20% CO_2. Thus, SA1 strain may prove to be a suitable candidate for biodiesel production due to the high lipid content. Table 4.4 shows a comparison of the pigment content of the CO_2 treated SA1 strain with other algal strains reported in literature (Bai et al., 2011). *S. obliquus* SA1 strain shows high content (6.03 ± 0.19%) of chlorophyll pigment after the CO_2 sequestration process as indicated in Table 4. Chlorophyll content in SA1 was 80% higher than the commercially used microalgae *Spirulina platensis* (1.20%), 52% higher than *Chlorella pyrenoidosa* (2.86%), and 83% higher than *Dunaliella salina* (1.01%). Chlorophyll is an effective source of nutrients that maintain human health, because its molecular form closely resembles that of hemoglobin. It is also a useful commercial pigment: it is the green dye, Natural green 3 (Bai et al., 2011). Thus *S. obliquus* SA1 could find commercial application due to its high chlorophyll content.

The CHN elementary analysis results were 44.43%, 48.20% of carbon, 6.40%, 7.08% of hydrogen, 5.74%, 6.67% of nitrogen and 43.41%, 38.03% of oxygen for the CO_2 treated and control SA1 respectively. This result was in accordance with

that obtained by Radmann et al. (2011) who reported 47.84% of carbon, 6.92% of hydrogen, 8.19% of nitrogen for *S. obliquus* cultivated with 12% CO_2.

4.4. Inoculum Size Optimization

Fig.4.7. shows the biomass concentration values obtained at different initial inoculum concentrations (13.3, 26.6, 40 and 53.3 mg L^{-1}) in presence of 13.8 ± 1.5% inlet CO_2 concentration. Highest biomass value of 4.75 ± 0.017 g L^{-1} was obtained on day 31 of incubation for 40 mg L^{-1} initial inoculum concentration. Since all the culture conditions employed during the inoculums size variation study were same (15% CO_2 supply, 25 °C culture temperature, 14 h:10 h light: dark and 6000 lux light intensity), 40 mg L^{-1} was inferred to be the optimum microalgal inoculum size and subsequently used in the bench scale and operational parameter optimization studies.

4.5. Bench Scale Open System Studies

4.5.1. Effect of varying CO_2 concentrations on growth kinetic parameters of SA1

Flue gas typically contains 12-15% CO_2. Growth studies of SA1 were carried out at varying CO_2 concentrations ranging from 0.03% to 35%. Since CO_2 was sparged at the bottom of the culture vessel, the concentration of CO_2 would gradually decline at the upper layers of the culture. Thus, the actual amount of CO_2 reaching the algal cells at the upper layers would be lower than the CO_2 concentration sparged in the medium (only intermittent stirring was provided). Hence, the CO_2 concentration was increased from 15% (flue gas concentration) to 35% for the growth parameter studies (at a culture depth of 0.17 m). The biomass concentration as a function of incubation time at varying CO_2 concentrations (ranging from 0.03% to 35%) and culture volumes at 15% concentration were plotted (Fig. 4.8.a and 4.8.b). SA1 showed an increase in maximum biomass value (X_{max}) with increase in CO_2 concentration at a culture depth of 0.17 m (Table 4.5.). Maximum biomass value (X_{max}) of 1.39 ± 0.023

g L^{-1} was obtained at 35% CO_2 concentration. This value was 2.5 times higher than the control culture (0.54 ± 0.003 g L^{-1}) which received ambient CO_2 (0.03%) for growth. The maximum specific growth rate (μ_{max}) (0.64 ± 0.040 d^{-1}) and maximum biomass productivity (P_{max}) (139.44 ± 1.12 mg L^{-1} d^{-1}) were also highest at 35% CO_2 concentration at a culture volume of 3.8 L. With *S. obliquus* SA1 cultivated at 35% CO_2, the maximum biomass value (1.39 ± 0.023 g L^{-1}) and maximum biomass productivity (139.44 ± 1.12 mg L^{-1} d^{-1}) obtained were 1.3 times and 1.7 times higher respectively than the reports of Tang et al. (2011), who reported a maximum biomass concentration of 1.03 ± 0.06 g L^{-1} and maximum biomass productivity of 81 ± 0.002 mg L^{-1} d^{-1} in *S. obliquus* SJTU-3 cultivated at 30% CO_2 concentration. The maximum biomass of 1.39 ± 0.023 g L^{-1} obtained with the SA1 strain at 35% CO_2 concentration was comparable to reports of of Ho et al. (2012) who obtained biomass concentration of 1.34 ± 0.12% using *S. obliquus* ESP-7 cultivated at 2.5% CO_2 feeding concentration. Thus, even at much higher inlet concentration of CO_2 as compared to literature reports, SA1 showed superior biomass production.

The depth of the culture in open pond system is a crucial factor since in open system there is considerable diffusion of CO_2 to the atmosphere. As the CO_2 gradually diffuses upwards in the algal pond, it is gradually used up by microalgae for photosynthesis and the remaining amount is released to the atmosphere. The culture depth was varied at 15% CO_2 concentration (typical flue gas CO_2 concentration) in the open system from 0.0425 m to 0.17m. Overall biomass productivity ($P_{overall}$) (54.33 ± 0.19 mg L^{-1} d^{-1}), CO_2 fixation rate (102.13 ± 0.36 mg L^{-1} d^{-1}) and maximum biomass productivity (P_{max}) (156.8 ± 4.37 mg L^{-1} d^{-1}) were the highest at a culture depth of 0.085m (Table 4.5.). These values decreased with further increase in depth of the culture. This may be accounted to increased light penetration at lesser culture depth

resulting in better light utilization by the algal cells leading to increased photosynthetic efficiency. de Morais and Costa (2007b) reported a maximum specific growth rate (μ_{max}) of 0.249 ± 0.021 d^{-1} and maximum biomass productivity of 76 mg L^{-1} d^{-1} in *S. obliquus* receiving 12% CO_2 concentration with a working volume of 1.8 L. The maximum specific growth rate (μ_{max}) (0.69 ± 0.049 d^{-1}) and maximum biomass productivity (P_{max}) (156.8 ± 4.37 mg L^{-1} d^{-1}) obtained for SA1 at a working volume of 1.9 L (culture depth of 0.085m) and a CO_2 concentration of 15% was 2.8 times and 2.0 times higher respectively than the reports of de Morais and Costa (2007b). SA1 thus proved to be a fast growing strain with high μ_{max} values of 0.64 ± 0.040 -- 0.70 ± 0.033 d^{-1} under high concentration of CO_2. Since the SA1 strain showed increased growth rates and biomass productivities under CO_2 concentrations ranging from 15% to 35% at large culture volume in open cultivation system, it could find potential application in open outdoor ponds.

4.5.2. Effect of varying CO_2 concentration on biochemical properties of SA1

The use of microalgae with high CO_2 concentration tolerance, fast growth rates and high CO_2 fixation rates for CO_2 sequestration process becomes economical provided the microalgal biomass obtained is a suitable feedstock for the generation of value added products. To this end, the biochemical composition of microalga strain SA1 was studied at varying concentrations of CO_2 (Table 4.6.). Microalgal biomass with high carbohydrate content could be used as feedstock for bioethanol production. Harun and Danquah (2011) used the microalgae *Chloroccum* sp. with a total carbohydrate content of 32.52% for considerable bioethanol production. The total carbohydrate content of control SA1 (37.09 ± 1.48%) and SA1 strain after 15% CO_2 treatment (32.60 ± 1.80%) were high enough to make this strain a suitable candidate for bioethanol production. Also the carbohydrate content of the 15% CO_2 treated SA1

strain (32.60 ± 1.80%) was 2 to 5 times higher than the reports of Ho et al. (2012) who reported carbohydrate content of 6.03% to 16.30% in different *S. obliquus* strains under 2.5% inlet CO_2 concentration. The total lipid content of the SA1 strain increased with increase in CO_2 concentration. The total lipid content of SA1 at 35% CO_2 concentration (41.17 ± 0.77%) was 49% higher than the control culture of SA1 (20.83 ± 2.30%). Tang et al. (2011) in their studies with *S. obliquus* SJTU-3 also found increasing trends of total lipids with increase in CO_2 concentration. Total lipid content in control culture (20.83 ± 2.30%) of SA1 was found to be 27% higher than reports of Tang et al. who reported a lipid content of 15.15% in *S. obliquus* SJTU-3 at a CO_2 concentration of 0.03%. Also total lipid obtained with the SA1 strain at higher CO_2 concentration were 39-51% higher than the reports of Tang et al. at increasing CO_2 level. Abou-Shanab et al. (2011) reported lipid content of 21 ± 1.1 % and 27 ± 1.9% in *S. obliquus* YSR04 and *S. obliquus* YSR05 respectively. The lipid content of 35% CO_2 treated SA1 strain (41.17 ± 0.77%) was 48% and 34% higher respectively than the reports of Abou-Shanab et al. (2011). The Gross calorific value (GCV) in algae which is linked to its lipid content (Phukan et al., 2011) also showed increasing trends with increase in CO_2 supply. These were as follows: 0.03% CO_2 (20.73 ± 1.24 MJ/Kg) < 15% CO_2 (23.09 ± 0.38 MJ/Kg) < 25% CO_2 (23.59 ± 0.23 MJ/Kg) < 35% CO_2 (24.46 ± 0.41 MJ/Kg). These values were higher than the value (18.59 ± 0.42 MJ/Kg) reported by Phukan et al. (2011) in *Chlorella* sp MP-1 having a total lipid content of 28.82 ± 0.72%. Total protein content in SA1 showed decreasing trends with increase in CO_2 concentration as follows: 0.03% CO_2 (10.22 ± 1.76%) > 15% CO_2 (6.71 ± 0.40%) > 25% CO_2 (4.47 ± 1.04%) > 35% CO_2 (3.76 ± 1.32%). The total chlorophyll content however increased with increase in CO_2 levels as follows: 0.03% CO_2 (3.83 ± 0.27%) < 15% CO_2 (6.32 ± 0.45%) < 25% CO_2 (7.29 ± 0.27%) < 35%

CO_2 (8.47 ± 0.15%). The increase in chlorophyll content with increase in CO_2 concentration implied that high CO_2 levels stimulated SA1 to synthesize chlorophyll to enhance the photosynthetic efficiency for subsequent conversion of CO_2 into carbohydrates. Our result corresponded with the study of Chiang et al. (2011) who reported an increase in chlorophyll content in the cyanobacteria *Anabaena* sp. CH1 from 1.55 ± 0.15% to 2.08% ± 0.11% with increase in CO_2 level from 5% to 15% respectively. Pigments like chlorophyll are high-value contents of algae cell regarded as economically valuable co-products of CO_2 sequestration process. Chlorophyll is an effective source of nutrients that maintain human health, because its molecular form closely resembles that of hemoglobin. It is also a useful commercial pigment: it is the green dye, Natural Green 3 (Bai et al., 2011). The chlorophyll yield of SA1 at 15% CO_2 concentration (6.32 ± 0.45%) was 2.5 times and 5.2 higher than chlorophyll content in the commercially used microalga *Chlorella pyrenoidosa* (2.48%) and the cyanobacterium *Spirulina platensis* (1.20%) respectively (Bai et al., 2011). Thus, SA1 can prove to be economical for CO_2 mitigation due to its potential for generation of value added products from the biomass.

4.5.3. Effect of varying CO_2 concentration on total CA activity

Fig.4.9. depicts the CO_2 concentrating mechanism (CCM) in eukaryotic algae. The working model for the CCM in unicellular green algae includes two different pathways that may or may not be present in the same algae. In some organisms, periplasmic carbonic anhydrase (CA) enhances the uptake of CO_2 from external HCO_3^- as CO_2 enters the cell. At intracellular pH, most of the CO_2 is converted to HCO_3^- at equilibrium, which is then actively concentrated in the chloroplast by a proposed HCO_3^- transporter at the chloroplast envelope. In other organisms, an ATP-dependent transporter has been proposed for HCO_3^- uptake at the plasmalemma as

well as the chloroplast envelope (Ghoshal et al., 2002). A pH gradient is set up across the chloroplast thylakoid membranes in the presence of light. The chloroplast stroma has a pH close to 8.0, whereas the thylakoid lumen has a pH around 4 to 5. Under these conditions, HCO_3^- is the predominant species of inorganic carbon (C_i) in the chloroplast stroma while CO_2 is the most abundant form of C_i in the thylakoid lumen. HCO_3^- transported into the acidic thylakoid lumen is rapidly converted to CO_2 by CA present in the thylakoid lumen thus elevating the CO_2 concentration above ambient levels and these CO_2 molecules are thereafter fixed by RuBisCo located in the pyrenoid for further formation of the organic compounds. Thus, the production and concentration of CO_2 in the acidic thylakoid lumen and thereafter fixation of these CO_2 molecules by RuBisCo increases the rate of photosynthesis. Since HCO_3^- cannot rapidly cross biological membranes, a transport protein or complex present at the thylakoid membrane facilitates the entry of HCO_3^- into the thylakoid lumen. Light driven photosynthetic electron transport is required to set up the mentioned pH gradients and also provides the H^+ ions for HCO_3^- to CO_2 conversion in the thylakoid lumen (Moroney & Ynalvez, 2007). Several isoforms of CA may exist which are localized in the periplasmic space, cytosol, chloroplast stroma and thylakoid lumen depending on the microalgal species which function in the CCM (Ghoshal et al., 2002).

The total CA activity was monitored periodically for SA1 receiving ambient (0.03%), 15% and 35% CO_2 for growth (Fig. 4.10.). The CA activity in the control culture receiving ambient CO_2 was higher than the CA activities of the 15% and 35% CO_2 treated culture throughout the incubation period except for days 15 and 17 of cultivation. Also, culture receiving 15% CO_2 for growth mostly showed higher CA activity than the 35% CO_2 treated culture. Previous reports (Aizawa & Miyachi, 1986)

indicate that the activity of CA in low-CO_2 cells is higher than that in high-CO_2 cells, while no difference has been confirmed in the activities of other photosynthetic enzymes between low- and high-CO_2 cells. Also induction of CA is regulated at transcriptional level and is strongly induced when algae are grown in a low-CO_2 environment (Jaiswal et al., 2005). Our result was in accordance to these reports as CA activity in the control culture was much higher than the CO_2 treated cultures except on day 15 and 17. The biomass concentration for control culture, 15% CO_2 and 35% CO_2 treated culture on day 15 were 0.27 ± 0.002 g L^{-1} < 0.31 ± 0.007 g L^{-1} < 0.94 ± 0.041 g L^{-1} respectively and the corresponding CA activities on the same day were 104.93 ± 2.45 E.U./mg chla < 145.68 ± 3.90 E.U./mg chla < 166.86 ± 1.90 E.U./mg chla respectively. During log phase (day 15), biomass concentration in 15% and 35% CO_2 treated culture were 13% and 71% higher than control respectively. During this period, the dissolved inorganic carbon (DIC) in the culture medium (comprised of CO_2, HCO_3^- and CO_3^{2-}) might have decreased due to huge amount of algal biomass generated and hence there was a subsequent increase in CA activity to facilitate the diffusion of DIC from outside the cells to the site(s) of the carboxylation reaction. As the biomass values gradually became more stable, the CA activity of the control increased as compared to the CO_2 treated cultures.

The total CA activity obtained with the cell homogenate on 15th day of cultivation for the 35% CO_2 treated SA1 was 166.86 ± 3.30 E.U./mg chla while the external (periplasmic) CA activity as determined using intact cells on the same day was found to be 101.01 ± 2.62 E.U./mg chla. Thus, intracellular CA activity was found to be 65.85 E.U./mg chla. Wang et al., 2006 obtained intracellular CA activities of 64.09 ± 5.83 E.U./mg chla, 61.55 ± 3.11 E.U./mg chla, 38.02 ± 2.03 E.U./mg chla and 19.99 ± 1.56 E.U./mg chla in the four cyanobacterial species, namely *Microcystis*

aeruginosa, *Microcystis viridis*, *Microcystis wesenbergii and Microcystis* sp. 573 respectively. CA activity obtained with a suspension of intact cells is due to the enzyme being located outside the plasmalemma, either in the periplasmic space or attached to the cell wall (Fig. 4.9.) (Aizawa & Miyachi, 1986). Since extracellular (periplasmic) CA functions to accelerate the equilibration of CO_2 and bicarbonate in alkaline medium so that CO_2 is formed (Fig. 4.9.), the SA1 strain possessing an extracellular CA activity of 101.01 ± 2.62 E.U./mg chla appears to utilize CO_2 as the preferred species of C_i from the medium.

4.5.4. Effect of inhibitors on CA activity

The effect of acetazolamide (AZ), a membrane-impermeant CA inhibitor was used to confirm the presence of periplasmic (external) CA, while ethoxyzolamide (EZ), a membrane-permeant CA inhibitor was used to confirm the presence of intracellular CA. AZ was added to a suspension of intact cells at increasing concentrations which inhibited the periplasmic (external) CA activity and the CA activities (E.U./mg chla) obtained were as follows: 94.87 ± 1.56(10 µM concentration) > 91.56 ± 1.40 (20 µM concentration) > 87 ± 2.51 (30 µM concentration) > 78.97 ± 3.04 (40 µM concentration) and 60.59 ± 2.16 (50 µM concentration). The membrane-permeant CA inhibitor, ethoxyzolamide (EZ) was added to the cell suspension at increasing concentrations prior to obtaining the cell homogenate and the CA activities (E.U./mg chla) obtained were as follows: 116.69 ± 4.21 (10 µM concentration) > 107.50 ± 2.41 (20 µM concentration) > 95.02 ± 2.92 (30 µM concentration) > 66.82 ± 2.35 (40 µM concentration) and 55.2 ±1.94 (50 µM concentration) (Fig. 4.11.a). AZ caused 40.01± 2.13% inhibition at 50 µM concentration, while 66.92 ± 1.16% inhibition was obtained with EZ at the same concentration (Fig. 4.11.b). Since AZ cannot penetrate into the cells and inhibits only

periplasmic (external) CA activity, while EZ penetrates into the cells and inhibits both external and internal CA (Mercado et al., 1998), higher inhibition of CA activity was obtained with EZ while the presence of both extracellular and intracellular CA were also confirmed in *S. obliquus* SA1.

4.6. Operational Parameter Optimization Studies

4.6.1. Effect of varying operational parameters on growth kinetic parameters of SA1

The operational parameters were varied to investigate the most suitable conditions for efficient CO_2 sequestration by microalga SA1 from flue gas (containing CO_2 in the range of 10-20%). Varying light intensities (2445, 2735 and 4351 lux) were employed and 15% CO_2 was continuously fed into the microalgal culture at a flow rate of 1.2 liter per hour (LPH). Light intensity controls photosynthetic growth in any microalgal system. It also affects CO_2 removal rates, biomass concentration and overall growth rate (Raeesossadati et al., 2014). In the present study it was found that SA1 showed improvement in all the growth kinetic parameters with increase in light intensity (Table 4.7.). The biomass concentration as a function of incubation time at varying light intensities were plotted (Fig. 4.12.a). The maximum biomass concentration (X_{max}) (3.15 ± 0.065 g L^{-1}) obtained at light intensity of 4351 lux was 38% higher than X_{max} obtained at 2445 lux (1.93 ± 0.009 g L^{-1}) and 17% higher than X_{max} obtained at 2735 lux (2.61 ± 0.015 g L^{-1}). Overall biomass productivity ($P_{overall}$), maximum specific growth rate (μ_{max}), maximum biomass productivity (P_{max}), maximum CO_2 fixation rate and maximum CO_2 utilization efficiency were highest at light intensity of 4351 lux with values of 202.67 ±1.31 mg L^{-1} d^{-1}, 0.89 ± 0.004 d^{-1}, 772.24 ± 7.77 mg L^{-1} d^{-1}, 1451.81 ± 14.62 mg L^{-1} d^{-1} and 2.57% respectively. Our result corresponded with that of Hulatt and

Thomas (2011) who reported an increase in biomass concentration (3.62 g L^{-1} > 3.18 g L^{-1} > 2.7 g L^{-1}) and CO_2 fixation rate (930 mg L^{-1} d^{-1}> 830 mg L^{-1} d^{-1}>720 mg L^{-1} d^{-1}) in microalga *Chlorella vulgaris* with increase in light intensity (50 W m^{-3}> 20 W m^{-3}> 10 W m^{-3}) under 4% CO_2 supply. Thus, light intensity of 4351 lux was employed in all subsequent experiments.

Continuous gas sparging into the cultivation media in the open cultivation system leads to considerable diffusion and escape of CO_2 to the atmosphere and thus less amount of CO_2 is utilized by microalgae. To further increase the CO_2 utilization efficiency by microalga SA1, CO_2 was supplied at varying time intervals: 6 h, 12 h and 18 h per day (Table 4.7.). A constant flow rate of 1.2 LPH was maintained. Time course profile of biomass concentration at varying gas sparging times is shown in Fig. 4.12.b. Highest value of maximum biomass concentration (X_{max}) (3.15 ± 0.065 g L^{-1}) was obtained at continuous gas supply of 24 h on day 14 of cultivation (the results discussed in 4351 lux light intensity studies). Highest value of maximum specific growth rate (μ_{max}) of 1.25 ± 0.034 d^{-1} was obtained at CO_2 supply of 18 h per day. Tang et al. (2011) reported μ_{max}value of 0.887 ± 0.012 $d^{-1}$and 0.780 ± 0.089 d^{-1} in S. *obliquus* SJTU-3 supplied with 10% CO_2 and 20% CO_2 respectively. The μ_{max} value (1.25 ± 0.034 d^{-1}) obtained at 15% CO_2 supply (18 h per day) with *S. obliquus* SA1 was 1.4 and 1.6 times higher respectively than the reports of Tang et al. (2011). Also the maximum CO_2 fixation rate (1113.51 ± 48.91mg L^{-1} d^{-1}) obtained at 15% CO_2 supply (18 h per day) with the SA1strain was 3.8 and 4.5 times higher respectively than that obtained by Tang et al. (2011) at 10% (288 ± 0.004 mg L^{-1} d^{-1}) and 20% (246 ± 0.002mg L^{-1} d^{-1}) CO_2 concentration. The maximum biomass concentration (X_{max}) obtained at 18 h per day CO_2 supply (2.36 ± 0.029g L^{-1}) and 12 h per day CO_2 supply (1.87 ± 0.006 g L^{-1}) were 4.7 and 3.7 times higher respectively than that

obtained at 6 h per day CO_2 supply (0.50 ± 0.005 g L^{-1}). SA1 showed increase in all the growth kinetic parameters with increase in the duration of CO_2 sparging. 6 h per day of CO_2 supply proved insufficient for biomass production and maximum biomass productivity (P_{max}) of 177.52 ± 6.31 was obtained which was 3.14 and 3.33 times lower than that obtained at 12 h/day (558.88 ± 3.87 mg L^{-1} d^{-1}) and 18 h/day (592.29 ± 26.01mg L^{-1} d^{-1}) CO_2 supply respectively. The maximum CO_2 utilization efficiency (3.73%) by SA1 was highest at 12 h per day of CO_2 supply at 1.2 LPH. The maximum biomass productivity (P_{max}) of 558.88 ± 3.87 mg L^{-1} d^{-1} obtained at 12 h per day of CO_2 supply was 7.35 and 3.6 times higher than that reported by de Morais and Costa (2007b) (76 ± 0.001mg L^{-1} d^{-1}) and Tang et al. (2011) (155 ± 0.004 mg L^{-1} d^{-1}) for *S. obliquus* strains cultivated under 12% and 10% CO_2 supply respectively. High biomass productivity could benefit downstream applications such as bio-energy production in addition to CO_2 biofixation. Moreover, by decreasing the CO_2 sparging duration from 24 h to 12 h per day the maximum CO_2 utilization efficiency by SA1 could be increased from 2.57% to 3.73% indicating less CO_2 escape at 12 h per day CO_2 supply.

The effect of varying flow rates of CO_2 were studied at 18 h per day and 12 h per day of gas supply to obtain the optimum flow rate in which retention time of CO_2 is increased leading to high maximum CO_2 utilization efficiency. Although the maximum biomass concentration (X_{max}) was highest (4.29 ± 0.022 g L^{-1}) (Fig. 4.12.c) at CO_2 flow rate of 3.68 LPH, the maximum CO_2 utilization efficiency (0.97%) was 7.6 times lower than that obtained at a flow rate of 0.43 LPH CO_2 (7.40%) at 18 h per day of gas supply. The maximum specific growth (μ_{max}) and maximum biomass productivity (P_{max}) obtained at 0.43 LPH CO_2 supply were 0.97 ± 0.043 d^{-1} and 597.70 ± 52.73 mg L^{-1} which indicated fast growth, good biomass productivity and

efficient CO_2 utilization by the SA1 strain at low flow rate of CO_2. Flow rate variations of CO_2 on growth kinetics and CO_2 utilization efficiency of SA1 were further studied at 12 h per day CO_2 supply. At 0.43 LPH CO_2 supply continuously for 12 h per day, maximum biomass concentration (X_{max}) of 3.32 ± 0.022 g $L^{-1}$was obtained after 13 days of cultivation (Fig. 4.12.d). Tang et al. (2011) obtained highest X_{max} value of 1.84 ± 0.01 g L^{-1} in *S. obliquus* SJTU-3 at CO_2 concentration of 10% after a cultivation period of 14 days. This value was 1.8 times lower than that obtained with SA1 after 13 day cultivation period. The maximum CO_2 utilization efficiency value (10.23%) was also highest under this condition. On decreasing the flow rate further to 0.143 LPH CO_2 (12 h per day supply) the maximum biomass productivity (P_{max}) and maximum biomass concentration (X_{max}) decreased to 125.81 ± 3.18 mg L^{-1} d^{-1} and 0.56 g L^{-1} respectively. These values were 77% and 83% lower respectively as compared to the P_{max} (550.66 ± 28.18 mg L^{-1} d^{-1})and X_{max} (3.32 ± 0.022 g L^{-1}) values obtained at 0.43 LPH CO_2 supply of 12 h per day (Table 4.7.). The maximum CO_2 utilization efficiency at 0.143 LPH CO_2 (12 h per day supply) was 7.03% which was 1.45 times lower than the highest value of maximum CO_2 utilization efficiency (10.23%) obtained at 0.43 LPH CO_2 (12 h/day supply). Also the maximum specific growth (μ_{max}) of 1.24 ± 0.028 d^{-1}obtained at 0.43 LPH CO_2 (12 h per day supply) was 1.27 times higher than μ_{max} (0.97 ± 0.043 d^{-1}) value obtained at 0.43 LPH CO_2 (18 h per day supply). Thus, the optimum condition for obtaining high biomass productivity and fast growth rate of SA1 along with high maximum CO_2 utilization efficiency was inferred as: 15% CO_2 supply at 0.43 LPH for 12 h per day at a light intensity of 4351 lux with light regime of 14 h:10 h (light: dark). SA1 with its high biomass yield, fast growth rates and improved CO_2 utilization efficiency (in open

cultivation system) at high CO_2 concentration thus proved to be a potential strain for CO_2 biofixation from industrial flue gas.

4.6.2. Periodic variations of CO_2 fixation rate and CO_2 utilization efficiency in SA1

The variation of the daily CO_2 fixation rate and CO_2 utilization efficiency under various operational conditions at 15% inlet CO_2 concentration were plotted (Fig. 4.13. a-j). The maximum CO_2 fixation rate (mg L^{-1} d^{-1}) and maximum CO_2 utilization efficiency (%) was highest at a light intensity of 4351 lux (24 h continuous CO_2 supply at 1.2 LPH) and the values were 1451.81 ± 14.62 mg L^{-1} $d^{-1}$and 2.57% respectively obtained on day 5 of incubation (Fig. 4.13.c). The maximum CO_2 fixation rate (1451.81 ± 14.62 mg L^{-1} d^{-1}) obtained was 62% higher than that obtained at light intensity of 2445 lux (548.85 ± 66.43 mg L^{-1} d^{-1}) and 42% higher than that obtained at light intensity of 2735 lux (833.11 ± 11.15 mg L^{-1} d^{-1}). Also, the maximum CO_2 fixation rate (2.57%) was 2.65 times higher than that obtained at light intensity of 2445 lux (0.97%) and 1.75 times higher than that obtained at light intensity of 2735 lux (1.47%). On decreasing the CO_2 sparging duration (at 1.2 LPH) from 24 h to 12 h per day, the maximum CO_2 utilization efficiency obtained on day 4 (3.73%) was 1.45 times higher (2.57% for 24 h supply at 1.2 LPH) (Fig. 4.13.e). The maximum CO_2 fixation rate under 12 h and 18 h of CO_2 supply at flow rate of 1.2 LPH were obtained on day 4 of incubation and the values were 1053.50 ± 10.59 mg L^{-1} d^{-1} and 1113.51 ± 48.91 mg L^{-1} d^{-1}respectively (Fig. 4.13. e and f). Although the maximum CO_2 fixation rate obtained at 6 h per day of CO_2 supply (333.73 ± 11.86 mg L^{-1} d^{-1}) (Fig. 4.13. d) was 3.15 times lower than that obtained at 12 h per day CO_2 (1053.50 ± 10.59 mg L^{-1} d^{-1}), the maximum CO_2 utilization efficiency under the same

condition (2.36%) was only 1.58 times lower than that obtained at 12 h per day CO_2 supply (3.73%).

The flow rate of CO_2 was decreased to 0.43 LPH at 18 h per day of CO_2 supply which increased the retention time of CO_2 in the culture increasing the maximum CO_2 utilization efficiency to 7.40% on day 6 (Fig. 4.13. g) which was 2.81 times higher than that (2.63%) obtained at 1.2 LPH. The maximum CO_2 fixation rate (1123.68 ± 99.14 mg L^{-1} d^{-1}) obtained under the same condition was also higher than that (1113.51 ± 48.91 mg L^{-1} d^{-1}) obtained at 1.2 LPH (18 h per day supply). On increasing the flow rate of CO_2 to 3.68 LPH, although the maximum CO_2 fixation rate (1263.36 ± 10.52 mg L^{-1} d^{-1}) obtained on day 5 (Fig. 4.13. h) was high due to the high biomass concentration achieved at high CO_2 flow rate, much CO_2 escaped from the system and the value of maximum CO_2 utilization efficiency (0.97%) was 7.62 times lower than that obtained at low flow rate of 0.43 LPH (7.40%) (Table 4.7.). Thus, supplying more CO_2 to the system (66.24 L per day) (Table 4.7.) keeping the concentration constant at 15% does not prove to be economical since much of the gas remains unutilized by microalgae. The flow rate of 0.43 LPH was employed at CO_2 supply of 12 h per day to study the utilization efficiency of strain SA1 under this condition. Maximum CO_2 utilization efficiency increased to 10.23% at 0.43 LPH CO_2 supply for 12 h per day (Fig. 4.13. i). Also high maximum CO_2 fixation rate (1035.25 ± 52.98 mg L^{-1} d^{-1}) and maximum biomass concentration (3.32 ± 0.022 g L^{-1}) were obtained under this condition. Maximum CO_2 utilization efficiency value of 10.23% in the open cultivation system under 15% inlet CO_2 concentration was 3.96 times higher than the value (2.58%) reported by De Morais and Costa (2007a) who cultivated *S. obliquus* in vertical tubular photobioreactor (closed system) under 12% inlet concentration. Westerhoff et al. (2010) reported maximum CO_2 utilization

efficiency of 3% by *Scenedesmus* + mixed culture under inlet CO_2 concentration of 12% in a helical tubular photobioreactor. This value was 3.41 times lower than that achieved by SA1 in the open system. Lv et al. (2010) reported a decrease in the maximum CO_2 biofixation ratio (%) from 35% to 2% with increase in inlet CO_2 concentration from 0.5% to 12% in microalga *Chlorella vulgaris* cultivated cylindrical photobioreactor. The maximum CO_2 utilization efficiency (10.23%) obtained with SA1 under 15% inlet CO_2 concentration supplied at a flow rate of 0.43 LPH for 12 h per day was 5 times higher than the value reported by Lv et al. at 12% inlet CO_2 concentration. Thus, SA1 proves to be a promising strain for CO_2 sequestration in open cultivation system since it shows high CO_2 utilization efficiency (10.23%) as compared to literature reports at 15% inlet CO_2 concentration. When the flow rate of CO_2 was decreased further to 0.143 LPH (1.71 L CO_2 per day) with 12 h per day supply, there was a drastic drop in the maximum biomass concentration (0.56 ± 0.002 g L^{-1}) (Fig. 4.12. d) and maximum CO_2 fixation rate (236.52 ± 5.98mg L^{-1} d^{-1}) (Fig. 4.13. j) which were 5.92 and 4.38 times lower respectively than that obtained at 0.43 LPH CO_2 supply of 12 h per day (Table 4.7.). However, the maximum CO_2 utilization efficiency of 7.03% obtained under this condition was only 1.46 times lower than that obtained at 0.43 LPH CO_2 supply of 12 h per day. Thus, CO_2 supply at very low flow rate of 0.143 LPH proved insufficient for microalgal growth and biomass production. Decreasing the flow rate of CO_2 from 1.2 LPH to 0.43 LPH (inlet CO_2 concentration being maintained constant at 15%) and reducing the CO_2 sparging duration to 12 h CO_2 per day thus proved to be the ideal condition for CO_2 sequestration by *S. obliquus* SA1.

4.6.3. Analysis of TIC and TOC in cultivation media

TIC (Mol L^{-1}) and TOC (ppm) values of the culture supernatant were obtained for the light intensity variation studies to investigate the variations of these parameters with continuous CO_2 supply of 15%. TIC values in the culture medium increased with progress in incubation time in all the 3 different light intensity studies until day 12 of incubation (Fig. 4.14. a). These values were 0.0175 Mol L^{-1}, 0.02 Mol L^{-1} and 0.0225 Mol L^{-1} respectively for light intensity of 2445 lux, 2735 lux and 4351 lux. Thus, continuous supply of CO_2 increased the TIC values in the media up to day 12 after which the value decreased due to increased uptake of CO_2 by the microalgae. Amount of CO_2 taken up by SA1 on day 14 of incubation was 0.082 Mol L^{-1}, 0.111 Mol L^{-1}, and 0.134 Mol L^{-1} respectively at light intensity of 2445 lux, 2735 lux and 4351 lux (Fig. 4.15. a-c). The biomass concentration on the 12th day of incubation was 1.47 ± 0.016 g L^{-1}, 1.85 ± 0.007 g L^{-1} and 2.75 ± 0.016 g L^{-1} for light intensities of 2445 lux, 2735 lux and 4351 lux respectively (Fig. 4.12. a). Peltier and Thibault (1985) demonstrated that respiration (especially photorespiration) of microalgae partly promotes the dissolved inorganic carbon (DIC) or CO_2 concentration in water. Increased biomass concentration of microalgae at light intensity of 4351 lux might have led to increased respiration leading to increased TIC value in the cultivation media. Also, the biomass concentration obtained at 2445 lux was lower than that obtained at 2735 lux leading to increased TIC levels in the cultivation media at 2735 lux. The TOC values in the cultivation media also showed increasing trends with increase in light intensity values. The highest value of TOC was obtained on day 15 of cultivation in all the cases. These were 182.32 ppm (4351 lux) >69.34 ppm (2735 lux) > 46.69 ppm (2445 lux) (Fig. 4.14. b). The biomass concentration on the same day was 3.08 ± 0.020 g L^{-1} (4351 lux) > 2.30 ± 0.014 g L^{-1} (2735 lux) > 1.83 ± 0.004 g

L^{-1} (2445 lux) (Fig. 4.12. a). Generally, microalgae can release some photosynthetically fixed organic carbon into the surrounding water through the leaky algal cells which are resulted from physical stress (Lin et al., 2012). Since high microalgal biomass was generated at light intensity of 4351 lux, photosynthetically fixed organic carbon was also highest at 4351 lux which must have been released in the culture media with increase in incubation time resulting in increased TOC concentration in the media at 4351 lux.

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Table 4.1. FTIR band assignments of CO_2 treated SA1 and control

Peaks obtained (Wavenumber cm^{-1})	Wavenumber range (cm^{-1})	Representative Group	Representative Biomolecules	CO_2 treated culture peaks (Wavenumber cm^{-1})	Control culture peaks (Wavenumber cm^{-1})
1028, 1049, 1053, 1074	1020-1085	P=O	Phospholipids, nucleic acids	1028, 1049, 1074	1053
1153	1120-1160	C-O-C stretching	Polysaccharides	1153	1153
1381, 1404, 1384	1380-1440	CH bending	Aliphatic groups	1381, 1404	1384
1458	1420-1480	C=O stretching	Carbonate ion	1458	-
1543, 1546	1540-1550	C-N stretching	Proteins (Amide II band)	1543	1546
1631, 1647, 1654	1610-1685	C=O stretching	Proteins (Amide I band)	1631, 1647, 1654	1654
1741	1730-1745	C=O stretching	Lipids (esters of fatty acids)and carbonyl groups of chlorophyll pigments	1741	-
3302, 3344	3300-3400	OH stretching	Carbohydrates, proteins, lipids (sterols and fatty acids), nucleic acids	3302, 3344	-

Table 4.2. Variation of growth kinetic parameters of *Scenedesmus obliquus* SA1 in the lab scale closed system studies (Data represents Mean ± standard deviation)

Culture Condition		Incubation Time (Days)	Maximum Biomass (X_{max}) (g L^{-1})	Overall Biomass Productivity ($P_{overall}$) (mg L^{-1} d^{-1})	CO_2 fixation rate (mg L^{-1} d^{-1})	Maximum Specific growth rate (μ_{max}) (d^{-1})	Maximum Biomass Productivity (P_{max}) (mg L^{-1} d^{-1})
Control		37	0.694 ± 0.002	16.82 ± 0.05	31.62 ± 0.096	0.25 ± 0.017	93.666 ± 0.577
Nitrate concentration (g L^{-1})	0.5	29	3.118 ± 0.003	94.756 ± 0.152	178.143 ± 0.285	0.57 ± 0.01	387 ± 2
	1	29	3.515 ± 0.004	110.243 ± 0.261	207.256 ± 0.493	0.58 ± 0.005	441.333 ± 4.16
	1.5	38	4.975 ± 0.003	119.863 ± 0.075	225.343 ± 0.145	0.67 ± 0.01	586 ± 7.21
	2	30	4.196 ± 0.002	134.513 ± 0.191	252.883 ± 0.361	0.42 ± 0.01	336.666 ± 3.51
	2.5	30	3.925 ± 0.002	123.466 ± 0.135	232.116 ± 0.255	0.36 ± 0.005	405 ± 2.645
	3	33	3.828 ± 0.001	109.566 ± 0.02	205.983 ± 0.041	0.25 ± 0.005	286 ± 1
Phosphate concentration (g L^{-1})	0.02	26	3.07 ± 0.015	108.96 ± 0.202	204.843 ± 0.383	0.63 ± 0.01	272 ± 4
	0.03	28	3.462 ± 0.001	117.99 ± 0.168	221.823 ± 0.315	0.57 ± 0.005	271.33 ± 2.08
	0.04	38	4.975 ± 0.003	119.863 ± 0.075	225.343 ± 0.145	0.67 ± 0.01	586 ± 7.21
	0.05	27	3.553 ± 0.002	122.183 ± 0.207	229.703 ± 0.394	0.57 ± 0.02	400.33 ± 6.42
	0.06	25	3.261 ± 0.006	122.8 ± 0.12	230.863 ± 0.225	0.55 ± 0.02	337 ± 6.92
	0.07	26	3.202 ± 0.002	120.22 ± 0.117	226.016 ± 0.220	0.55 ± 0.01	241.33 ± 10.06
Photoperiod Light : Dark (h)	12:12	29	2.013 ± 0.002	56.636 ± 0.124	106.476 ± 0.236	0.63 ± 0.017	141.33 ± 3.21
	14:10	38	4.975 ± 0.003	119.863 ± 0.075	225.343 ± 0.145	0.67 ± 0.01	586 ± 7.21
	16:8	28	3.288 ± 0.002	106.31 ± 0.168	199.863 ± 0.315	0.74 ± 0.005	233 ± 4.35
	24:0	24	3.176 ± 0.002	125.806 ± 0.145	236.516 ± 0.270	0.66 ± 0.032	381.66 ± 2.08
Temperature (°C)	25	38	4.975 ± 0.003	119.863 ± 0.075	225.343 ± 0.145	0.67 ± 0.01	586 ± 7.21
	30	31	3.658 ± 0.001	109.083 ± 0.110	205.076 ± 0.210	0.70 ± 0.015	346 ± 2.64
	35	30	3.038 ± 0.001	92.443 ± 0.125	173.79 ± 0.236	0.75 ± 0.026	241 ± 1
	40	28	0.883 ± 0.001	30.3 ± 0.017	56.966 ± 0.028	0.54 ± 0.020	59 ± 3

Table 4.3. Biochemical properties of *Scenedesmus obliquus* SA1 obtained in the lab scale closed system studies (Data represents Mean ± standard deviation)

Properties		CO_2 treated SA1	Control SA1
Elemental analysis (wt. %)	Carbon	44.43	48.20
	Hydrogen	6.40	7.08
	Nitrogen	5.74	6.67
	Oxygen (by difference)	43.41	38.03
Biochemical analysis (% of DCW)	Total carbohydrate content	30.87 ± 0.64	39.77 ± 0.67
	Total protein content	9.48 ± 1.65	12.92 ± 0.78
	Total lipid content	33.04 ± 0.46	23.44 ± 1.08
	Total chlorophyll content	6.03 ± 0.19	4.92 ± 0.37
	Carotenoid Content	0.02 ± 0.005	0.06 ± 0.02

Table 4.4. Comparison of the chlorophyll and carotenoid yield of *Scenedesmus obliquus* SA1 with previously reported studies (Data represents Mean ± standard deviation)

Strain	Chlorophyll content (%)	Carotenoid content (%)
S. obliquus SA1 (CO_2 treated)	6.03 ± 0.19	0.02 ± 0.005
Chlorella sp.	1.7	0.2
Dunaliella primolecta	5.3	2.6
Chlorella minutissima	3.1	0.3
Isochrysis aff. galbana	5.6	2.0
Nannochloropsis oculata	2.9	0.5
Chlorella pyrenoidosa	2.86	0.05
Spirulina platensis	1.20	0.43
Chlorella sp.	0.38	0.02
Dunaliella salina	1.01	7.25
Chlamydomonas acidophila	-	1.56

Table 4.5. Variation of growth kinetic parameters of *Scenedesmus obliquus* SA1 in bench scale open system studies (Data represents Mean ± standard deviation)

Input CO_2 (% v/v)	Culture Depth (m)	Incubation Time (Days)	Maximum Biomass (X_{max}) ($g\ L^{-1}$)	Overall Biomass Productivity ($P_{overall}$) ($mg\ L^{-1}\ d^{-1}$)	CO_2 fixation rate ($mg\ L^{-1}\ d^{-1}$)	Maximum Specific growth rate (μ_{max}) (d^{-1})	Maximum Biomass Productivity (P_{max}) ($mg\ L^{-1}\ d^{-1}$)
0.03	0.17	28	0.54 ± 0.003	18.54 ± 0.111	34.85 ± 0.20	0.10 ± 0.011	42.33 ± 3.05
15	0.0425	25	1.42 ± 0.003	54.27 ± 0.49	102.02 ± 0.93	0.59 ± 0.046	146.16 ± 11.65
15	0.085	24	1.36 ± 0.018	54.33 ± 0.19	102.13 ± 0.36	0.69 ± 0.049	156.8 ± 4.37
15	0.1275	24	1.19 ± 0.002	47.97 ± 0.25	90.18 ± 0.48	0.70 ± 0.033	108.82 ± 4.69
15	0.17	29	1.11 ± 0.038	36.58 ± 1.77	68.78 ± 3.34	0.18 ± 0.01	92 ± 15.71
25	0.17	22	1.24 ± 0.079	53.77 ± 4.17	101.08 ±7.84	0.55 ± 0.089	122.33 ±7.09
35	0.17	25	1.39 ± 0.023	51.94 ± 0.55	97.65 ± 1.03	0.64 ± 0.040	139.44 ± 1.12

Table 4.6. Biochemical properties of *Scenedesmus obliquus* SA1 obtained in the bench scale open system studies (Data represents Mean ± standard deviation)

Properties		Control SA1	15% CO_2 treated SA1	25% CO_2 treated SA1	35% CO_2 treated SA1
Gross calorific value (GCV) (MJ/Kg)		20.73 ± 1.24	23.09 ± 0.38	23.59 ± 0.23	24.46 ± 0.41
Biochemical analysis (% of DCW)	Total carbohydrate content	37.09 ± 1.48	32.60 ± 1.80	24.86 ± 1.59	27.8 ± 1.11
	Total protein content	10.22 ± 1.76	6.71 ± 0.40	4.47 ± 1.04	3.76 ± 1.32
	Total lipid content	20.83 ± 2.30	31.59 ± 1.32	35.39 ± 1.44	41.17 ± 0.77
	Total chlorophyll content	3.83 ± 0.27	6.32 ± 0.45	7.29 ± 0.27	8.47 ± 0.15

Table 4.7. Variation of growth kinetic parameters of *S. obliquus* SA1 in the optimization studies (Mean ± standard deviation)

Parameters Studied		Incubation Time (Days)	Input CO_2/day (L)	Maximum Biomass (X_{max}) (g L^{-1})	Overall Biomass Productivity ($P_{overall}$) (mg L^{-1} d^{-1})	Maximum Specific growth rate (μ_{max}) (d^{-1})	Maximum Biomass Productivity (P_{max}) (mg L^{-1} d^{-1})	Maximum CO_2 fixation rate (mg L^{-1} d^{-1})	Maximum CO_2 utilization efficiency (%)
Light Intensity (Lux) 24 h CO_2 supply at 1.2 LPH	2445	15	28.8	1.93 ± 0.009	119.34 ± 0.254	0.78 ± 0.023	291.94 ± 35.33	548.85 ± 66.43	0.97
	2735	15	28.8	2.61 ± 0.015	150.66 ± 0.814	0.88 ± 0.038	443.14 ± 5.93	833.11 ± 11.15	1.47
	4351	15	28.8	3.15 ± 0.065	202.67 ± 1.31	0.89 ± 0.004	772.24 ± 7.77	1451.81 ± 14.62	2.57
CO_2 sparging Duration (h) CO_2 supply at 1.2 LPH 4351 lux	6	8	7.2	0.50 ± 0.005	52.64 ± 0.546	1.11 ± 0.028	177.52 ± 6.31	333.73 ± 11.86	2.36
	12	8	14.4	1.87 ± 0.006	193.76 ± 14.16	1.20 ± 0.031	558.88 ± 3.87	1053.50 ± 10.59	3.73
	18	8	21.6	2.36 ± 0.029	222.20 ± 5.77	1.25 ± 0.034	592.29 ± 26.01	1113.51 ± 48.91	2.63
	24	15	28.8	3.15 ± 0.065	202.67 ± 1.31	0.89 ± 0.004	772.24 ± 7.77	1451.81 ± 14.62	2.57
CO_2 flow rate (LPH) 18 h CO_2 supply 4351 lux	0.43	8	7.74	1.85 ± 0.019	214.83 ± 2.68	0.97 ± 0.043	597.70 ± 52.73	1123.68 ± 99.14	7.40
	1.2	8	21.6	2.36 ± 0.029	222.20 ± 5.77	1.25 ± 0.034	592.29 ± 26.01	1113.51 ± 48.91	2.63
	3.68	14	66.24	4.29 ± 0.022	294.14 ± 1.24	1.07 ± 0.002	672 ± 5.60	1263.36 ± 10.52	0.97
CO_2 flow rate (LPH) 12h CO_2 supply 4351 lux	0.143	10	1.71	0.56 ± 0.002	53.51 ± 0.252	0.78 ± 0.018	125.81 ± 3.18	236.52 ± 5.98	7.03
	0.43	13	5.16	3.32 ± 0.022	253.27 ± 1.25	1.24 ± 0.028	550.66 ± 28.18	1035.25 ± 52.98	10.23
	1.2	8	14.4	1.87 ± 0.006	193.76 ± 14.16	1.20 ± 0.031	558.88 ± 3.87	1053.50 ± 10.59	3.73

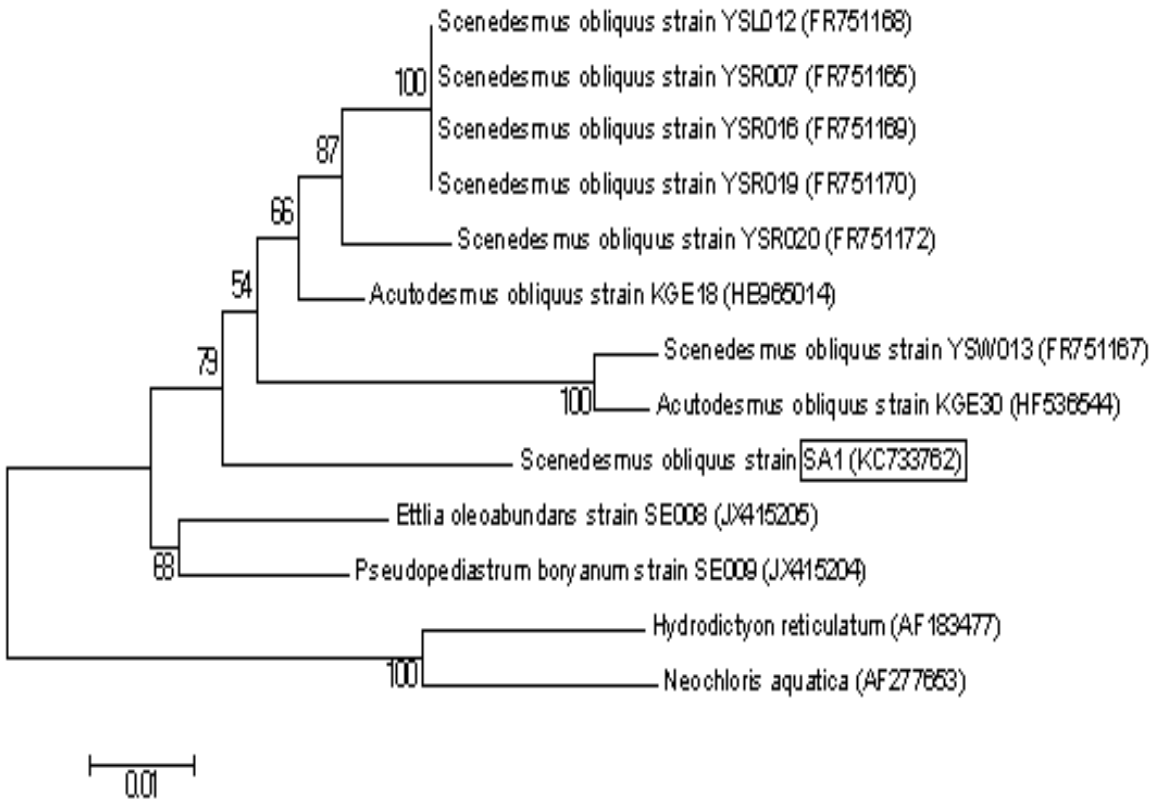
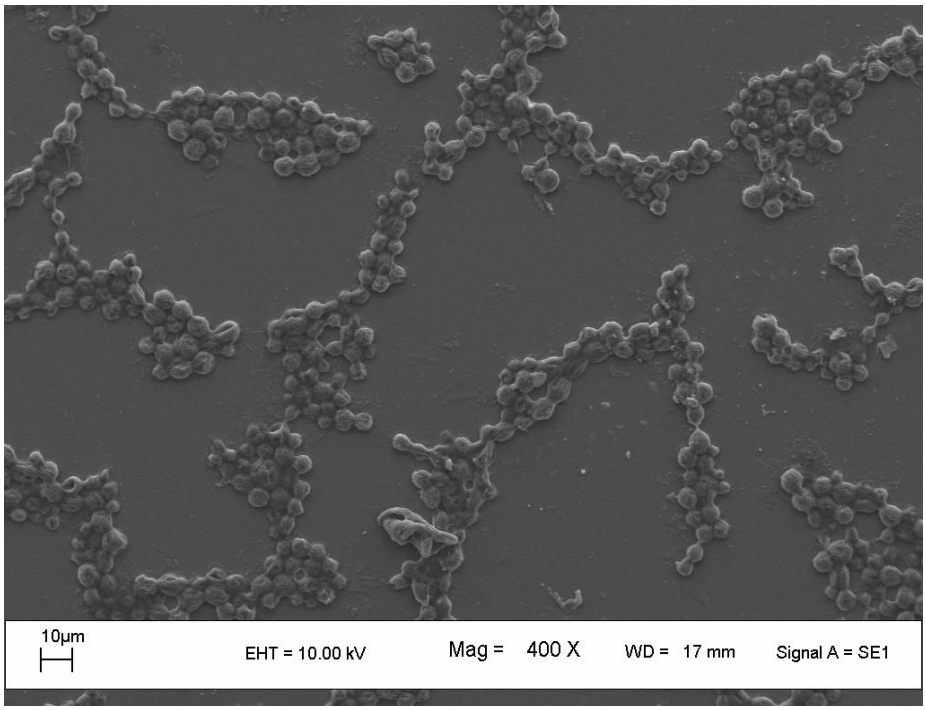
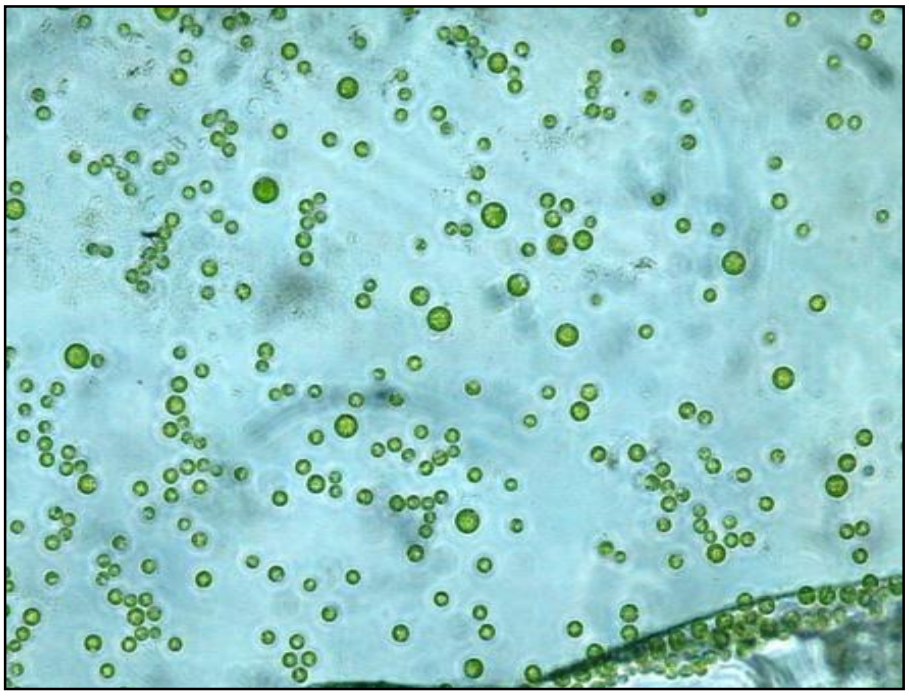


Fig. 4.1. Neighbour-joining showing phylogenetic position of *S. obliquus* SA1 and related taxa based on partial 28s rRNA gene sequence comparisons. Bootstrap values are indicated at nodes. Scale bar (=0.01) represents nucleotide substitution per 100 nucleotide. Representative sequences in the dendogram were obtained from GenBank (accession number is in parentheses).

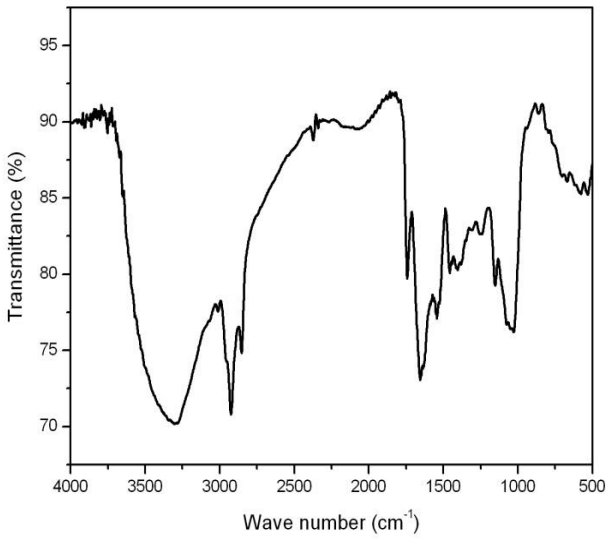


(a)

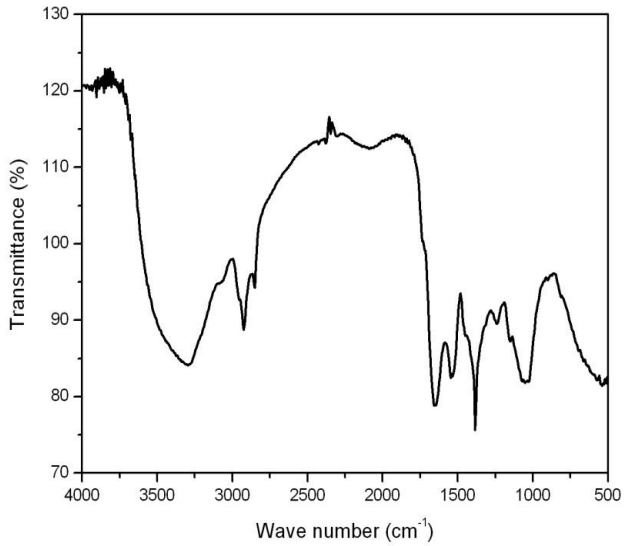


(b)

Fig. 4.2. (a) SEM image and (b) Light Microscopic image (40X) of *S. obliquus* SA1



(a)



(b)

Fig. 4.3. FTIR spectra of (a) 13.8 ± 1.5% CO_2 treated *S. obliquus* SA1 (b) Control

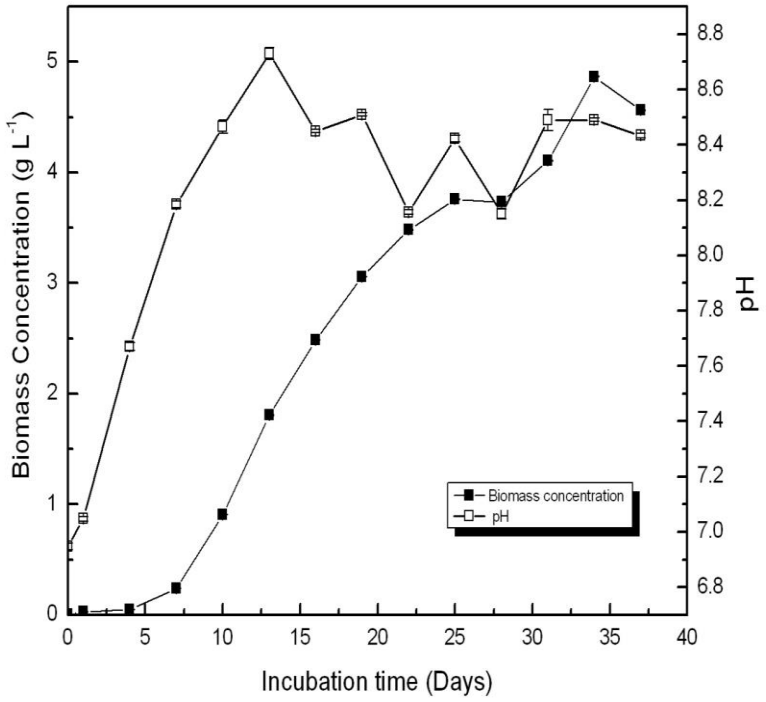
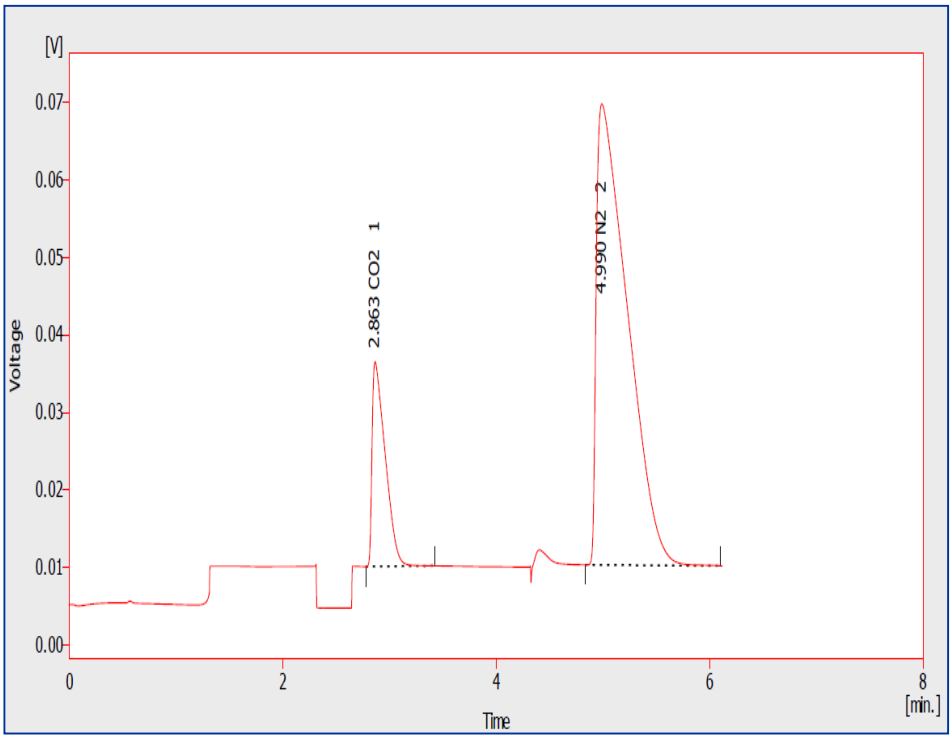
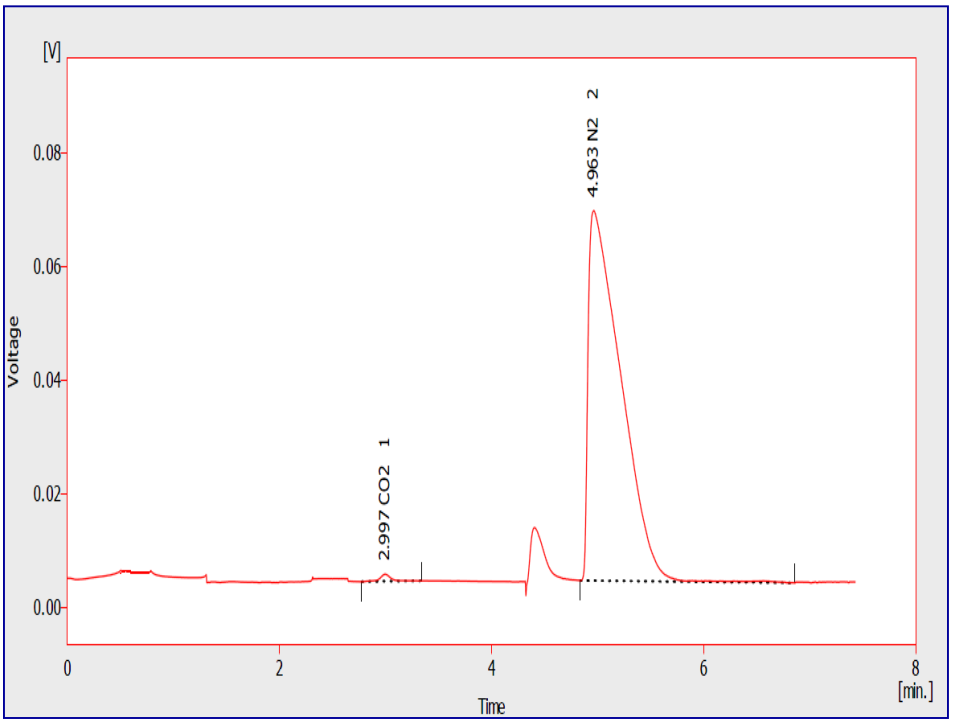


Fig. 4.4. Medium pH as a function of time for *S. obliquus* SA1 cultivated in BG 11 supplemented with 13.8 ± 1.5% CO_2.



(a)



(b)

Fig. 4.5. GC data obtained of (a) inlet and (b) outlet gas streams

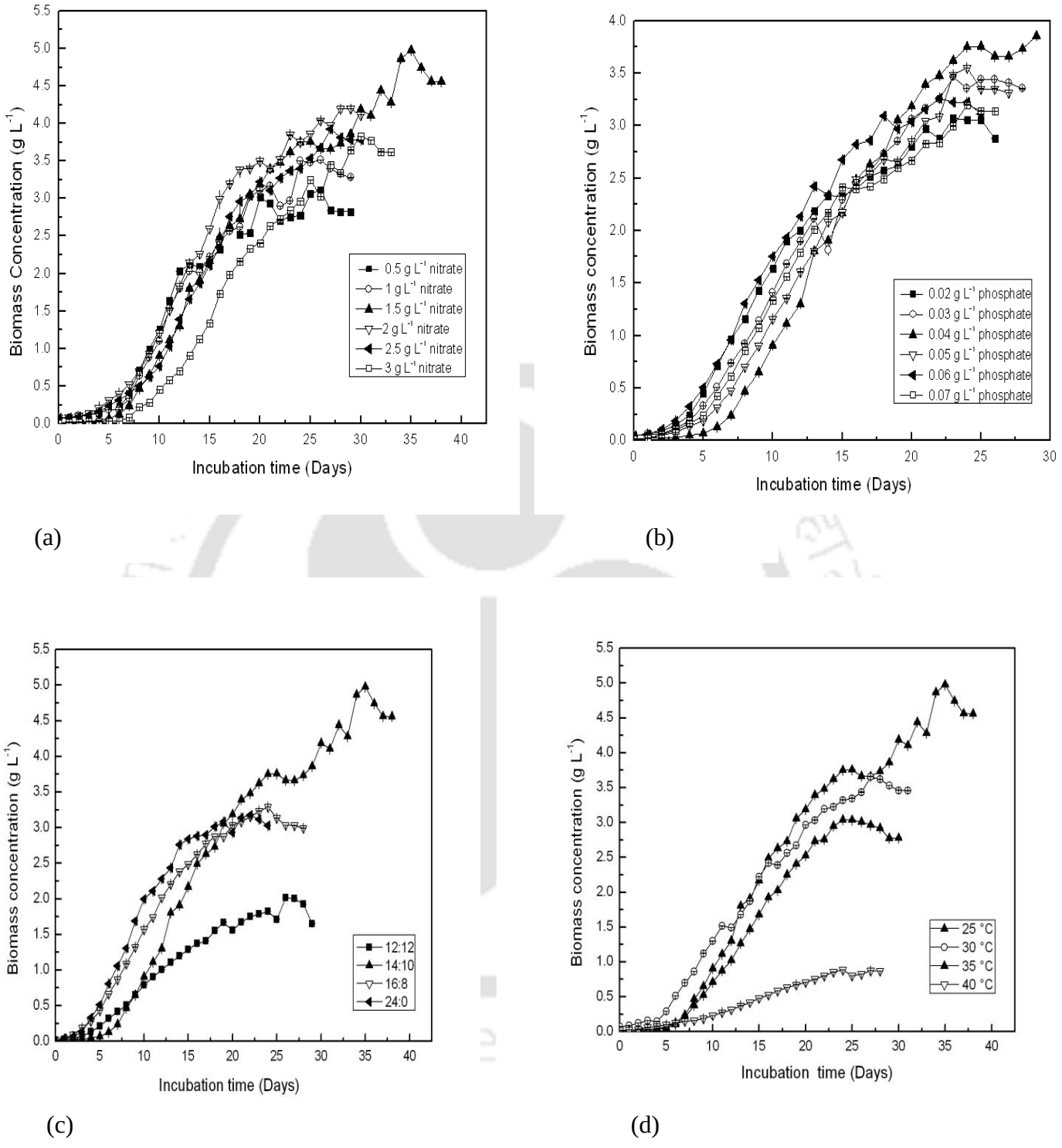


Fig. 4.6. Time course profile of biomass concentration for *S. obliquus* SA1 cultivated with 13.8 ± 1.5% CO_2 at varying (a) nitrate concentrations (b) phosphate concentrations (c) photoperiods and (d) temperatures

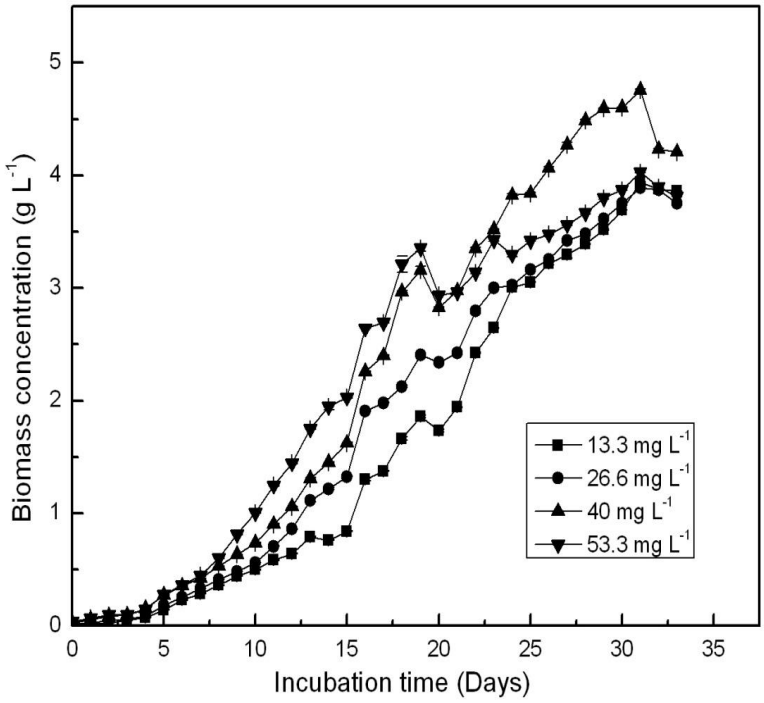
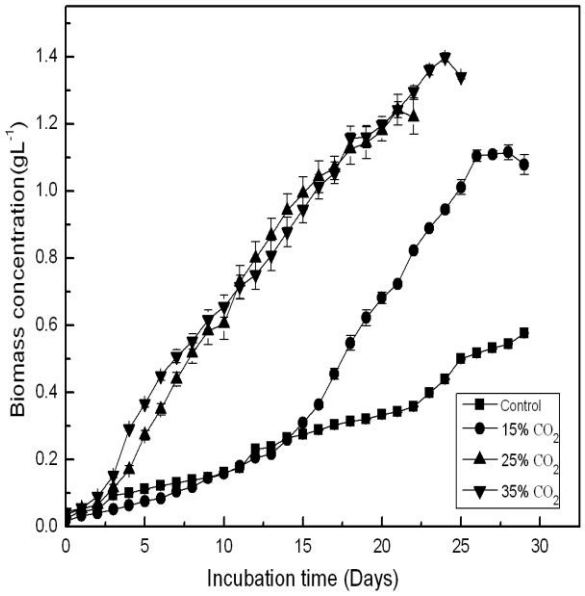
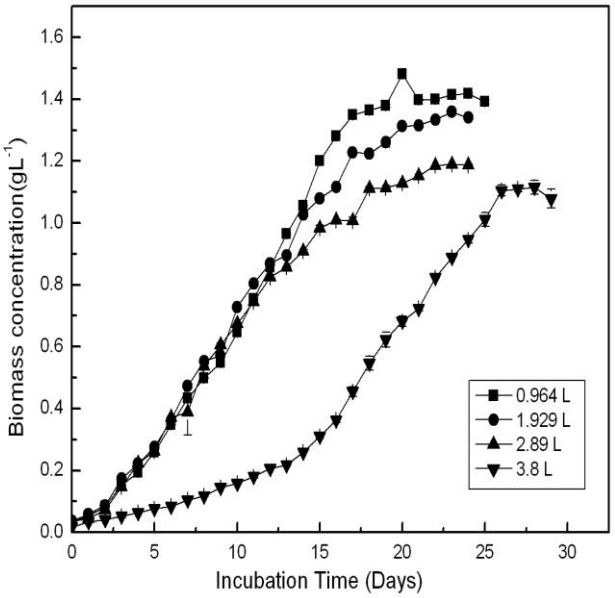


Fig. 4.7. Time course profile of biomass concentration for *S. obliquus* SA1 cultivated with 13.8 ± 1.5% CO_2 at varying initial inoculum concentration



(a)



(b)

Fig. 4.8. Time course profile of biomass concentration for *S. obliquus* SA1 at varying (a) CO_2 concentrations (b) culture volume in presence of 15% inlet CO_2

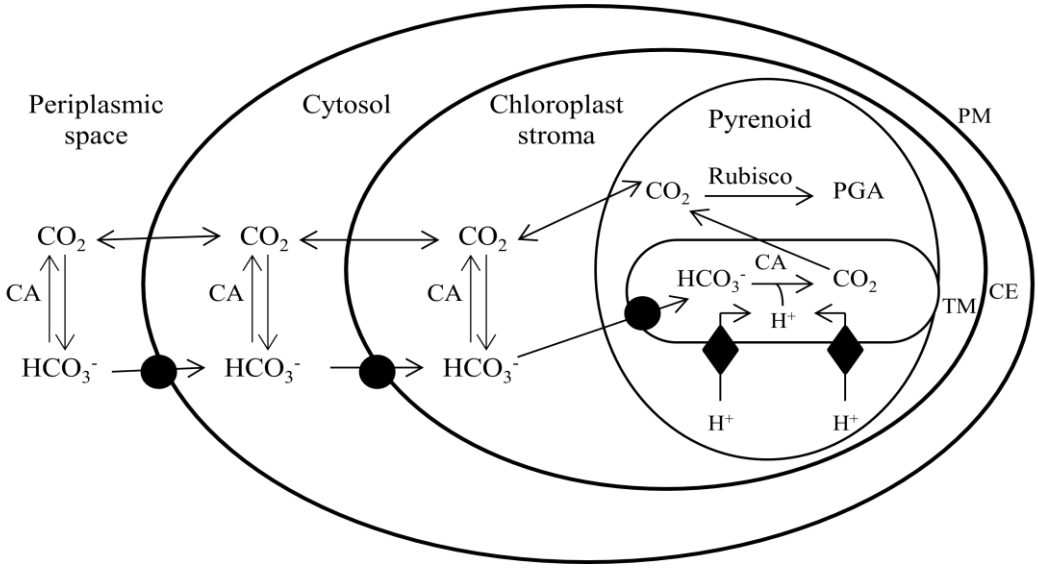


Fig. 4.9. CO_2 concentrating mechanism **(**CCM comprising CA) in eukaryotic algae. PGA, 3-phosphoglyceric acid; PM, plasma membrane; CE, chloroplast envelope; TM, thylakoid membrane. The filled circles indicate possible bicarbonate transporters, and the closed diamonds indicate the photosynthetic electron transport chain.

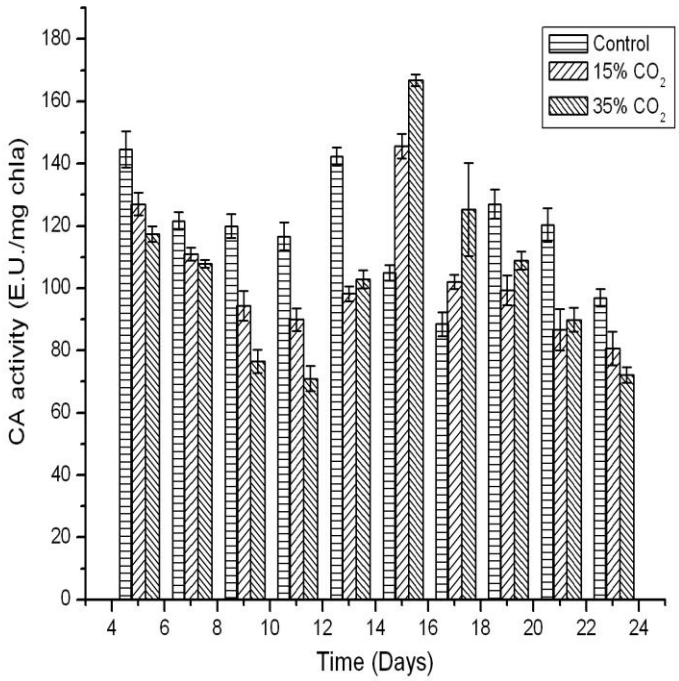
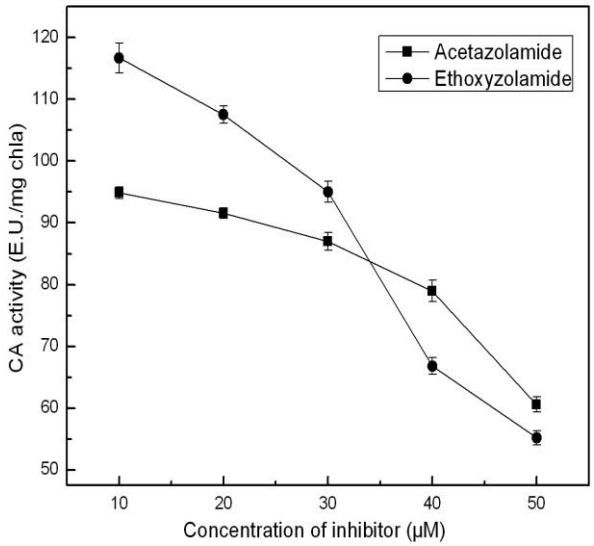
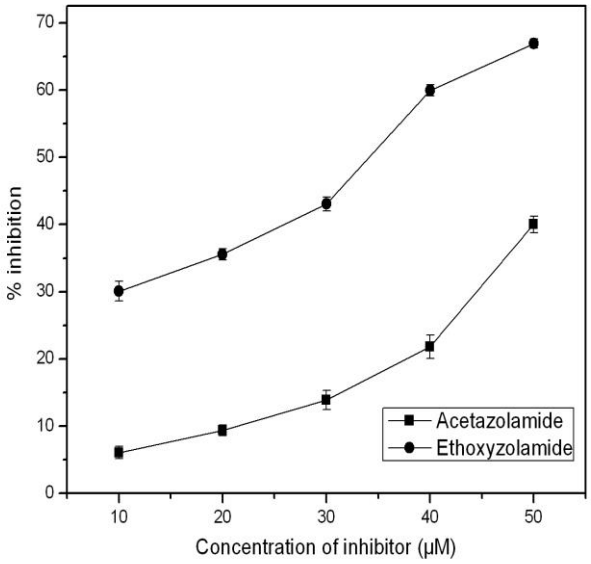


Fig. 4.10. CA activity of *S. obliquus* SA1 at varying inlet CO_2 concentrations



(a)



(b)

Fig. 4.11. (a) CA activity of *S. obliquus* SA1 at varying inhibitor concentrations (b) Percent Inhibition of CA activity at varying inhibitor concentrations

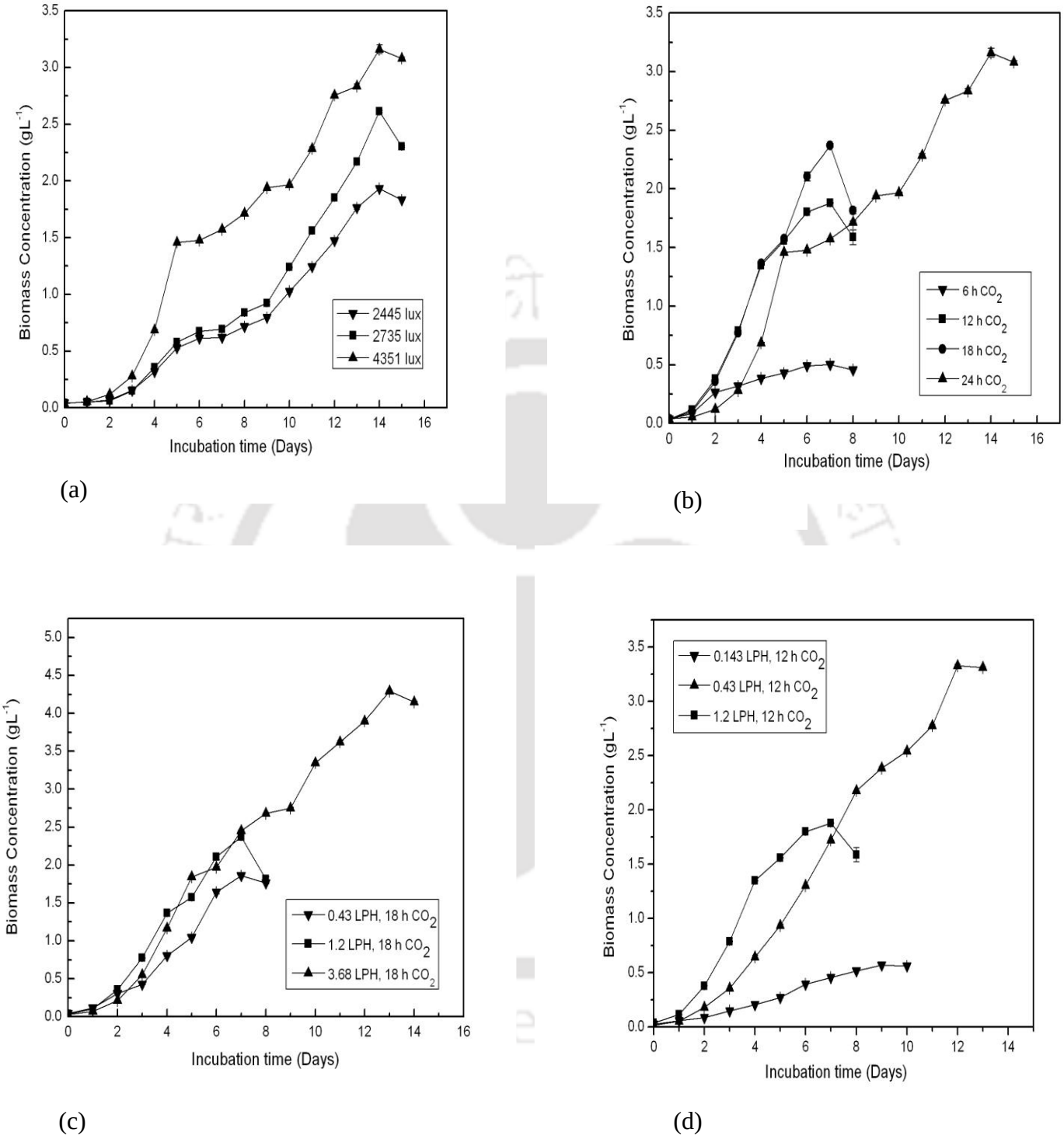
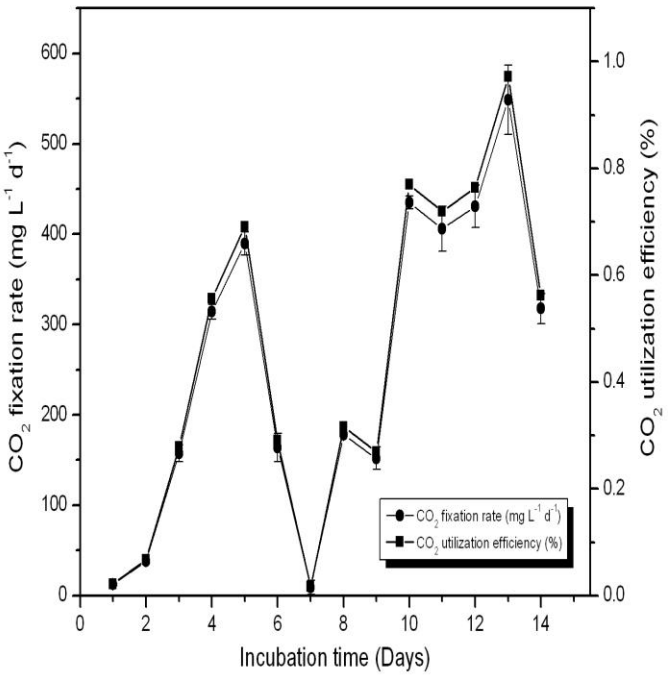
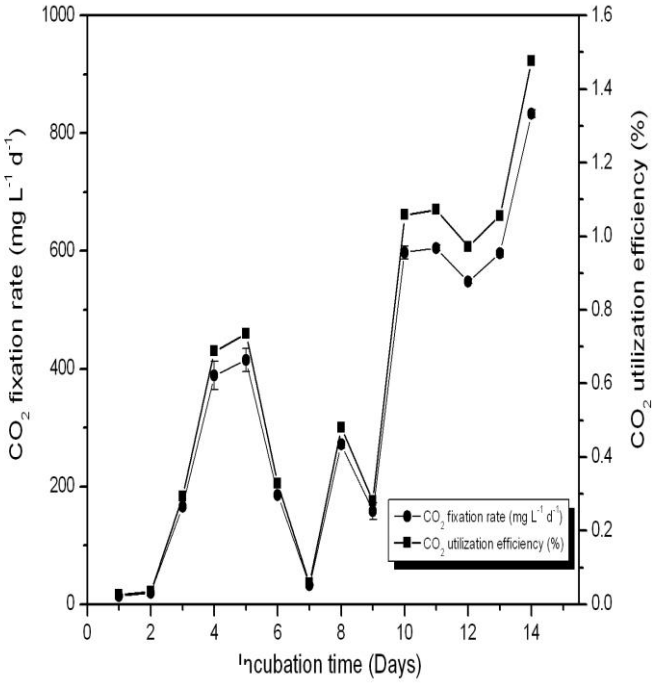


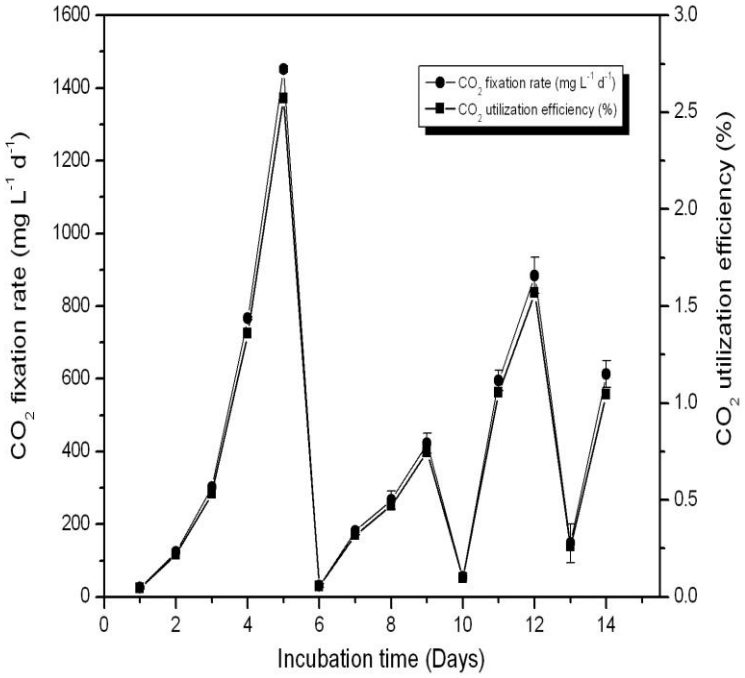
Fig. 4.12. Time course profile of biomass concentration for *S. obliquus* SA1 at varying (a) Light intensities (b) CO_2 sparging durations (c) Flow rates at 18 h per day CO_2 supply and (d) Flow rates at 12 h per day CO_2 supply


CO_2 fixation rate (mg L^{-1} d^{-1})
CO_2 utilization efficiency (%)
Incubation time (Days)
CO_2 fixation rate (mg L^{-1} d^{-1})
CO_2 utilization efficiency (%)

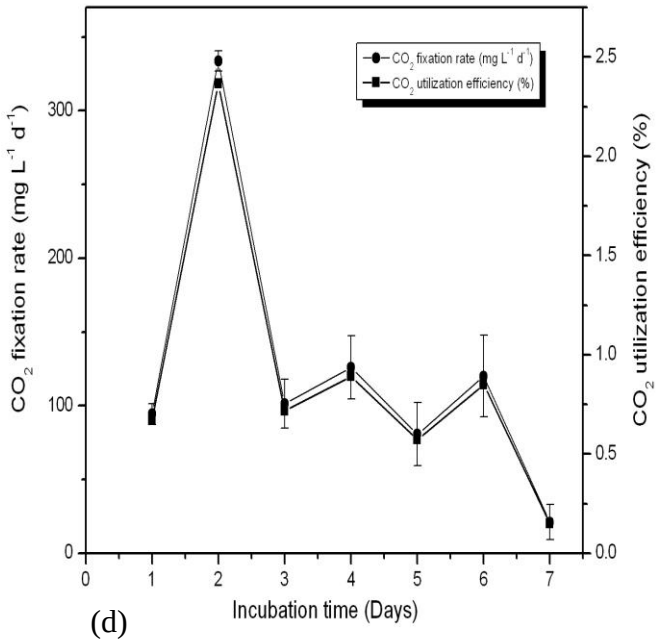
(a)


CO_2 fixation rate (mg L^{-1} d^{-1})
CO_2 utilization efficiency (%)
Incubation time (Days)
CO_2 fixation rate (mg L^{-1} d^{-1})
CO_2 utilization efficiency (%)

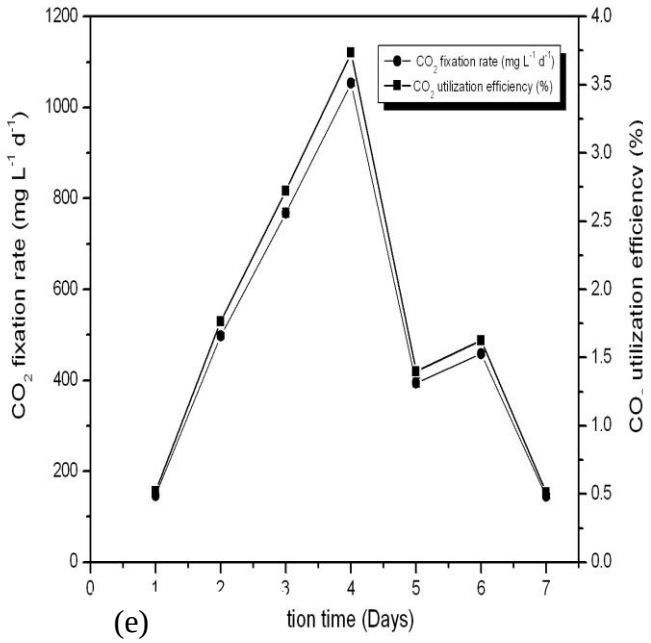
(b)


CO_2 fixation rate (mg L^{-1} d^{-1})
CO_2 utilization efficiency (%)
Incubation time (Days)
CO_2 fixation rate (mg L^{-1} d^{-1})
CO_2 utilization efficiency (%)

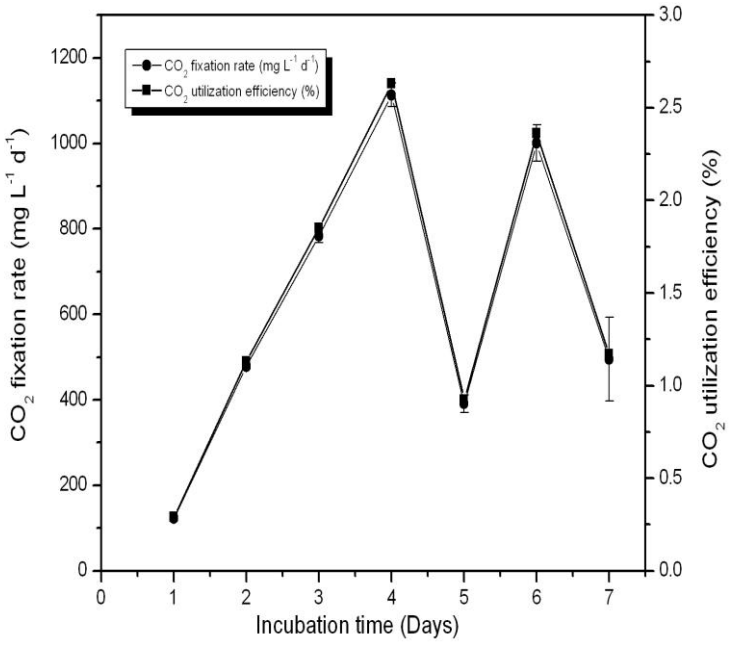
(c)


CO2 fixation rate (mg L-1 d-1)
CO2 utilization efficiency (%)
CO2 fixation rate (mg L-1 d-1)
CO2 utilization efficiency (%)
Incubation time (Days)

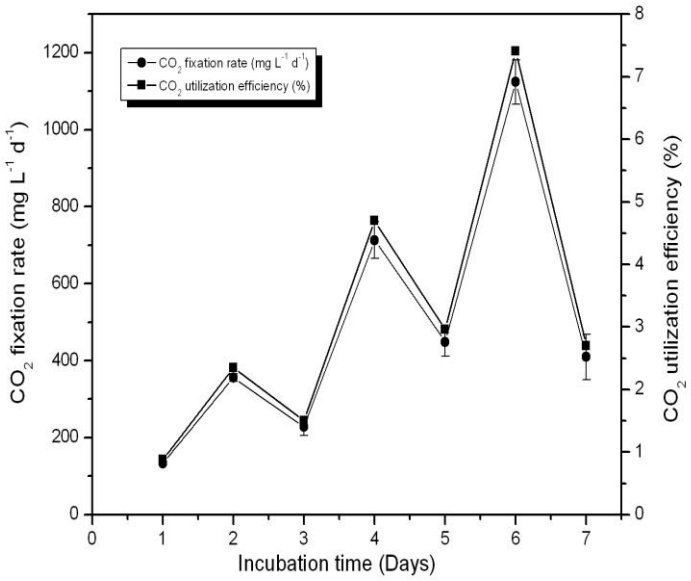
(d)


CO2 fixation rate (mg L-1 d-1)
CO2 utilization efficiency (%)
CO2 fixation rate (mg L-1 d-1)
CO2 utilization efficiency (%)
tion time (Days)

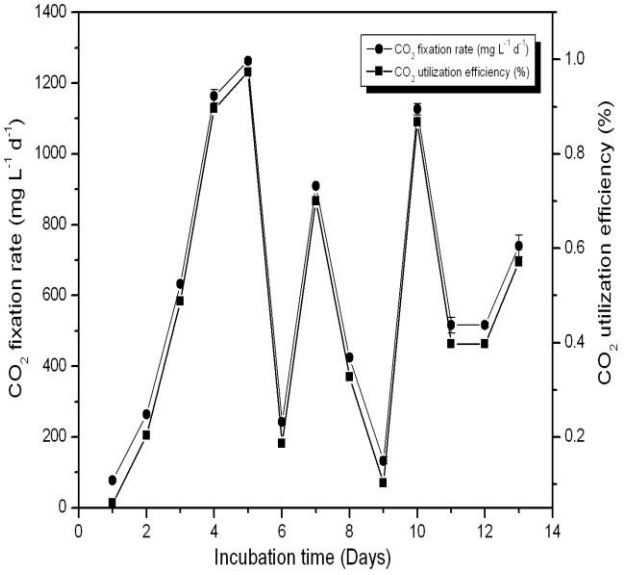
(e)


CO2 fixation rate (mg L-1 d-1)
CO2 utilization efficiency (%)
CO2 fixation rate (mg L-1 d-1)
CO2 utilization efficiency (%)
Incubation time (Days)

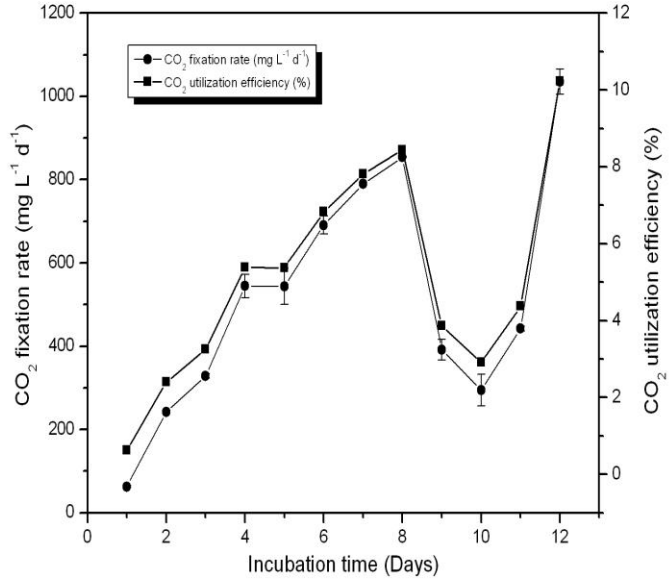
(f)



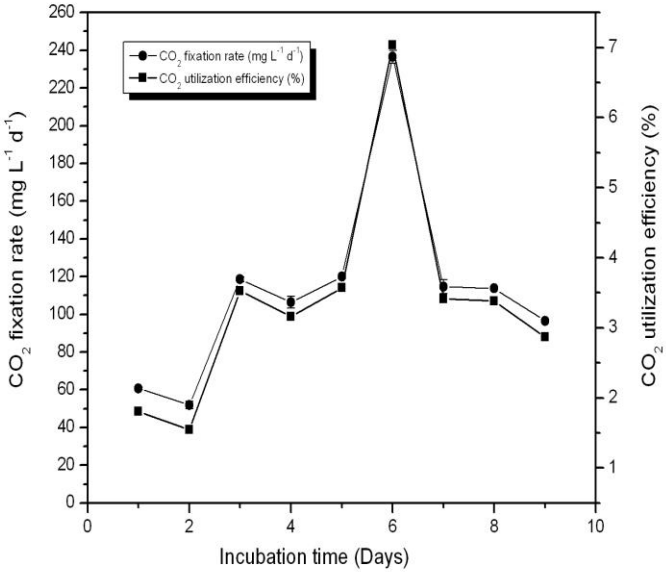
(g)



(h)

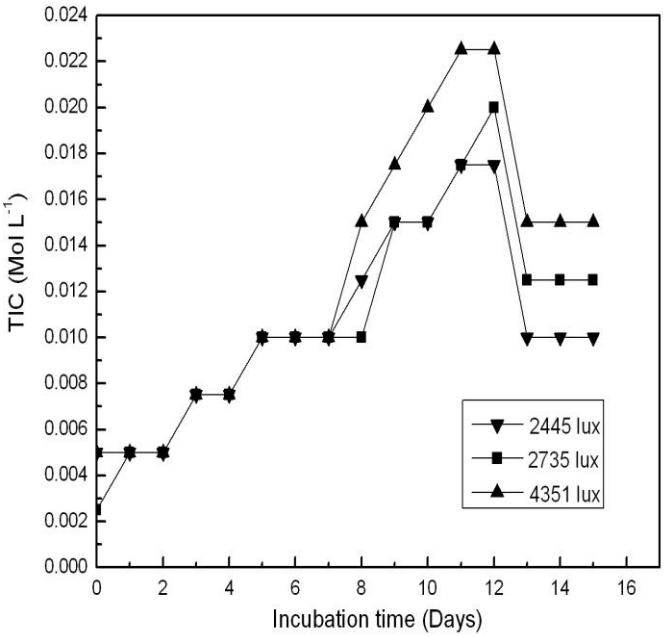


(i)

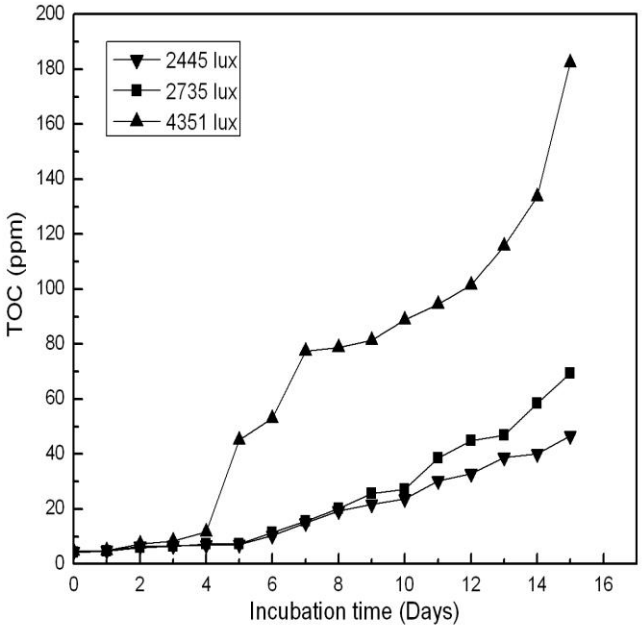


(j)

Fig. 4.13. Variation of CO_2 fixation rate and CO_2 utilization efficiency with incubation time at (a) 2445 lux (b) 2735 lux (c) 4351 lux (d) 6 h per day CO_2 supply (e) 12 h per day CO_2 supply (f) 18 h per day CO_2 supply (g) 0.43 LPH at 18 h per day CO_2 supply (h) 3.68 LPH at 18 h per day CO_2 supply (i) 0.43 LPH at 12 h per day CO_2 supply and (j) 0.143 LPH at 12 h per day CO_2



(a)



(b)

Fig. 4.14. Variation of (a) total inorganic carbon (TIC) and (b) total organic carbon (TOC) concentration in cultivation medium at varying light intensities with incubation time.

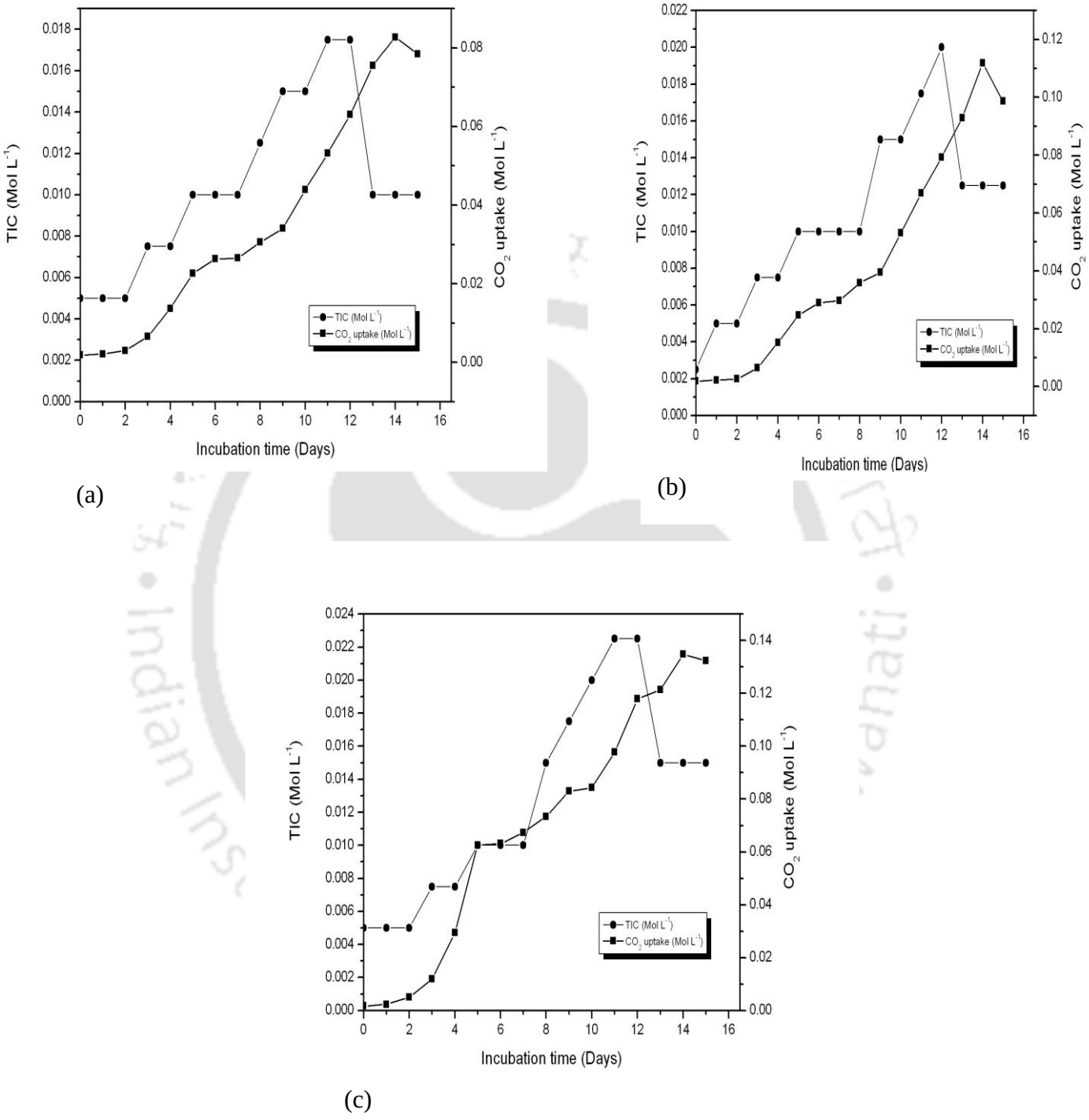


Fig. 4.15. Variation of TIC in cultivation medium and CO_2 uptake by SA1 at (a) 2445 lux (b) 2735 lux and (c) 4351 lux

CHAPTER V

CONCLUSIONS AND FUTURE SCOPE

This chapter summarizes the inferences drawn from the present work and recommendations for future work.

5.1. Conclusions

The present study concluded that microalga *Scenedesmus obliquus* SA1 is tolerant to flue gas CO_2 concentration (15%) and high temperature of around 40 °C (flue gas temperature after desulfurization process). SA1 showed high growth rates, high biomass concentration, high CO_2 fixation rates and high biomass productivities in open culture system in the bench scale studies thus making this strain an appropriate one for CO_2 sequestration from flue gas in open outdoor ponds. The strain also possesses an attractive biochemical profile and thus could find commercial application after CO_2 sequestration.

SA1 strain was isolated from a freshwater body (bio-diversity hotspot region of Guwahati, Assam, India) and identified as the green microalgae *Scenedesmus obliquus* based on 28S rRNA gene sequencing. Scanning electron microscopic analysis showed the globular cell morphology of SA1. FTIR spectra of 13.8 ± 1.5% CO_2 treated SA1 showed fourteen distinct peaks over the wavenumber range 4000-900 $cm^{-1}$and control (receiving atmospheric CO_2 for growth) SA1 showed five distinct peaks in the same range. Peaks representing nucleic acids, polysaccharides,

and proteins were obtained in case of both control and CO_2 treated SA1. However, peak at 1741cm^{-1} obtained in case of the CO_2 treated SA1 was attributed to C=O stretching of lipids (esters of fatty acids) and carbonyl groups of chlorophyll pigments. This peak was not observed in case of control which indicated that lipid and pigment accumulation in the present microalgal strain was being facilitated by the supply of CO_2. A peak of particular interest at 1458 cm^{-1} corresponding to C=O stretching of carbonate ion was observed in case of the CO_2 treated culture only indicating that HCO_3^- might be accumulated and stored in chloroplast stroma as a result of high CO_2 supply.

SA1 strain was found to completely fix the supplied CO_2 to organic carbon in the lab scale closed system studies since the inlet CO_2 concentration of 13.8 ± 1.5% was reduced to 0.5% after 14 days of incubation as evident from Gas Chromatography analysis of inlet and outlet gas streams. Nitrate and phosphate concentrations, photoperiod regime and culture temperatures were varied in the lab scale closed system studies to find out the optimum condition for obtaining maximum biomass concentration, biomass productivity and CO_2 fixation rates. At 13.8 ± 1.5% CO_2 with nitrate and phosphate concentration of 1.5 g L^{-1} and 0.04 g L^{-1} respectively, photoperiod regime of 14:10 h (light: dark) and 25 °C, maximum biomass (4.975 ± 0.003 g L^{-1}), maximum biomass productivity (586 ± 7.21 mg L^{-1} d^{-1}) and highest CO_2 fixation rate (252.883 ± 0.361 mg L^{-1} d^{-1}) were obtained which were higher than most of the relevant studies. At elevated temperature (40 °C) and 13.8 ± 1.5% CO_2 maximum biomass concentration of 0.883 ± 0.001 g L^{-1} and maximum specific growth rate of 0.54 ± 0.020 d^{-1} were obtained which were 1.27 and 2.16 times higher respectively than that obtained for the control culture incubated at 25 °C. Thus, even at high temperature, SA1 showed improved growth when supplied with 13.8 ± 1.5%

CO_2 supply. The optimized conditions obtained in lab scale closed system were employed in the subsequent studies. The carbohydrate, protein, lipid, and chlorophyll content of the CO_2 treated SA1 were 30.87 ± 0.64%, 9.48 ± 1.65%, 33.04 ± 0.46 and 6.03 ± 0.19% respectively in the lab scale studies, which were higher than previous reports. Also, the lipid and chlorophyll content of the CO_2 treated SA1 strain were 1.41 and 1.23 times higher respectively than that of the control culture which corresponded to the data obtained in FTIR.

SA1 strain was cultivated in bench scale open system at varying CO_2 levels ranging from 0.03-35% (v/v) and subsequently the carbonic anhydrase activity (CA) and the biochemical properties were monitored. Highest value of maximum biomass concentration (1.39 ± 0.023 g L^{-1}), and maximum specific growth rate (0.64 ± 0.040 d^{-1}) were obtained at 35% CO_2 concentration at a culture depth of 0.17 m. Culture depth was varied from 0.0425m to 0.17 m at CO_2 concentration of 15%. Overall biomass productivity ($P_{overall}$) (54.33 ± 0.19 mg L^{-1} d^{-1}), CO_2 fixation rate (102.13 ± 0.36 mg L^{-1} d^{-1}) and maximum biomass productivity (P_{max}) (156.8 ± 4.37 mg L^{-1} d^{-1}) were the highest at a culture depth of 0.085m. These values decreased with further increase in depth of the culture. This may be accounted to increased light penetration at lesser culture depth resulting in better light utilization by the algal cells leading to increased photosynthetic efficiency. Since the SA1 strain showed increased growth rates and biomass productivities under CO_2 concentrations ranging from 15% to 35% at large culture volume in open cultivation system, it could find potential application for CO_2 sequestration in open outdoor ponds. The total carbohydrate content of control SA1 (37.09 ± 1.48%) and SA1 strain after 15% CO_2 treatment (32.60 ± 1.80%) were high enough to make this strain a suitable candidate for bioethanol production. The total lipid content of the SA1 strain increased with increase in CO_2

concentration. The total lipid content of SA1 at 35% CO_2 concentration (41.17 ± 0.77%) was 49% higher than the control culture of SA1 (20.83 ± 2.30%). Thus, the strain could find application for production of biodiesel due to high content of lipids at increased CO_2 concentration. The total chlorophyll content increased with increase in CO_2 levels as follows: 0.03% CO_2 (3.83 ± 0.27%) < 15% CO_2 (6.32 ± 0.45%) < 25% CO_2 (7.29 ± 0.27%) < 35% CO_2 (8.47 ± 0.15%). The increase in chlorophyll content with increase in CO_2 concentration implied that high CO_2 levels stimulated SA1 to synthesize chlorophyll to enhance the photosynthetic efficiency for subsequent conversion of CO_2 into carbohydrates.

The total carbonic anhydrase (CA) activity obtained with the cell homogenate on 15^{th} day of cultivation for the 35% CO_2 treated SA1 was 166.86 ± 3.30 E.U./mg chla while the external (periplasmic) CA activity as determined using intact cells on the same day was found to be 101.01 ± 2.62 E.U./mg chla. Thus intracellular CA activity was found to be 65.85 E.U./mg chla. Since extracellular (periplasmic) CA functions to accelerate the equilibration of CO_2 and bicarbonate in alkaline medium so that CO_2 is formed, the SA1 strain possessing an extracellular CA activity of 101.01 ± 2.62 E.U./mg chla appears to utilize CO_2 as the preferred species of inorganic carbon from the medium. The effect of acetazolamide (AZ), a membrane-impermeant CA inhibitor was used to confirm the presence of periplasmic (external) CA, while ethoxyzolamide (EZ), a membrane-permeant CA inhibitor was used to confirm the presence of intracellular CA. AZ and EZ inhibited the external and internal enzyme activity in SA1 respectively thus confirming the presence of both extracellular and intracellular CA in SA1.

SA1 strain was cultivated in a cylindrical glass photobioreactor under 15% CO_2 concentration at varied operational conditions (light intensity, CO_2 sparging duration

and CO_2 flow rates). At light intensity of 4351 lux, CO_2 sparging duration of 12 h per day and flow rate of 0.43 liter per hour, maximum biomass concentration of 3.32 ± 0.022 g L^{-1}, maximum specific growth rate of 1.24 ± 0.028 d^{-1}, maximum CO_2 fixation rate of 1035.25 ± 52.98 mg L^{-1} d^{-1} and maximum CO_2 utilization efficiency of 10.23% were obtained which were higher than most of the relevant literature reports. Total inorganic carbon and total organic carbon concentration of the cultivation medium was monitored with time which showed increasing trends with increase in biomass concentration. Thus, residence time of CO_2 in the cultivation medium could be maximized by adopting the aforementioned operational conditions.

5.2. Future Scope

Further research is needed for the performance evaluation of microalgae under harsh process parameters such as high temperature and toxic pollutants present in flue gas. Furthermore, cultivation systems and photobioreactor design need to studied and optimized and further optimization of important process parameters need to be done prior to using microalgae-based CO_2 fixation and biomass production in the industrial fields.

LIST OF PUBLICATIONS

Peer Reviewed Journals

- Basu, S., Roy, A.S., Mohanty, K., Ghoshal, A.K., **2013**. Enhanced CO_2 sequestration by a novel microalga*: Scenedesmus obliquus* SA1 isolated from bio-diversity hotspot region of Assam, India. ***BioresourceTechnology*** 143, 369-377.
- Basu, S., Roy, A.S., Mohanty, K., Ghoshal, A.K., **2014**. CO_2 biofixation and carbonic anhydrase activity in *Scenedesmus obliquus* SA1 cultivated in large scale open system, ***BioresourceTechnology*** 164, 323-330.
- Basu, S., Roy, A.S., Ghoshal, A.K., Mohanty, K., 2015. Operational strategies for maximizing CO_2 utilization efficiency by the novel microalga *Scenedesmus obliquus* SA1 cultivated in lab scale photobioreactor. ***Algal Research*** 12, 249-257.

Publications in Conference Proceedings

- Basu, S., Mohanty, K., Ghoshal, A.K,. "Algal Route to CO_2 capture", One day Symposium on Environment and Us, IIT Guwahati, 5th June, 2012.
- Basu, S., Ghoshal, A.K, Mohanty, K. "CO_2 biofixation by a novel microalgal strain *Scenedesmus obliquus* SA1 isolated from biodiversity hotspot region of Assam", International Conference on Algal Biorefinery, IIT Kharagpur, 11-12 January, 2013.
- Basu, S., Roy, A.S., Mohanty, K., Ghoshal, A.K. "Optimization of growth conditions of the high CO_2 tolerant microalga: *Scenedesmus obliquus* SA1",

2nd International Conference on Algal Biorefinery, Technical University of Denmark, 27-29 August, 2014.

- Basu, S., Roy, A.S., Mohanty, K., Ghoshal, A.K. "CO_2 biofixation by the novel microalga: *Scenedesmus obliquus* SA1 cultivated in large scale open system", National Conference on Sustainable Development of Environmental Systems, IIT Guwahati, 20-21 June, 2014.
- Basu, S., Roy, A.S., Ghoshal, A.K, Mohanty, K. "Optimization of operational parameters for efficient CO_2 sequestration by the novel microalga: *Scenedesmus obliquus* SA1", National Conference on Challenges in Environmental Research, IIT Guwahati, 04-06 June, 2015.