2013

Induction of Microalgal Lipids for Biodiesel Production in Tandem with Sequestration of High Carbon Dioxide Concentration

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INDUCTION OF MICROALGAL LIPIDS FOR BIODIESEL PRODUCTION IN TANDEM WITH SEQUESTRATION OF HIGH CARBON DIOXIDE CONCENTRATION

By

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A THESIS

Submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

In Chemical Engineering

MICHIGAN TECHNOLOGICAL UNIVERSITY

2013

This thesis has been approved in partial fulfillment of the requirements for the Degree of MASTER OF SCIENCE in Chemical Engineering.

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Acknowledgements

- First of all, I am grateful to the almighty God**,** who has blessed and guided me throughout these years, giving me the strength to finish this thesis and all of my accomplishments.

- My thesis advisor, Dr. Ching-An Peng for his guidance and support throughout my graduate study at Michigan Tech University. Thank you for allowing me to join your research group. Your mentorship, advice and encouragement on both my research and personal career growth.

- Sincere thanks to Drs. John Sandell and Haiying Liu for serving as my committee members.

- Michigan Tech Graduate School, Chemical Engineering Department, King-Chavez-Parks future faculty Initiative, Sustained Support to Ensure Engineering Degrees (S-SEED) Grant and Gary & Judy Anderson Endowed Scholarship for funding.

- My colleagues at lab Alicia Sawdon, Ethan Wedemeyer, Jiesheng Wang, Jun Zhang, Nasirn Salehi, Ornella Nkurunziza and Sushi Pachpinde, and all of friends at Michigan Tech for their constant engagement, entertainment, friendship and advice along the way.

- Lastly but most importantly, my mother, Mabel Watson for her unconditional love, care and support throughout my life. Thank you for giving me the motivation and confidence to set higher targets and chase after it. To my sisters, little brother, aunties and uncles, thank you all for your cheering love and words of encouragement over the past year.

Abstract

There is no doubt that sufficient energy supply is indispensable for the fulfillment of our fossil fuel crises in a stainable fashion. There have been many attempts in deriving biodiesel fuel from different bioenergy crops including corn, canola, soybean, palm, sugar cane and vegetable oil. However, there are some significant challenges, including depleting feedstock supplies, land use change impacts and food use competition, which lead to high prices and inability to completely displace fossil fuel [1-2]. In recent years, use of microalgae as an alternative biodiesel feedstock has gained renewed interest as these fuels are becoming increasingly economically viable, renewable, and carbon-neutral energy sources. One reason for this renewed interest derives from its promising growth giving it the ability to meet global transport fuel demand constraints with fewer energy supplies without compromising the global food supply.

In this study, *Chlorella protothecoides* microalgae were cultivated under different conditions to produce high-yield biomass with high lipid content which would be converted into biodiesel fuel in tandem with the mitigation of high carbon dioxide concentration. The effects of CO_2 using atmospheric and 15% CO_2 concentration and light intensity of 35 and 140 µmol $m^{-2}s^{-1}$ on the microalgae growth and lipid induction were studied. The approach used was to culture microalgal *Chlorella protothecoides* with inoculation of 1×10^5 cells/ml in a 250-ml Erlenmeyer flask, irradiated with cool white fluorescent light at ambient temperature. Using these conditions we were able to determine the most suitable operating

conditions for cultivating the green microalgae to produce high biomass and lipids. Nile red dye was used as a hydrophobic fluorescent probe to detect the induced intracellular lipids. Also, gas chromatograph mass spectroscopy was used to determine the CO_2 concentrations in each culture flask using the closed continuous loop system. The goal was to study how the 15% CO_2 concentration was being used up by the microalgae during cultivation. The results show that the condition of high light intensity of 140 µmol $m^{-2}s^{-1}$ with 15% CO_2 concentration obtain high cell concentration of 7 x 10^5 cells mL^{-1} after culturing *Chlorella protothecoides* for 9 to 10 day in both open and closed systems respectively. Higher lipid content was estimated as indicated by fluorescence intensity with 1.3 to 2.5 times CO_2 reduction emitted by power plants. The particle size of *Chlorella protothecoides* increased as well due to induction of lipid accumulation by the cells when culture under these condition (140 µmol $m^{-2}s^{-1}$ with 15% CO_2 concentration).

Chapter 1 Introduction

1.1 Research Objective

To investigate a new alternative of growing microalgae, *Chlorella protothecoides*, under different conditions to obtain high density biomass accumulated with high lipid contents for biodiesel production while reducing high concentration of carbon dioxide gas. The effect of CO_2 and light intensity on the microalgae growth and lipid induction were studied.

1.2 Research Aim

- ✓ Determine the most optimum combination (CO_2 concentration plus light intensity) for culturing *Chlorella protothecoides* with high cell density
- ✓ To evaluate the most suitable growing conditions which will optimize the induction process of accumulating lipid yield contents of *Chlorella protothecoides* for biodiesel production
- ✓ To sequester CO_2 with the concentration commonly detected in the flue gas of power plants.

1.3 Biodiesel from Microalgae

Due to increasing combustion of fossil carbon footprint, higher fuel prices and depleting feedstock supplies to produce energy in a more stainable fashion, it is understood that biofuel from first and second generation feedstock has the inability to fulfill of our fossil fuel crises, ensure sustainable production and minimum lifecycle GHG emission reduction [1-2, 55]. There are several alternatives which are under consideration to replace current

global transport fuel without compromising global food supply, ecological stability and with minimum environmental impact. One of these alternatives includes third generation biofuel such as microalgae. In recent years, the use of microalgae for production of biofuel such as biodiesel has held huge interest due to their renewable and sustainable features [1-4, 6]. Like many plants, microalgae use sunlight, water and carbon sources to produce oil-like substances which can be converted to biodiesel through photosynthesis [1, 3]. This process involves the reduction of CO_2 by utilizing light and water through photoautotrophs (unusually plants and algae) which help to produce energy storage in the form of reduced carbon components, mostly lipid oil and carbohydrates which are extracted for biodiesel production [3,4]. Biodiesels derived from microalgae have several advantages as compared to current first generation feedstock crops like corn, canola, soybeans, palm, sugar cane, maize, wheat and vegetable oil [1, 7]. Some of these advantages include: the potential to meet global fossil fuel crises using limited land and water resources, no need to compromise global food supply, easy harvesting technique, faster growth rate, higher photosynthetic efficiency, reduction of nitrous oxide and CO_2 gas emissions which are major contributors to serious global warming resulting in higher temperatures of the surface air [7-9]. With new energy independence policy and legislation, such as sustainable biofuel targets in the U.S Energy Policy Act (EPA 2005), Energy Independence and Security Act (EISA 2007), and the European Union (EU 2020), use of microalgae is expected to ensure a safe, reliable living environment by reducing atmospheric CO_2 and increasing energy security [7-8]. Microalgae are considered to be suitable alternative feedstock for biofuel production such as biodiesel.

Microalgae are a diverse group of photosynthetic unicellular microorganisms which grow at a much faster growth rate than plants in most conditional weather condition [2, 9]. They can be cultured in seawater which contained a high amount of CO_2 [2]. The algae can utilize CO_2 fixation by consuming it and releasing oxygen which can be used in the development of life support systems as oxygen producer or food substitute [1, 7-9]. There are different types of microalgae which can be used in the process of making biodiesel production (see some listed in Table 1). Depending on the type of microalgae species, the algae can produce different lipids, hydrocarbons and other complex oil content which is suitable for the production of biodiesel. However, the known total lipid content of microalgae varies from 1-77% and can yield 10-30 times higher the amount of biodiesel production than any other biofuel from the first generation feedstock crops [8, 11]. It was estimated that about 58,700 and 136,900 L/ha of oil annually can be obtained from using microalgae species alone for biodiesel production, occupying 1.1 to 2.5% of the total land area of the U.S while replacing 50% of current fossil fuel as shown in Table 2 [1, 4,10].

Algae lipid contents can be increased under stressful conditions usually caused by light, CO_2, and a shortage of nutrients like nitrogen or phosphate and then converted to biofuel through a transesterification reaction [1, 5-7]. The lipid content present in microalgae consists of neutral lipid, polar lipid, hydrocarbons, as well as percentages of triglycerides and ester which are comprised of free fatty acids and glycerol [11, 55]. In the transesterification reaction, the triglycerides are reacted with methanol to produce methyl esters of free fatty acids that are biodiesel and glycerol in the presence of a catalyst, usually sodium hydroxide, potassium hydroxide or sodium methylate. The catalyst act in converting the methanol to

form strong nucleophiles which react well with the triglycerides to form three new methyl esters as a fuel and glycerol as a byproduct as shown in Figure 1 [11- 14].

In this study, microalgae, *Chlorella protothecoides* was chosen due to its faster growth, easier cultivation and ability to produce lipid content up to 58% of dry weight biomass [1, 4, 8]. *Chlorella protothecoides* is a unicellular green alga of genus Chlorella which contains chlorophyll that can be used for energy and making processed foods more visually appealing [3]. In the cultivation process of the chlorophyll, the microalgae *Chlorella protothecoides* require carbon dioxide, water, sunlight and nutrients to reproduce. *Chlorella protothecoides* has a spherical size about 2 to 10 µm in diameter without flagella as shown in Figure 2. It can be grown in either photoautotrophically or heterotrophically under different culture conditions resulting in higher biomass or lipid content [14].

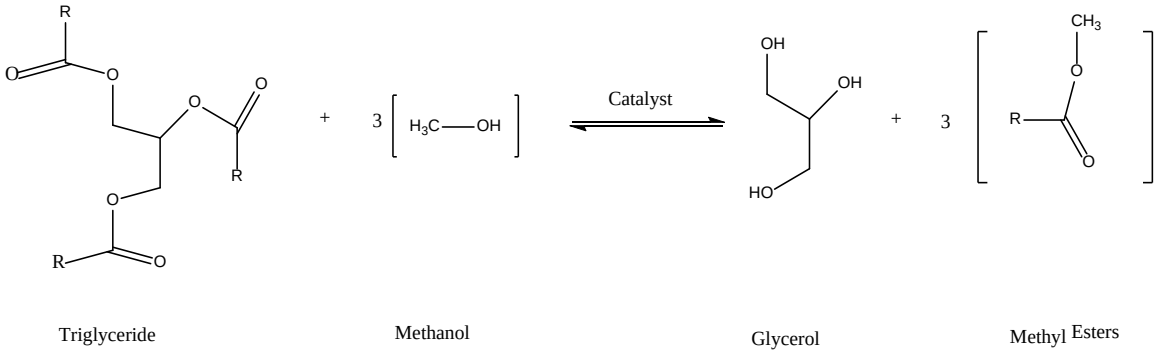

Figure 1. Transesterification reaction process diagram (adapted from [11]).

Table 1. Lipid oil contents of some microalgae [1, 4, 8].

Microalgae Type	Lipid Oil Content (% dry weight)
Ankistrodesmus sp.	24-31
Botryococcus braunii	25-75
Chaetoceros muelleri	33.6
Chaetoceros calciltrans	15-40
Chlorella emersonii	25-63
Chlorella protothecoides	15-58
Chlorella sorokiniana	19-22
Chlorella vulgaris.	5-58
Chlorella sp.	10-48
Crypthecodinium cohnii	20-51
Cylindrotheca sp.	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	>20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzchia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp.	50-77

Table 2. Comparison of biodiesel feedstock sources for meeting 50% of U.S transport fuel needs [8, 10].

Crop Type	Oil Yield (L/ha)	Total Land Area Based on the US (Mha)	Percent of US Existing Crop
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Palm	5950	45	24
Microalgae[a]	136,900	2	1.1
Microalgae[b]	58,700	1.5	2.5

a. *70% of oil by weight in biomass*

b. *30% of oil by weight in biomass*

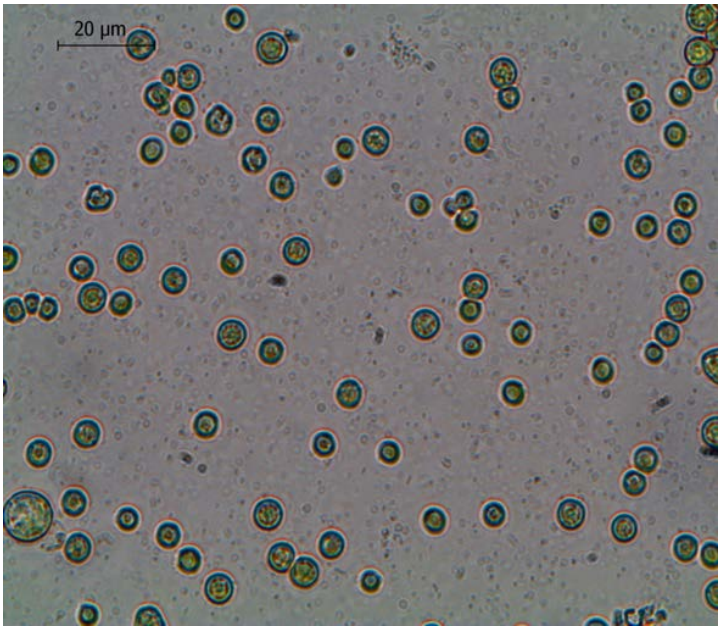

Figure 2. Image of *Chlorella Protothecoides* under light microscopy.

1.4 Carbon Dioxide Sequestration

Carbon dioxide sequestration refers to the removal or reduction of CO_2 from the atmosphere which is generated from fossil fuels being burned by industries related to natural gas processing, iron and steel manufacturing, electricity generation, cement and combustion of municipal solid waste [15, 19, 27]. Typically this is done by photosynthetic organisms such as green plants, algae or bacteria to capture most of the CO_2 emitted by power plants, usually 15%-20% v/v [15, 28, 30]. Flue gases generated from industrial power plants consist of nitrogen (N_2), carbon dioxide (CO_2), oxygen (O_2), water vapor, minor amounts of carbon monoxide (CO), sulfur oxides (SO_x) and nitrogen oxides (NO_x) [25-26]. Among all these flue gases the most global environmental concern is the enormously increased amount of CO_2 concentration in the atmosphere. CO_2 is considered one of the major contributors to "global warming" or "greenhouse effect" which causes extreme weather changes, increase in global temperature, arise in sea level, acidification of the ocean, loss of ecosystems, melting of glaciers and health hazardous to humans [16-18, 26-27].

It was estimated by EPA that in 2011 in the United States, CO_2 accounted for 84% of all U.S greenhouse gas emission, about 6, 0702 million metric tons of CO_2, a 10% increase from 1990-2011 and 31% increase of all level of CO_2 in the atmosphere from since 1750 to 2010 as shown in Figure 3. The waste CO_2 generated in the U.S is shown in Table 3. There has been a lot of efforts to reduce greenhouse gases, helping to make industry processes more sustainable and environmental friendly. Some of these methods include the capture and subsequent sequestration of CO_2 in deep oceans, aquifers, or depleted oil and gas wells, utilization of CO_2 in industrial application, and utilization of other alternative

fuels (such as natural gas and hydrogen) or renewable energy sources (such as wind and solar) that result in the reduction of CO_2 emissions generated [28]. All of these have disadvantage associated with them. Some include higher production cost, inability to consume all or most of the CO_2 generated into the atmosphere, space requirement per unit of energy produced, expense to switch from current system to newest technology, safety issues and waste disposal. Among all these methods, researchers around the world have looked at other alternatives which are more efficient in reducing CO_2 emission from most industry processes and in the atmosphere. Although they found out that biological fixation of CO_2 using microalgae via photosynthesis is more promising in solving the global warming problem [25, 28-29]. With the biological approach, CO_2 is captured by algae and converted into carbon molecules via photosynthetic processes which use light to reduce carbon from CO_2 to complex carbon molecules. These molecules usually act as stored energy such as fuels or fuel precursors.

Table 3. U.S carbon dioxide emissions by source [18].

Factory	Increasing rate from 1990-2011 (%)
Commercial and Residential	11
Agriculture	8
Industry	20
Transportation	33
Electricity	28

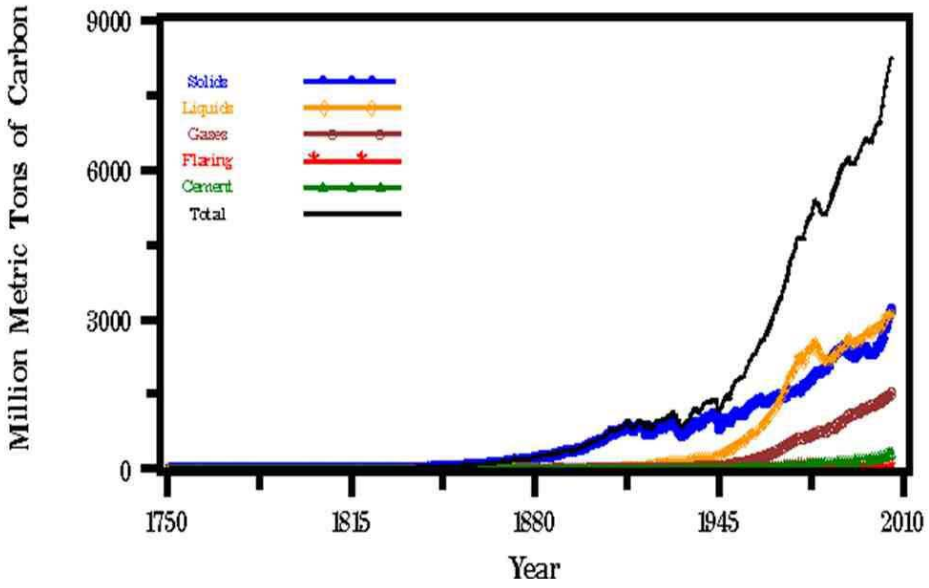

Figure 3. Increasing level of CO_2 in the atmosphere since 1750 [27].

1.5 CO_2 Effect on Microalgae

The growth of microalgae requires CO_2 as one of the main nutrients to carry out photosynthesis. As reported from previous research studies, CO_2 can tune the pH of culture medium and act as the carbon source for microalgal growth [16, 31]. Typically microalgae biomass consists of 40% to 50% carbon by dry weight, meaning that to grow 1.0 kg of algae biomass, it required 1.5-2.0 kg of CO_2 [32]. In the cultivation of microalgae, it is important to know the right amount of CO_2 concentration that is suitable for the different types of microalgae. Different species have various CO_2 tolerances. High CO_2 concentration may result in growth inhibition while lower concentration could limit microalgae cell growth [16, 32-33]. Atmospheric CO_2 of 0.0387% v/v is too low for microalgae growth, therefore requiring to supplement with carbon sources [15, 28, 30]. The carbon sources

include CO_2, H_2CO_3, HCO_3^-, and CO_3^{2-}, but for the cultivation of microalgae only CO_2 and $HCO3^-$ are used. Although high CO_2 concentrations can cause a narcotic effect, some species can tolerate CO_2 concentrations greater than 15% (shown in Table 4).

Table 4. CO_2 tolerance of various algae species (adapted from [16, 34])

Microalgae Species	Maximum tolerable CO_2 Concentration (%)	Reference #
Cyanidium caldarium	100	35
Scenedesmus sp.	80	36
Chlorococcum littorale	60	37
Synechococcus elongatus	60	38
Euglena gracilis	45	39
Chlorella sp.	40	40
Eudorina spp.	20	41
Dunaliella tertiolecta	15	42
Nannochloris sp.	15	43
Chlamydomonas sp.	15	44
Tetraselmis sp.	14	45

In algae photosynthesis, CO_2, water and minerals are converted into oxygen and energy rich organic compounds by utilizing captured light energy [21-22, 28]. The process utilizes photons to produce oxygen, carbohydrates and other compounds into chemical energy such as fuel. The general equation that describes photosynthesis is shown in Equation 1.

$$6\ CO_2 + 12\ H_2O + \text{light source} + \text{green plant} \rightarrow (CH_2O)_6 + 6\ O_2 + 6\ H_2O \qquad (1)$$

This process of photosynthesis involves a light-independent reaction, where carbon dioxide and other compounds are converted into carbohydrates [23-24]. In this process, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH) produced from the light-dependent reaction are utilized, reacting with CO_2 and hydrogen ions to form three-carbon sugar via the Calvin Cycle, newly ADP and NADP are formed. The produced sugar during the light-independent reaction produces a carbon structure which can be used in the production of amino acid and lipids. The overall equation for the light-independent reactions in green plants like microalgae is given in Equation 2.

$$3CO_2 + 9ATP + 6NADPH + 6H^+ \rightarrow C_3H_6O_3 - \text{phosphate} + 9ADP + 8P_i + 6\ NADP + 3H_2O \quad (2)$$

1.6 Light Effect on Algae

Apart from carbon sources, light intensity is necessary for microalgae growth. Light is the limiting factor for both the microalgae growth and lipid composition. It affects directly the growing and photosynthesis of the microalgae. Many microalgae species perform well in different light intensities in order to produce ATP and NADPH. This occur in the present of light via the photosynthesis where photons of light energy are absorbed by chlorophyll molecules and converted into ATP, NADPH and oxygen is released [24]. During the reaction, light energy is used to remove water from the algae via transpiration as shown in Figure 4. In this process of transpiration, the energy source activates the chloroplast in the algae which causes enzyme to diffuse from the water. Then the water is reacted in the

presence of light energy to release oxygen, hydrogen and electrons as shown in Equation 3. After the oxidation of water is accomplished, the produced hydrogen is bonded to form NADPH and produces oxygen as a waste product through a reduction reaction as shown in Equation 4. Finally, in both equations (Equation 2-3), the free electrons form chemical bonds by the reduction of nicotinamide adenine dinucleotide phosphate (NADPH) to NADPH oxidase and adenosine diphosphate (ADP) to adenosine triphosphate (ATP) during the light reaction. The overall equation or the light dependent reaction is shown in Equation 5. Figure 5 show the chemically reactions stages of the photosynthesis process in algae cultivation.

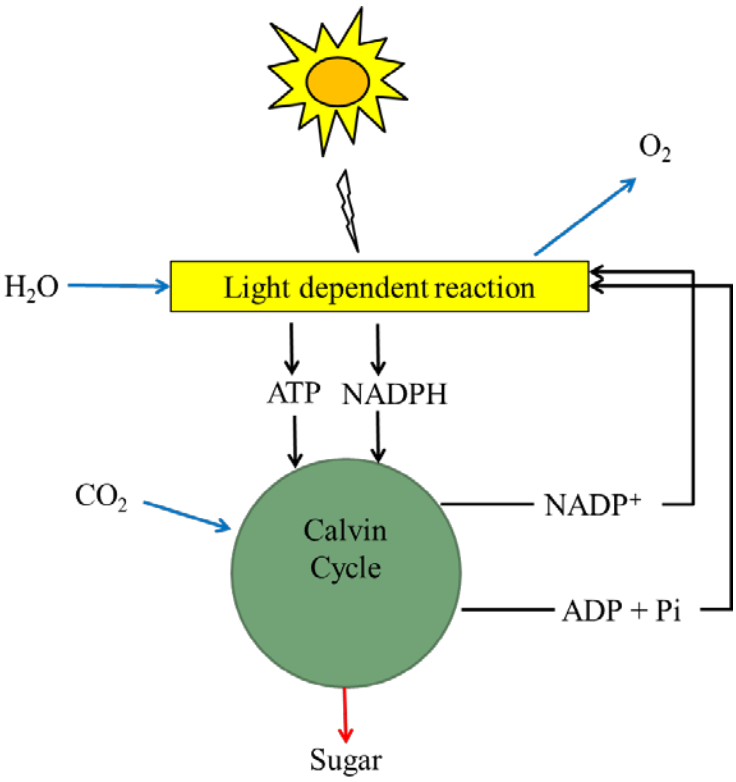

Figure 4. Photosynthesis process that converts photon into chemical energy, splitting water to liberate O_2 via oxidation reaction and fixing CO_2 into sugar.

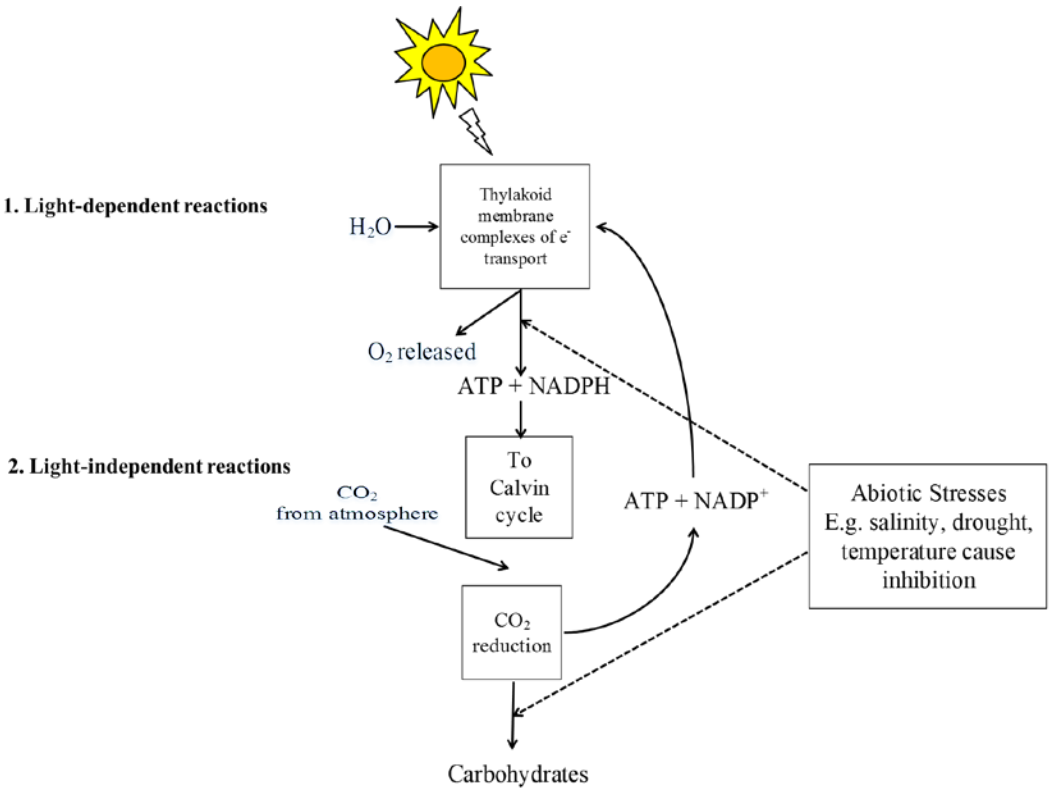

Figure 5. Two chemical reaction stages of photosynthesis (adapted from [23]).

$$12\ H_2O + \text{light source} \rightarrow 6\ O_2 + 24\ H^+ + 24\ e^- \quad (3)$$

$$NADP + H_2O \rightarrow NADP + H^+ + O \quad (4)$$

$$2\ H_2O + 2\ NADP + 2\ ADP + 2\ P_i + \text{light} \rightarrow O_2 + 2\ NADPH + 2H^+ + 2\ ADP \quad (5)$$

As reported from previous research, when increasing light intensity, the growth of microalgae growth is directly proportional to the increased light intensity. When the microalgae cells are exposed to a high light intensity for a long period it causes

photoinhibition. This is due to damage of the repair mechanism of photosystem II which leads to inactivation of the oxygen evolving system and electron carriers, although the light intensity required for most microalgae is relatively low compared to that of higher plants [25, 33, 47]. As reported by Ling et al. (2009), C*hlorella vulgaris* was cultured using different light intensities ranging from 0-185 µmol $m^{-2}s^{-1}$, showing that light intensity of 90 µmol $m^{-2}s^{-1}$ and anything above will cause photoinhibition. Most microalgae have different chlorophyll types which are dependent on different absorption wavelength. Typically, all chlorophylls have absorption wavelength of 450-475 nm and 630-675 nm. Also it is important to know the type of light to use for different algae species. Since algae contain a variety of pigments such as chlorophyll a, lutein, phycobiliproteins, red and blue phycoerythrin and zeaxanthin which react differently to different light sources. Scientifically, it has been suggested to used blue and red light for microalgae cultivation because it penetrates little on the algae suspension than green light [25].

Chapter 2 Materials and Methods

2.1 Microalgae and Medium

The unicellular alga *Chlorella protothecoides* was purchased from the Culture Collection of Algae at University of Texas (Austin, TX, USA). The culture medium used was Bristol's medium which contained 0.25 g $NaNO_3$, 0.025 g $CaCl_2.2H_2O$, 0.075g $MgSO_4.7H_2O$, 0.075 g K_2HPO_4, 0.175 g KH_2PO_4, and 0.025 g NaCl. The pH of the medium was adjusted to 6.83 after sterilization, using 0.1 M NaOH, then 1 g of proteose peptone was added to the final solution and adjusted to one liter solution. The solution was autoclaved at 121°C for 45 min and stored in a refrigerator.

2.2 Cultivation

Chlorella protothecoides was cultivated at a room temperature of 25°C with inoculation of $1x10^5$ cells per mL in a 250-mL Erlenmeyer flask, irradiated with fluorescence light bulbs and cultured at room temperature (25°C). All glassware used in the experiments were cleaned and autoclaved (2340 M Tuttnauer Brinkman Autoclave, Rochester, NY) at 121°C for 45 min before use. Then an initial starter culture solution was made using 200 mL of media, exposed to 2.4 W/m^2 (800 lux) of fluorescent light and allowed to culture for 3 weeks. Later, 106 mL of the starting solution was diluted with 494 mL Bristol medium with a total solution culture of 600 mL. The culture was then divided into four flask of A, B, C and D. Each had 150 mL, carried out in 250-mL Erlenmeyer flasks with constant mixing using magnetic stirring bar and orbital shaker with the speed of 40 rpm, exposed to fluorescent light intensity, normal room air (containing 0.0387% CO_2) and CO_2 (15% CO_2), in an open and closed system as shown in Figure 6-8 respectively.

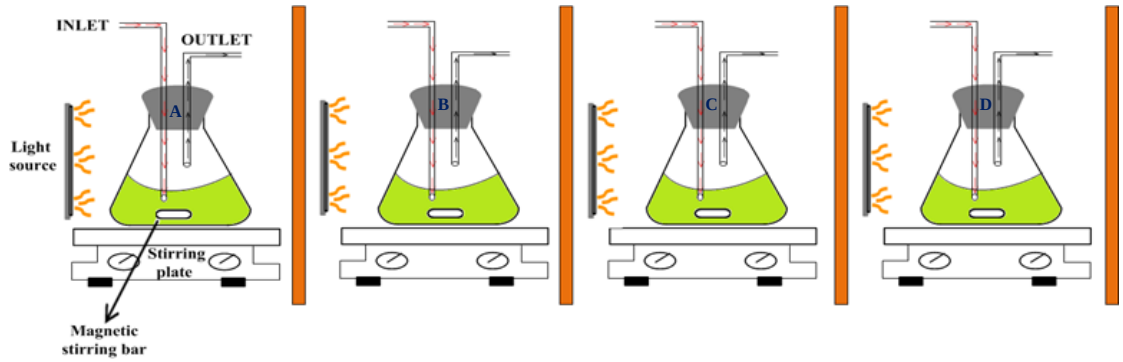

Figure 6. Description of equipment set-up for *Chlorella prototheco*ides cultivation exposed to fluorescent light intensity and normal room air containing 0.037% CO_2 in an open system.

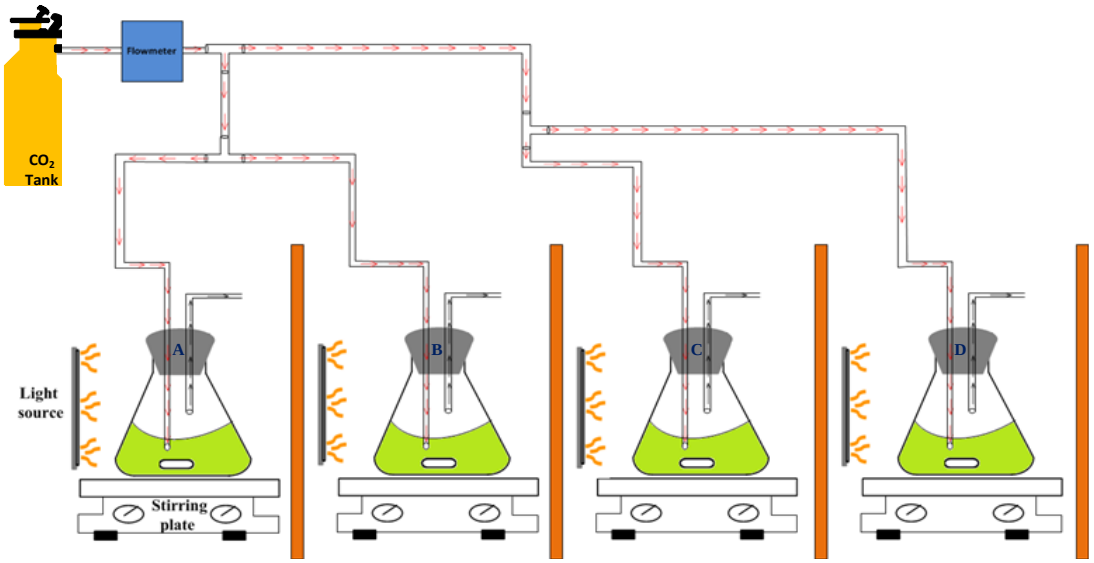

Figure 7. Description of equipment set-up for *Chlorella prototheco*ides cultivation exposed to fluorescent light intensity and 15% CO_2 in an open system.

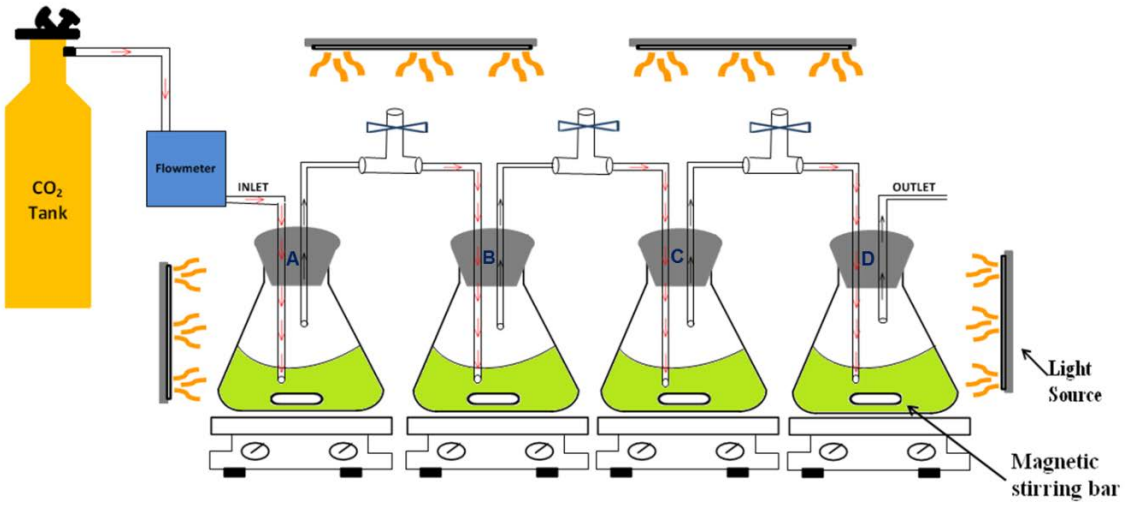

Figure 8. Description of equipment set-up for *Chlorella protothecoides* cultivation exposed to fluorescent light intensity using 15% carbon dioxide in a closed continuous loop system.

2.3 Light Intensity Studied

Each cultured sample was exposed to fluorescent light intensity of 35, 70, 140, and 210 µmol $m^{-2}s^{-1}$ (detected by 3251 Traceable® Dual-Range Light Meter, Fisher Scientific) for flasks A, B, C and D using atmospheric and 15% CO_2, respectively in an open system as described in Figures 6-7 above. The main goal was to study the light effect on the growth of *Chlorella protothecoides*. After studying the initial light effect, light intensity of 35 and 140 µmol $m^{-2}s^{-1}$ were chosen for further investigation due to its higher kinetic growth and cultured lipid content. Further investigation was carried out using 15% CO_2 in a closed continuous loop system shown in Figure 8.

2.4 Carbon Dioxide Studied

The cells were cultivated with inoculation of $1x10^5$ cells per mL in a 250-mL Erlenmeyer flask, irradiated with fluorescent light bulbs and cultured at room temperature (25°C). 15% CO_2 balanced with 85% nitrogen and normal room air containing 0.0387% CO_2 were used. The volumetric flowrate of 15% CO_2 was control at 70 mL/min using a flow meter (Gilmont Industrial Flowmeter, Fisher Scientific). This was regulated at such flow rate (70 mL/min) to ensure equal bubbling in each culture flasks.

2.5 Determination of Cells Growth

A 1 mL sample was taken from each of the stock cultures into 250 ml flask solution, placed into an Eppendorf tube, diluted with one drop of iodide solution (I_2KI) and mixed well. Later a 20 µL Eppendorf droplet of immersion solution was placed on a microscope hemocytometer containing 9 squares. The cells in 5 of the hemocytometer squares were averaged and the total cell counts were obtained. Each sample taken from the culture was used for counting cell concentration and measuring pH readings. The procedure was repeated on a daily and every other day basis.

2.6 Determination of Cells Diameter

A 1 mL sample was taken from each cultured algae solution, placed into cuvette and the average cells diameter was measured with a Zetasizer Nano ZS (Malvern Instrument, Westborough, UK).

2.7 Determination of Cells Imaging

Regular and fluorescent cell image was obtained using a microscope equipped with LAS EZ color and fluorescent camera (Leica EZ DMI3000 B, Buffalo Grove, IL) with objective lenses of 10, 20, & 40X. The microscope also had a shutter UV lamp box. For regular cell imaging, 1 mL sample was taken from each cultured algae solution, placed into an eppendorf tube and mixed well. Later a 20 µL Eppendorf droplet of immersion solution was placed on a microscope slip, attached to the microscope and the cell image was acquired.

2.8 Gas Chromatography Mass Spectrometer (GC/MS)

The CO_2 concentration in each cell culture flask was analyzed by a gas chromatography mass spectrometer (GCMS QP5050,Shimadzu, Canby, OR) using a column of DB-5MS UI with dimension of 25 m x 0.25 mm x 0.25 µm and a flame ionization detector (FID). A sample was taken from each flask as shown in Figure 9. About 0.25 µL of each sample were injected into the column. The parameters for the program were set at 200°C injection temperature of 250°C interface temperature, 32.2 kPa column inlet pressure. One mL per min of column flow and a nitrogen split ratio of 99:1 was used as the carrier.

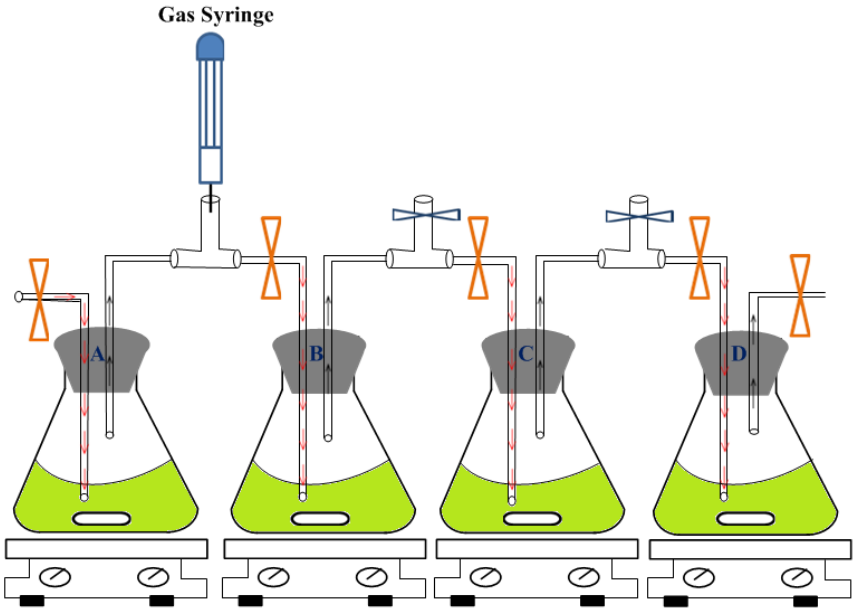

Figure 9. GCMS sampling equipment setup.

2.9 Determination of Lipid Content

The lipid content of the microalgae was detected through the use of Nile red dye (Sigma Aldrich, St Louis, MO). This approach was utilized to study the amount of lipid being produced each day under the different cell cultivation conditions. The dye was used as a hydrophobic fluorescent probe for the detection of lipid deposits in the cell. A stock solution was prepared using 0.001 g of the Nile red in 3 mL of dimethyl sulfoxide (DMSO), stored and protected from light. To stain the algae cells, 1 mL of the cultured algae solution was obtained, centrifuged at 3500 rpm at 4°C for 5 min. The supernatant liquid was separated from the solid cell pellet and discarded. One drop of the Nile red solution was added to the solid cell pellet for 10 min for the dye to enter into the cells wall. Then the mixture was centrifuged, the cell pellets were washed with distilled water, centrifuged

again, 1 mL of culture media added and mixed well. The mixture was examined by a fluorescence microscope. Depending on the amount of cell lipid present in the solution, one could observe the fluorescence under the microscope and determine the cell fluorescence intensity. In addition, cell fluorescence intensity was detected by a spectrofluorometer (Synergy M_x, Biotek,Winooski, VT). This procedure was repeated daily for each culture condition.

For fluorescent imaging, 1 ml sample was taken from each cultured algae solution, placed into an eppendorf tube and centrifuged at 1200 rpm at 4 ^oC for 10 min. The supernatant liquid was separated from the solid cell pellet and discarded. One drop of the Nile red solution was added to the solid cell pellet for 10 min for the dye to enter into the cells wall. Then the mixture was centrifuged, the cell pellets were washed with distilled water, centrifuged again, 1 mL of culture media added and mixed well. A 20 µL Eppendorf droplet of the immersion solution was placed on a microscope slide, attached to the microscope and the fluorescent cells image was acquired. The desired camera objective lenses used for all imaging were 20X and 40X. The procedure was repeated on a daily and every other day basis.

Chapter 3 Results and Discussion

3.1 Growth Kinetics

In Figure 10, it gives the effect of light on the growth of C. *prototheco*ides under a variety of light intensities ranging from 30 to 210 µmol $m^{-2}s^{-1}$ in an open batch culture system exposed to normal room air for a total cultivation period of 8 days (Figure 6). As reported by Ling et al. (2009), C. *vulgaris* was cultured using different light intensities ranging from 0-185 µmol $m^{-2}s^{-1}$. It was found that using light intensity of 0-90 µmol $m^{-2}s^{-1}$ and anything above these conditions could result in photoinhibition. However in this study, the maximum cell density of *C.prototheco*ides obtained was 2.5 x 10^6 cells mL^{-1} using a light intensity of 210 µmol $m^{-2}s^{-1}$ as shown in Figure 11. The average cell sizes obtained were 1.66, 1.18, 1.13 & 1.11 µm for light intensity of 210, 140, 70 and 35 µmol $m^{-2}s^{-1}$, respectively after 8 days of culture (see Figure 12).

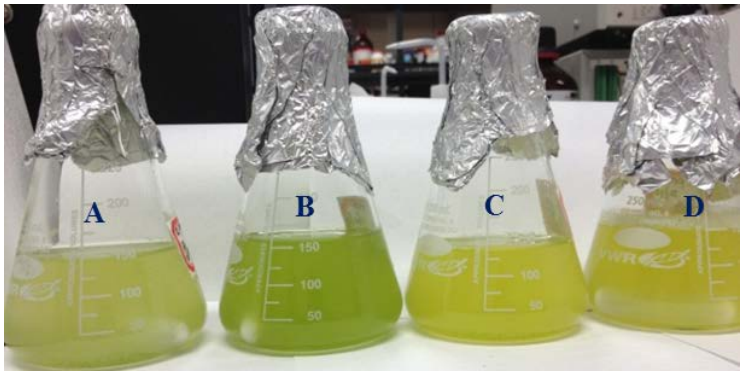

Figure 10. Effect of light intensity on the growth of C. *prototheco*ides. Flask A, B, C & D were irradiated respectively with light intensity of 35, 70, 140 & 210 µmol $m^{-2}s^{-1}$ and exposed to normal room air at ambient temperature. The cultures were inoculated with 1.4×10^5 cells mL^{-1} and grown for 8 days.

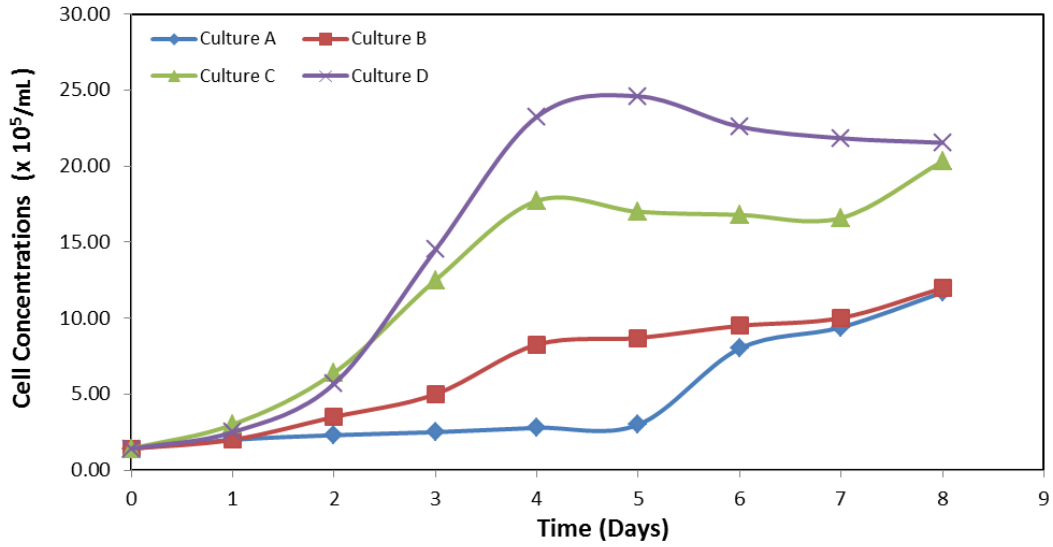

Figure 11. Growth kinetics of *C. prototheco*ides cultures A, B, C & D exposed to normal room air, light intensity of 35, 70, 140 and 210 µmol $m^{-2}s^{-1}$ and ambient temperature with initial cell concentration of 1.4 $\times 10^5$ cells mL^{-1}.

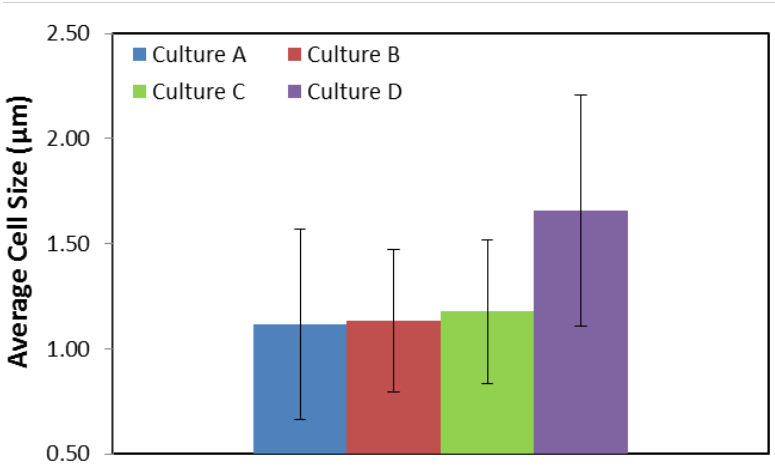

Figure 12. Average cell size of *C. prototheco*ides cultured at (A) 35 (B) 70 [1] (C) 140 and (D) 210 µmol $m^{-2}s^{-1}$and exposed to normal room air with initial cell concentration of 1.4 $\times 10^5$ cells mL^{-1}.

After studying the effect of light on the growth of *C. prototheco*ides under the four light intensities and normal room air, two of the four light intensities (35 and 140 µmol $m^{-2}s^{-1}$)

were chosen for further investigation using 15% CO_2 concentration due to its higher lipid content produced. The primary objective was to study the effect on the growth kinetic of *C. prototheco*ides using both light and CO_2 concentration. Figure 13 shows the combination effect of light and CO_2 on the growth kinetic of *C. prototheco*ides using light intensities of 35 and 140 µmol $m^{-2}s^{-1}$ in a batch culture incubated with 15% CO_2 above for a total cultivation period of 9 days in an open batch system (Figure 7). The maximum cell density of *C. prototheco*ides obtained was 17 × 10^5 cells mL^{-1} using a light intensity of 140 µmol $m^{-2}s^{-1}$as shown in Figure 14. The average cell sizes obtained were 1.69 and 1.50 µm for light intensity of 140 and 35 µmol $m^{-2}s^{-1}$, respectively as shown in Figure 15.

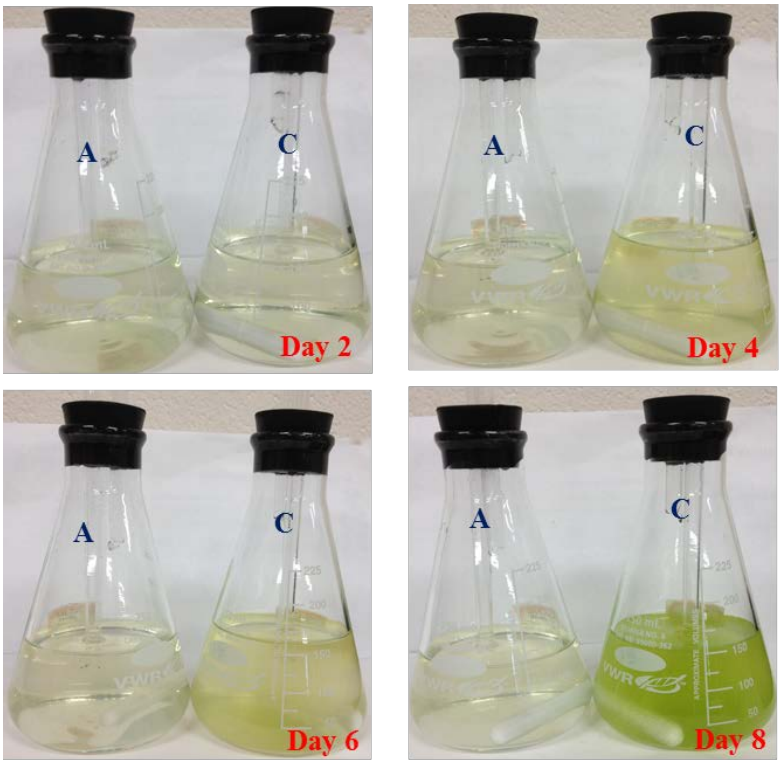

Figure 13. Effect of light intensity and CO_2 on the growth of *C. prototheco*ides. Flasks A & C are exposed to light intensity of 35 & 140 µmol $m^{-2}s^{-1}$, respectively while injecting 15% CO_2 concentration with initial cell concentration of 3.5 ×10^5 cells mL^{-1} for 9 days of cultivation.

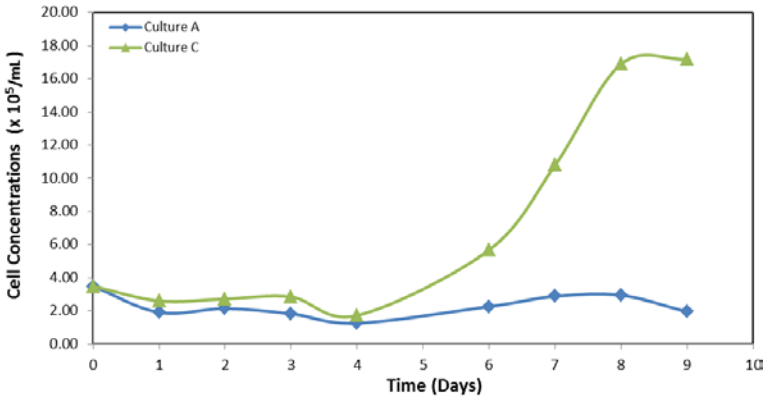

Figure 14. Growth kinetics of *C. protothecoides*. Flasks A & C are exposed to light intensity of 35 & 140 µmol $m^{-2}s^{-1}$, respectively while injecting 15% CO_2 concentration with initial cell concentration of 3.5 ×10^5 cells mL^{-1}.

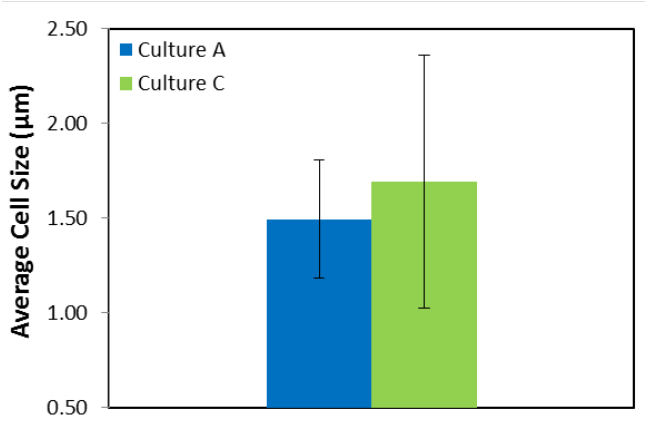

Figure 15. Average cell size of C. *protothecoides* cultured at (A) 35 and (C) 140 µmol $m^{-2}s^{-1}$and 15% CO_2 concentration with initial cells concentration of 3.5 ×10^5 cells mL^{-1}.

As show in Figures 16-17, the effect of light and CO_2 on the growth kinetic of *C. protothecoides* using light intensities of 35 and 140 µmol $m^{-2}s^{-1}$ with 15% CO_2 in a closed continuous loop system (as described in Figure 8) was studied. To study the sequestration of CO_2 concentration by microalgae at each cultivation stage, four new flasks were made and cultured for a total cultivation period of 7 and 10 days for light intensities of 35 and140 µmol $m^{-2}s^{-1}$, respectively.

Figure 16. Effect of light intensity and CO_2 on the growth of *C. protothecoides*. Flasks A, B, C & D were exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the closed continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1} for 7 days of cultivation.

Figure 17. Effect of light intensity and CO_2 on the growth of *C. protothecoides*. Flasks A, B, C & D exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 2×10^5 cells mL^{-1} for 10 days of cultivation.

The maximum cell densities of *C. protothecoides* obtained were 1.3 ×10^6 and 1.1 × 10^6 cells mL^{-1} as shown in Figures 18 and 19, respectively. The average cell size obtained were 2.02, 1.98, 1.39, 1.43, 1.43 µm for light intensity of 35 $m^{-2}s^{-1}$ and 1.83, 1.69, 2.46, 2.44µm for light intensity of 140 µmol $m^{-2}s^{-1}$ as shown in Figures 20 and 21, respectively.

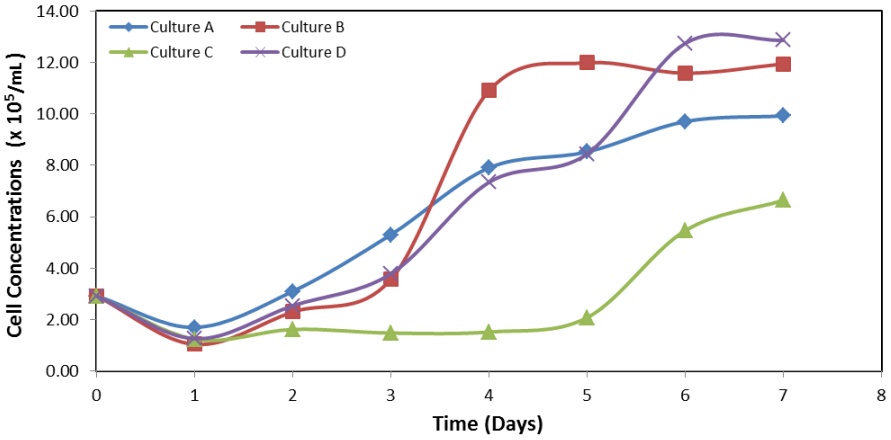

Figure 18. Growth kinetics of *C. protothecoides*. Flasks A, B, C & D were exposed to the same light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the closed continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1}.

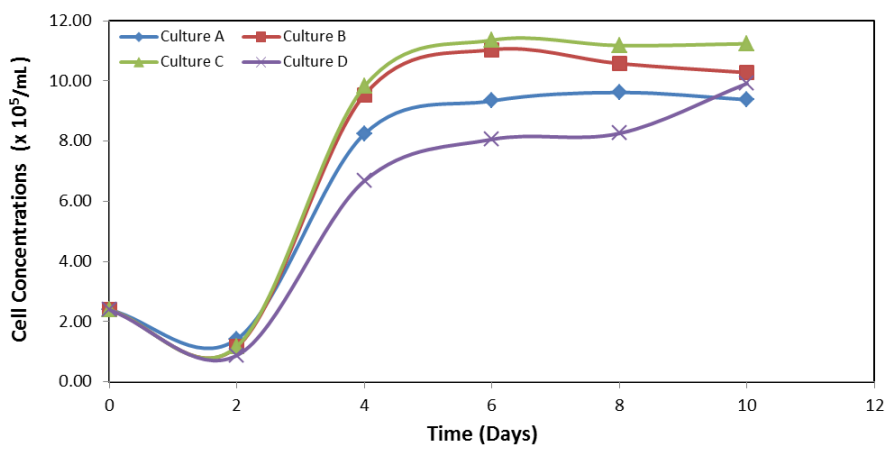

Figure 19. Growth kinetics of *C. protothecoides*. Flasks A, B, C & D were exposed to the same light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the closed continuous loop system with initial cell concentration of 2 × 10^5 cells mL^{-1}.

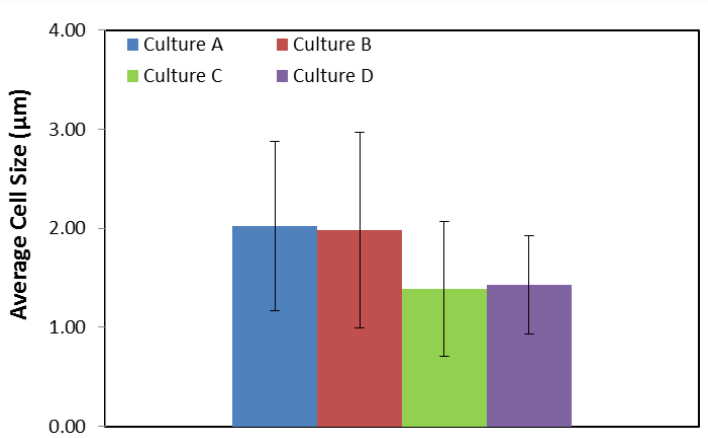

Figure 20. Average cell size of C. *protothecoides* cultured at light intensity of 35 µmol $m^{-2}s^{-1}$ using 15% CO_2 concentration in the continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1}.

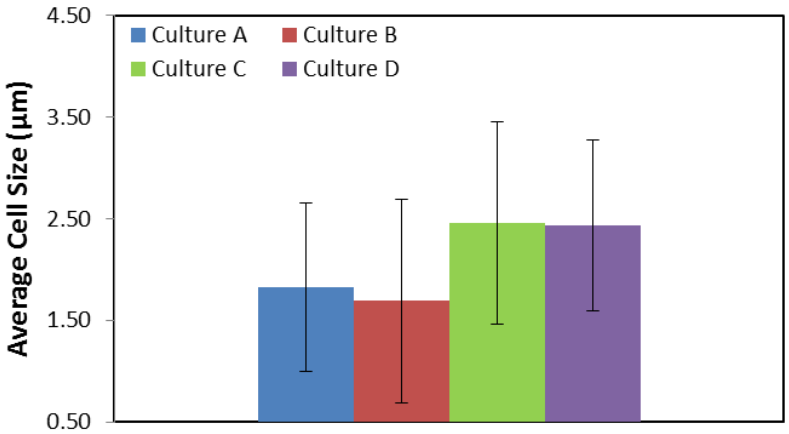

Figure 21. Average cell size of *C. protothecoides* cultured at light intensity of 140 µmol $m^{-2}s^{-1}$ using 15% CO_2 concentration in the continuous loop system with initial cell concentration of 2×10^5 cells mL^{-1}.

The results suggested as the light intensity increases, the cell concentration increases exponentially and photoinhibition begin to occur. Increased light intensity causes the algae cultures to obtain a yellowish color in the open system when exposed to normal atmospheric CO_2. This effect was probably because the cells were under too much

photoinhibition stress with reduced carbon and nutrient source which resulted in pH change. These different findings on the effects of the light intensity on cell growth kinetics could have been due to the fact that, as photoinhibition occurred, the cell multiplication became stagnant because the cells closer to the light source were inactive and the cells at the center were less affected. It was also observed that with high light and high CO_2 concentration in both open and closed systems, the microalgae cultures obtained a darker green color. The result illustrates that with high light and high CO_2 concentration, the cell growth responded well with increased cell concentration after day 5 of cultivation stage without any photoinhibition effect. The increase in light played an important role in the photosynthesis of the microalgae. As the light increases, the photosynthesis and photosystem 2 (PSII) efficiency declines due to photo damage of the cell wall caused by absorption of photon energy to accumulate lipid [51]. The electron acceptor which is needed for the photosynthetic reaction decreases as the light increases, causing an oxidative damage to the polyunsaturated fatty acid (PUFA) [55].

3.2 pH Effect on Growth Kinetics

In order to study the carbon and nutrient effect on the algae, pH was measured daily for each experiment. The initial pH for the medium was 6.83 for all algae culture. Figures 22 and 23 give the pH profile of *C. protothecoides* cultured at different light intensities, exposed to normal room air and 15% CO_2 concentration, respectively cultured in an open system. Figures 24 and 25 show the pH profile of *C. protothecoides* at light intensities of 35 and 140 µmol $m^{-2}s^{-1}$ using 15% CO_2 concentration cultured in a closed continuous loop system.

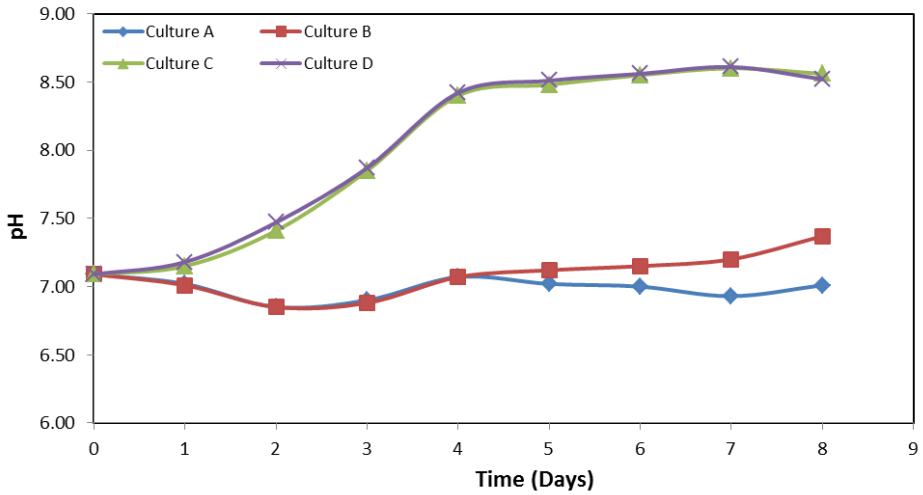

Figure 22. pH measurement of *C. prototheco*ides. Flasks A, B, C & D were exposed to normal room air and light intensity of 35, 70, 140 and 210 µmol $m^{-2}s^{-1}$, respectively in an open system with initial cell concentration of 1.4 ×10^5 cells mL^{-1}.

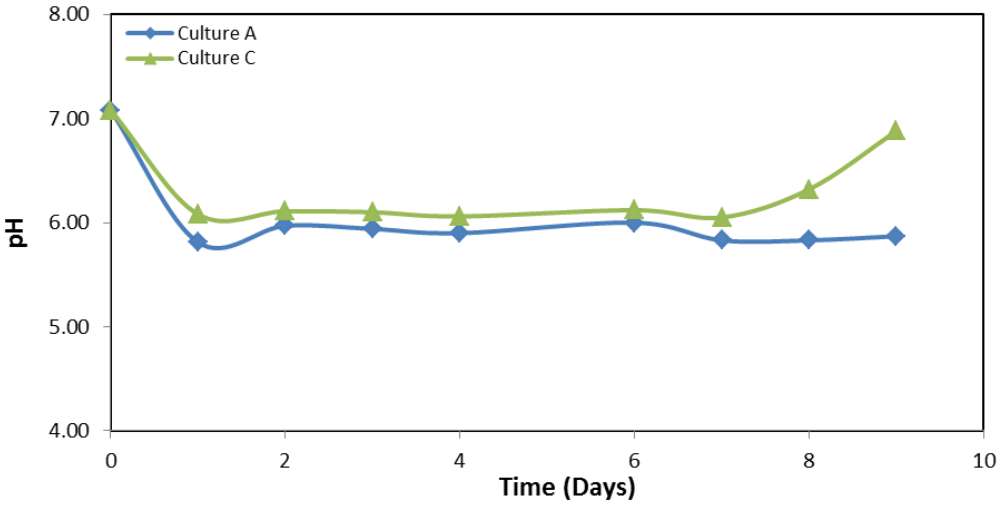

Figure 23. pH measurement of *C. prototheco*ides. Flasks A & C were exposed to light intensity of 35, & 140 µmol $m^{-2}s^{-1}$, 5% CO_2 concentration in an open system with initial cell concentration of 3.5 × 10^5 cells mL^{-1}.

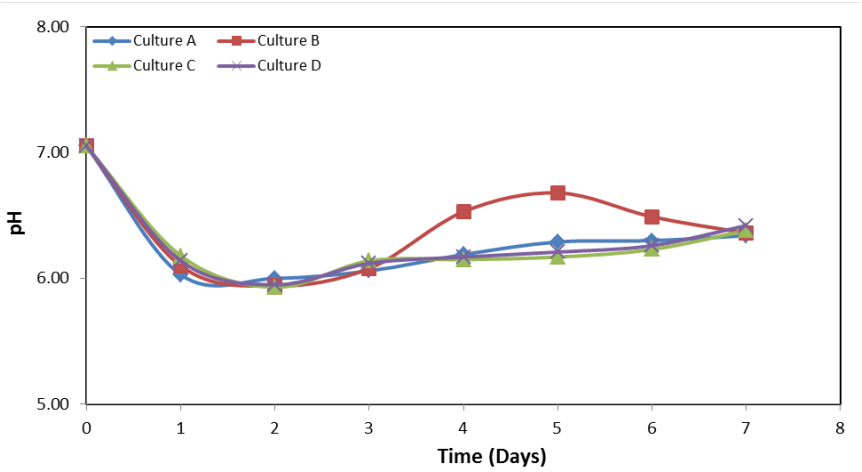

Figure 24. pH measurement of C*hlorella prototheco*ides cultures A, B, C & D exposed to light intensity of 35 µmol $m^{-2}s^{-1}$ and 15% CO_2 concentration cultured in the continuous loop system with initial cell concentration of 3 x 10^5 cells mL^{-1}.

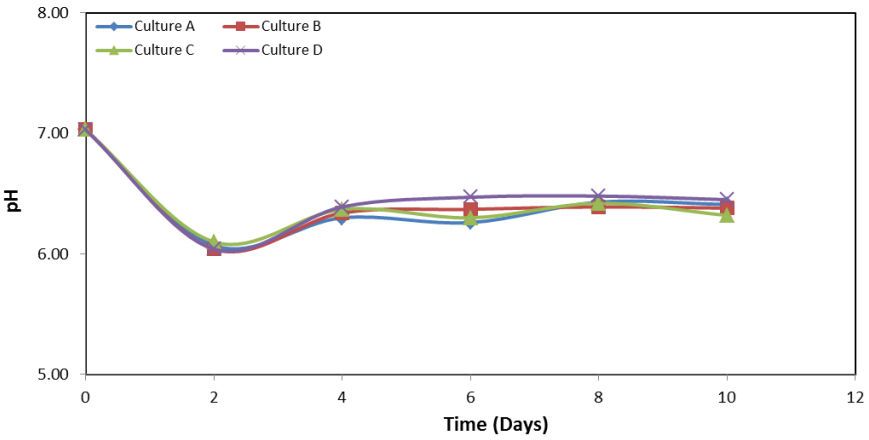

Figure 25. pH measurement of *C. prototheco*ides. Flasks A, B, C & D were exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration cultured in the continuous loop system with initial cells concentration of 2 ×10^5 cells mL^{-1}.

The results indicate that, as the light intensity increased when exposed to normal room air, the pH increased. When the microalgae culture was exposed to light intensities of 35 and 140 µmol $m^{-2}s^{-1}$ using 15% CO_2 concentration and cultured in a closed continuous loop system, the pH decreased. As the microalgae grew, the faster they consumed CO_2, the

higher pH was obtained. As reported by Chen et al. (1994), high pH results in higher carbonate, lower bicarbonate and molecular CO_2 level in the microalgae culture. In such condition where there is less carbon dioxide available for photosynthesis in water, it decreases the microalgae abundance over time due to high alkalinity [53, 54]. In the photosynthesis process, the CO_2 reacts with the water to form H^+ and H CO_3- or CO_3^{2-}.

3.3 Lipid Induction

The lipid contents of *C. prototheco*ides were compared using different light intensities and carbon dioxide concentrations. Figures 26 and 27 give the total relative fluorescence intensity relating to lipid content of *C. prototheco*ides at different light intensities, exposed to normal room air and 15% CO_2 concentration, respectively cultured in an open system. Figures 28 and 29 shows the total relative fluorescence intensity relating to lipid contents of *C. prototheco*ides at light intensities of 35 and 140 µmol $m^{-2}s^{-1}$ using 15% CO_2 concentration cultured in a closed continuous loop system.

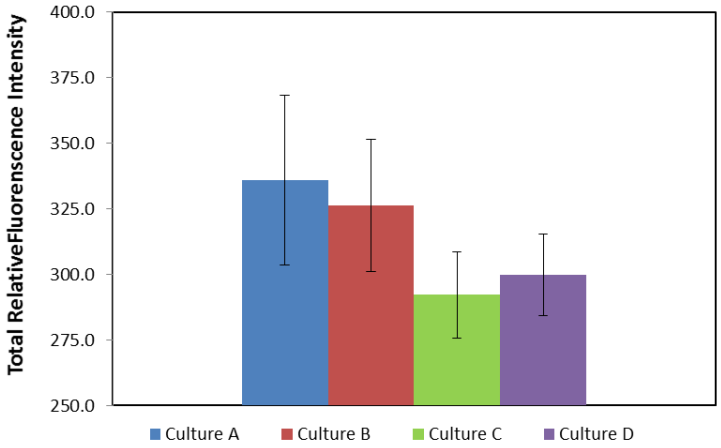

Figure 26. Lipid concentration as indicated by fluorescence of *C. prototheco*ides. Flasks A, B, C & D were exposed to normal room air and light intensities of 35, 70, 140 and 210 µmol $m^{-2}s^{-1}$, respectively in an open system with initial cell concentration of 1.4 ×10^5 cells mL^{-1} for 8 days of cultivation.

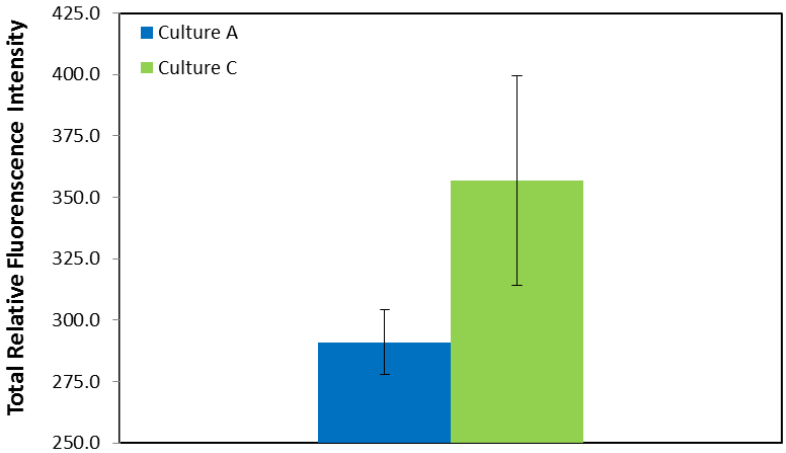

Figure 27. Lipid concentration as indicated by fluorescence of *C. prototheco*ides. Flasks A & C were exposed to light intensity of 35 & 140 µmol $m^{-2}s^{-1}$, respectively while using 15% CO_2 concentration in an open system with initial cell concentration of 3.5×10^5 cells mL^{-1} for 9 days of cultivation.

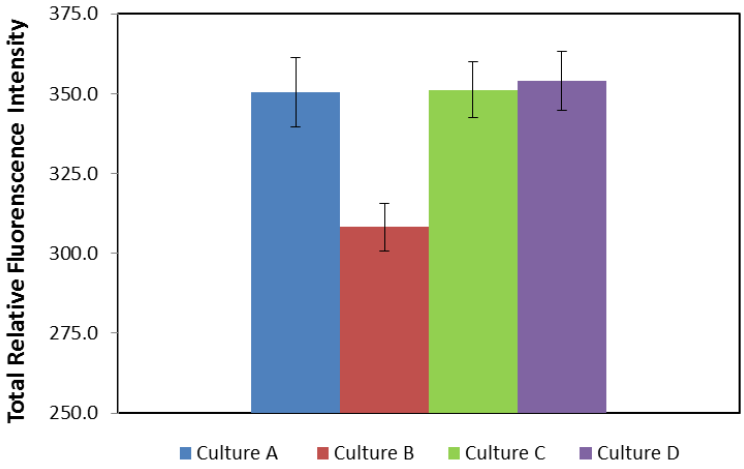

Figure 28. Lipid concentration as indicated by fluorescence of *C. prototheco*ides. Flasks A, B, C & D were exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1} for 7 days of cultivation.

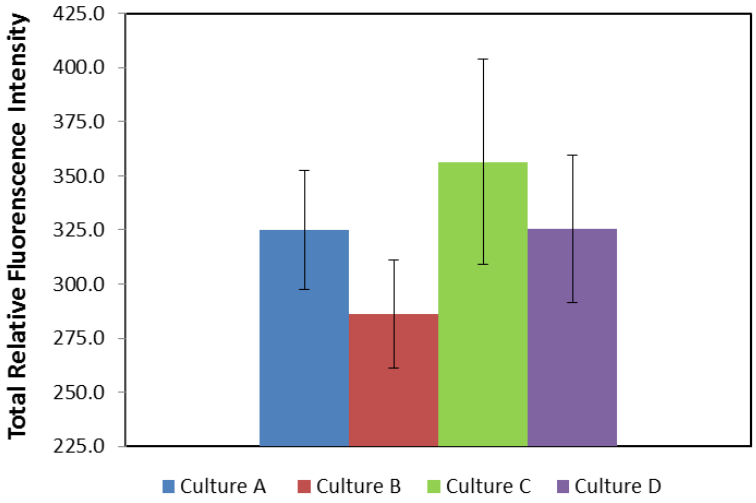

Figure 29. Lipid concentration as indicated by fluorescence of *C. protothecoides*. Flasks A, B, C & D were exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 2×10^5 cells mL^{-1} for 10 days of cultivation.

Figures 30 and 31 give the total relative fluorescence intensity per cells relating to lipid content of *C. protothecoides* at different light intensities, exposed to normal room air and 15% CO_2 concentration, respectively culture in an open system. Figures 32 and 33 shows the total relative fluorescence intensity per cells relating to lipid contents of *C. protothecoides* at light intensities of 35 and 140 µmol $m^{-2}s^{-1}$ using 15% CO_2 concentration cultured in a closed continuous loop system.

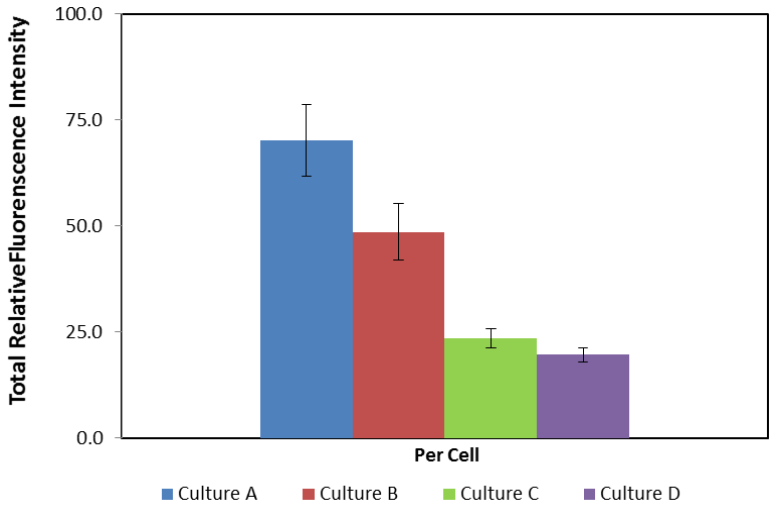

Figure 30. Lipid concentration per cell as indicated by fluorescence of *C. prototheco*ides. Flasks A, B, C & D were exposed to normal room air and light intensities of 35, 70, 140 and 210 µmol $m^{-2}s^{-1}$, respectively in an open system with initial cell concentration of 1.4 $\times 10^5$ cells mL^{-1} for 8 days of cultivation.

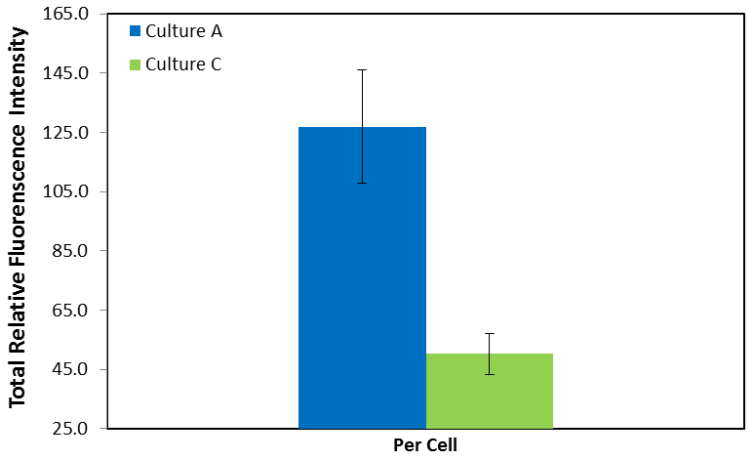

Figure 31. Lipid concentration per cell as indicated by fluorescence of *C. prototheco*ides. Flasks A & C were exposed to light intensity of 35 & 140 µmol $m^{-2}s^{-1}$, respectively while using 15% CO_2 concentration in an open system with initial cell concentration of 3.5 $\times$ 10^5 cells mL^{-1} for 9 days of cultivation.

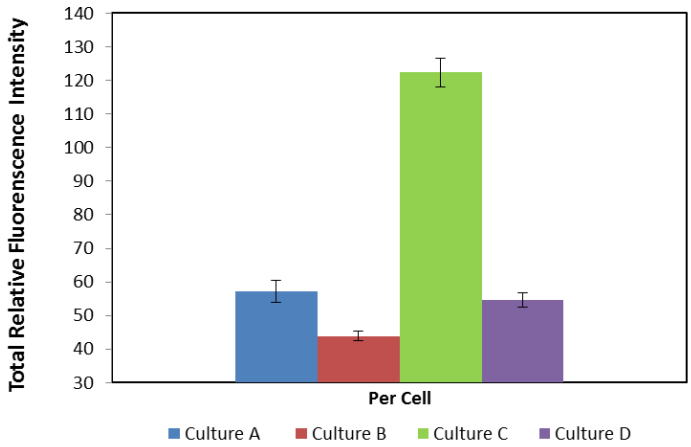

Figure 32. Lipid concentration per cell as indicated by fluorescence of *C.prototheco*ides. Flasks A, B, C & D were exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1} for 7 days of cultivation.

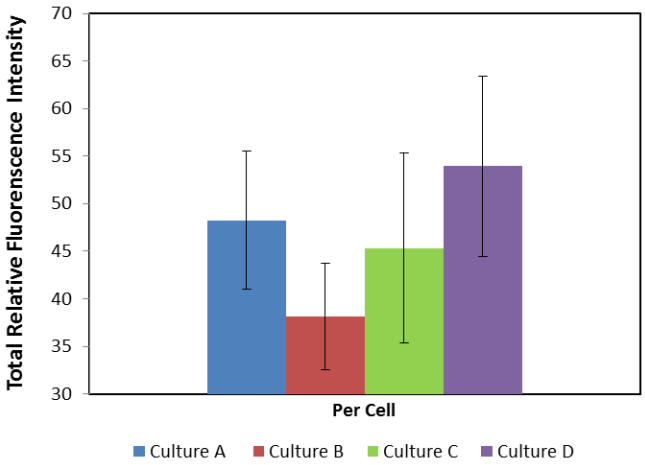

Figure 33. Lipid concentration per cell as indicated by fluorescence of *C. prototheco*ides. Flasks A, B, C & D were exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 2×10^5 cells mL^{-1} for 10 days of cultivation.

The results show that the microalgae produce higher lipid contents under the light intensity of 30 µmol $m^{-2}s^{-1}$ when exposed to normal atmospheric CO_2 cultured in the open system. The maximum fluorescence intensity of *C. prototheco*ides obtained under this condition was 336 (Figure 26). With high light and high CO_2 concentration in both open and closed

systems, the microalgae performed well, producing higher lipid contents indicated my fluorescence. Under this condition (high light and high CO_2 concentration), the total lipid content increases while the lipid per cell decreases. The maximum fluorescence intensity of *C. protothecoides* obtained was 356.8 (Figure 27). As reported from previous research studies, it showed that an increase in carbon source helps accumulation of higher lipid contents in microalgae cells [50]. It was also reported, low light intensity, induces the formation of the polar lipids membranes which are associated with chloroplasts whereas high light decreases the total polar lipid content, increasing the level of neutral lipid storage of triacylglycerols (TAGs) [55-61]. Under high light and high CO_2 concentration in microalgae cultivation, it helps to protect the mechanism of the cells while producing higher fatty acid in stored TAG [55]. The differences in results were believed to be due to complete photosynthesis, consumption of CO_2 by the cells and synthesizing higher lipid content by the effect of the light.

3.4 CO_2 Sequestration

Carbon dioxide consumption by *C. protothecoides* under different light intensities and CO_2 concentration was measured using a GCMS for each cell cultures in both open and closed systems. The primary goal was to monitor the uptake of CO_2 and the amount of oxygen released in each culture flask by the microalgae. The result was analyzed using the GCMS average relative CO_2 and O_2 percent intensity for the injected 15% CO_2 balanced with 85% nitrogen in each algae culture. As show in Figures 34 -36, the effluent CO_2 concentration for *C. protothecoides* culture at light intensities of 35 & 140 µmol $m^{-2}s^{-1}$ using 15% CO_2 concentration cultured both in open and closed systems.

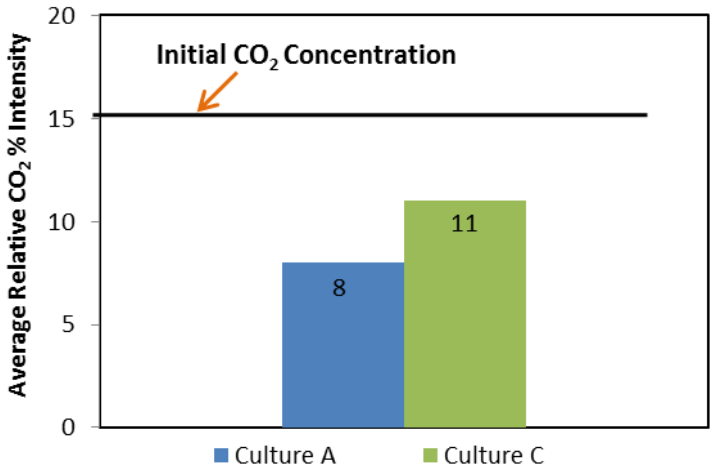

Figure 34. Effluent CO_2 concentration released in the cultures A & C of *C. protothecoides* when exposed to light intensities of 35 & 140 µmol $m^{-2}s^{-1}$, respectively using 15% CO_2 concentration cultured in an open system with initial cells concentration of 3.5×10^5 cell mL^{-1} for9 days of cultivation.

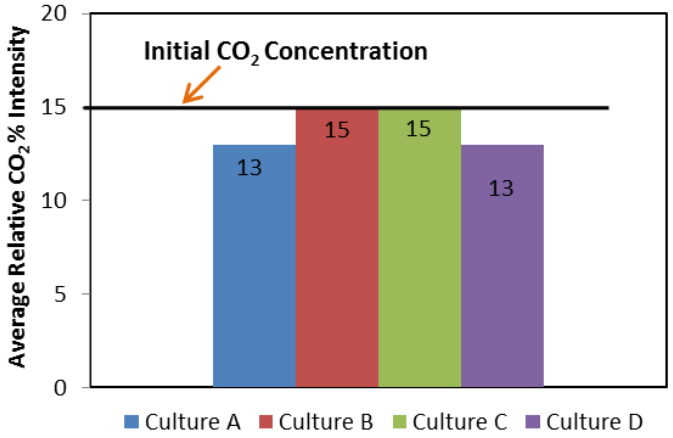

Figure 35. Effluent CO_2 concentration released in the cultures A, B, C & D of *C. protothecoides* when exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1} for 7 days of cultivation.

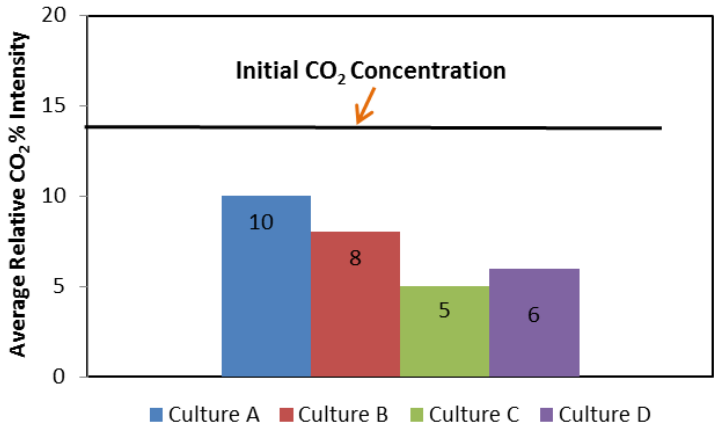

Figure 36. Effluent CO_2 concentration released in the cultures A, B, C & D of *C. prototheco*ides when exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1} for 10 days of cultivation.

Figures 37-39, show the effluent O_2 concentration intensity of *C. prototheco*ides at light intensities of 35 & 140 µmol $m^{-2}s^{-1}$ using 15% CO2 concentration cultured both in open and closed systems.

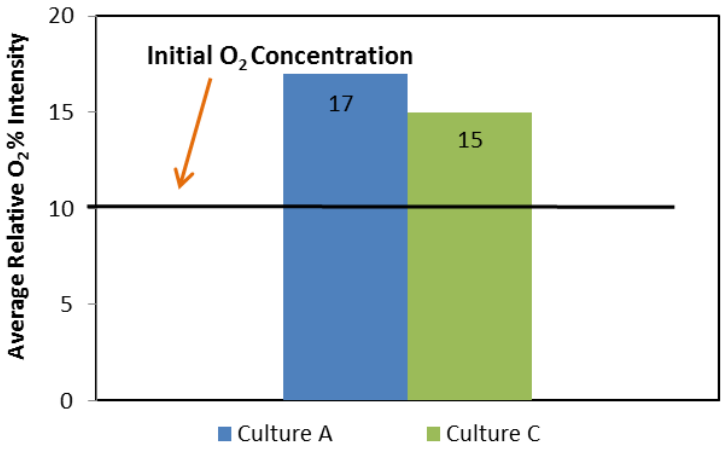

Figure 37. Effluent O_2 concentration released in the cultures A & C of *C. prototheco*ides when exposed to light intensities of 35 & 140 µmol $m^{-2}s^{-1}$, respectively using 15% CO2 concentration cultured in an open system with initial cells concentration of 3.5×10^5 cell mL^{-1} for 9 days of cultivation.

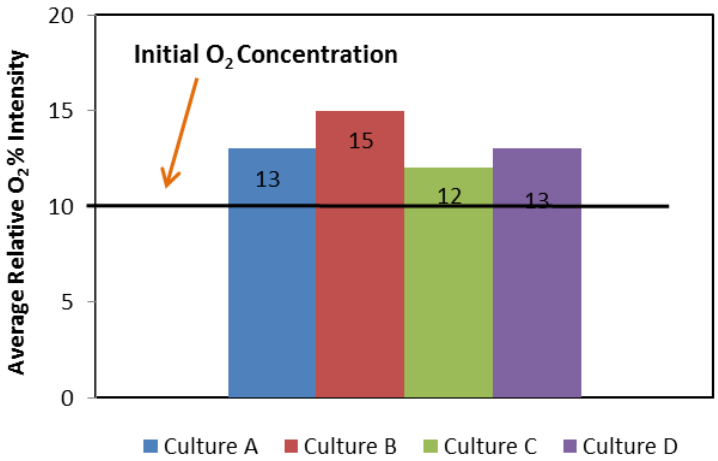

Figure 38. Effluent O_2 concentration released in the cultures A, B, C & D of *C. protothecoides* when exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1} for 7 days of cultivation.

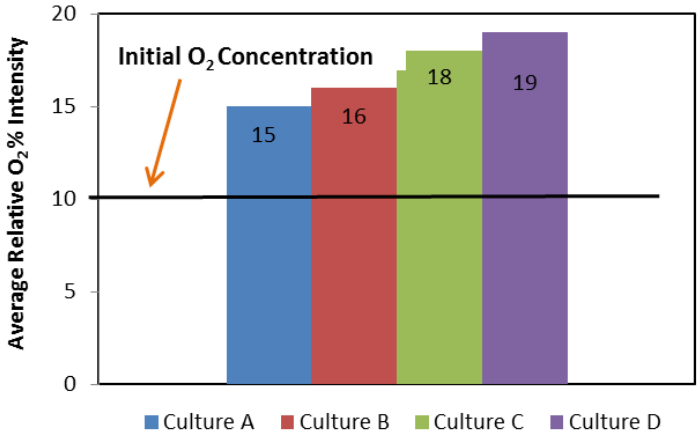

Figure 39. Effluent O_2 concentration released in the cultures A, B, C & D of *C. protothecoides* when exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system with initial cell concentration of 3×10^5 cells mL^{-1} for 10 days of cultivation.

The results show that under light intensity of 35 µmol $m^{-2}s^{-1}$ and high CO_2 concentration in both open and closed systems, the microalgae did not performed well. The algae did not grown until after day 5 of cultivating resulting in consumption of the CO_2 due to oxygen

built up in the each culture flask. The CO_2 concentration in the culture was still high, allowing the microalga to produce less lipid contents as compared to the case using high light and high CO_2 concentration. Under light and high CO_2 concentration in the closed continuous loop system, the microalgae consumed 1.3 to 2.5 times of the initial 15% CO_2 concentration after 10 days of cultivation.

Chapter 4 Conclusion

As demonstrated in this research, microalgae *Chlorella prototheco*ides was grown in an open, closed continuous loop system, exposed to different light intensities (35, 70, 140, 210 $m^{-2}s^{-1}$) with the used of normal room air and 15% CO_2 concentration. The primary goals was to increase the algae biomass and lipid accumulation for biodiesel production in tandem with sequestration of high CO_2 concentration. The results showed that the optimum growth condition of *Chlorella prototheco*ides were estimated using a light intensity of 140 µmol $m^{-2}s^{-1}$ and 15% CO_2 concentration. Under such condition (140 µmol $m^{-2}s^{-1}$ and 15% CO_2 concentration), photoinhibition of the microalgae *Chlorella prototheco*ides was observed. High average cell concentrations of 7×10^5 cells mL^{-1} were obtained when cultured in both open and close system. The particle size of the microalgae, *Chlorella prototheco*ides increases, total lipid accumulation were increased with increasing light intensity and use of 15% CO_2 concentration as indicated by fluorescence intensity under the light microscopy using Nile Red dye. Using both experimental method of culturing *Chlorella prototheco*ides in an open and closed continuous loop system with 15% CO_2 concentration. The results indicated that *Chlorella prototheco*ides consumed the CO_2 faster in the closed continuous loop system reducing the CO_2 concentration from 15% to 5% overall, about 1.3% to 2.5% CO_2 reduction.

Chapter 5 Future Work

- Use upper limit of CO_2 concentration (> 20%) to study the effect on the growth of *Chlorella protothecoides* under light intensities higher than 140 µmol $m^{-2}s^{-1}$.

- Establish an efficient model on carbon dioxide sequestration using the closed continuous loop system.

- Develop lipid extraction process which is suitable for extracting the algae oil and compared with the results obtained by Nile red dye.

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Appendix A

Table 5. Raw data of *C. prototheco*ides. Flask A was exposed to light intensity of 35 µmol $m^{-2}s^{-1}$ and normal room air at ambient temperature. The culture were inoculated with 1.4 × 10^5 cells mL^{-1} and grown for 8 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.09	56.6	1.42	298	0.8713
1	7.02	10	2.00	320	0.6109
2	6.85	115	2.30	340	0.7893
3	6.90	12.5	2.50	337	1.1885
4	7.07	139	2.78	336	2.2070
5	7.02	15	3.00	340	1.1680
6	7.00	40	8.00	375	1.0808
7	6.93	470	9.40	389	0.9801
8	7.01	585	11.70	288	1.1310
		Average	**4.8**	**335.9**	**1.11**

Table 6. Raw data of *C. prototheco*ides. Flask B was exposed to light intensity of 70 µmol $m^{-2}s^{-1}$ and normal room air at ambient temperature. The culture were inoculated with 1.4 × 10^5 cells mL^{-1} and grown for 8 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.09	56.6	1.42	298	0.8713
1	7.01	10	2.00	310	0.9693
2	6.85	175	3.50	349	0.8992
3	6.88	25	5.00	305	0.8147
4	7.07	413	8.26	320	0.8010
5	7.12	43.5	8.70	300	1.2231
6	7.15	47.5	9.50	335	1.3866
7	7.20	501	10.02	363	1.4970
8	7.37	599	11.98	356	1.7330
		Average	**6.7**	**326.2**	**1.13**

Table 7. Raw data of *C. protothecoides*. Flask C was exposed to light intensity of 140 µmol $m^{-2}s^{-1}$ and normal room air at ambient temperature. The culture were inoculated with 1.4×10^5 cells mL^{-1} and grown for 8 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cell size (µm)
0	7.09	56.6	1.42	298	0.8713
1	7.15	15	3.00	290	0.7719
2	7.41	320	6.40	278	0.7768
3	7.85	62.5	12.50	274	1.0531
4	8.40	886	17.72	279	1.4330
5	8.48	85	17.00	285	1.5028
6	8.55	84	16.80	290	1.2119
7	8.60	830	16.60	321	1.2425
8	8.56	1016	20.32	315	1.7435
		Average	**12.4**	**292.2**	**1.18**

Table 8. Raw data of *C. protothecoides*. Flask D was exposed to light intensity of 210 µmol $m^{-2}s^{-1}$ and normal room air at ambient temperature. The culture were inoculated with 1.4×10^5 cells mL^{-1} and grown for 8 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.09	56.6	1.42	298	0.8713
1	7.18	12.5	2.50	286	0.9612
2	7.47	284	5.68	284	1.0905
3	7.87	72.5	14.50	310	1.6428
4	8.42	1163	23.26	336	1.9255
5	8.51	123	24.60	300	2.1291
6	8.56	113	22.60	292	2.2143
7	8.61	1092	21.84	299	2.2545
8	8.52	1077	21.54	293	1.8335
		Average	**15.3**	**299.8**	**1.66**

Appendix B

Table 9. Raw data on *C. prototheco*ides. Flask A was exposed to light intensity of 35 µmol $m^{-2}s^{-1}$ and 15% CO_2 concentration. The culture were inoculated with 3.5×10^5 cells mL^{-1} and grown for 9 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.08	17.45	3.49	279	1.4515
1	5.81	95	1.90	292	1.8268
2	5.97	107	2.14	273	1.1100
3	5.94	91	1.82	276	1.6755
4	5.90	63	1.26	292	2.0230
6	6.00	113	2.26	298	1.3200
7	5.83	144	2.88	296	1.6330
8	5.83	147	2.94	297	1.2220
9	5.87	97	1.94	315	1.1945
		Average	**2.3**	**290.9**	**1.50**

Table 10. Raw data on *C. prototheco*ides. Flask C was exposed to light intensity of 140 µmol $m^{-2}s^{-1}$ and 15% CO_2 concentration. The culture were inoculated with 3.5×10^5 cells mL^{-1} and grown for 9 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.08	17.45	3.49	279	1.4515
1	6.08	130	2.60	315	1.3045
2	6.11	136	2.72	340	1.0912
3	6.10	142	2.84	347	1.0323
4	6.06	86	1.72	354	1.3340
6	6.12	284	5.68	371	1.3095
7	6.05	540	10.80	395	2.6230
8	6.32	845	16.90	395	2.4865
9	6.88	859	17.18	415	2.5895
		Average	**7.1**	**356.8**	**1.69**

Appendix C

Table 11. Raw data on *C. protothecoides*. Flask A was exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 3×10^5 cells mL^{-1} and grown for 7 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cell size (µm)
0	7.05	117	2.93	354	0.9076
1	6.03	85	1.70	336	1.3514
2	6.00	155	3.10	333	0.7703
3	6.06	265	5.30	348	2.6670
4	6.19	395	7.90	354	2.6059
5	6.29	426	8.52	358	2.6435
6	6.30	485	9.70	360	2.7138
7	6.34	496	9.92	362	2.5393
		Average	**6.1**	**350.6**	**2.02**

Table 12. Raw data on *C. protothecoides*. Flask B was exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 3×10^5 cells mL^{-1} and grown for 7 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cell size (µm)
0	7.05	117	2.93	354	0.9076
1	6.10	52	1.04	345	1.0159
2	5.94	115	2.30	334	0.9850
3	6.08	178	3.56	339	1.5905
4	6.53	545	10.90	342	2.1118
5	6.68	599	11.98	347	2.9650
6	6.49	579	11.58	350	3.0470
7	6.36	597	11.94	356	3.2123
		Average	**7.0**	**345.9**	**1.98**

Table 13. Raw data on *C. protothecoides*. Flask C was exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 3×10^5 cells mL^{-1} and grown for 7 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cell size (µm)
0	7.05	117	2.93	354	0.9076
1	6.18	62	1.24	338	1.6951
2	5.93	81	1.62	341	1.0541
3	6.14	74	1.48	361	0.8609
4	6.15	76	1.52	360	0.8851
5	6.17	104	2.08	358	1.0353
6	6.23	273	5.46	352	1.8687
7	6.38	332	6.64	346	2.7763
		Average	**2.9**	**351.3**	**1.39**

Table 14. Raw data on *C. protothecoides*. Flask D was exposed to light intensity of 35 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 3×10^5 cells mL^{-1} and grown for 7 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cell size (µm)
0	7.05	117	2.93	354	0.9076
1	6.14	63	1.26	348	1.8015
2	5.95	127	2.54	335	0.9731
3	6.12	189	3.78	365	1.2931
4	6.17	367	7.34	360	1.1095
5	6.21	422	8.44	358	1.1635
6	6.26	637	12.74	353	1.8932
7	6.42	643	12.86	360	2.2830
		Average	**6.5**	**354.1**	**1.43**

Appendix D

Table 15. Raw data on *C. prototheco*ides. Flask A was exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 2×10^5 cells mL^{-1} and grown for 10 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.03	96	2.40	290	1.8005
2	6.07	71	1.42	303	0.8750
4	6.30	412	8.24	319	2.4858
6	6.26	467	9.34	342	1.1499
8	6.43	481	9.62	330	1.5800
10	6.41	469	9.38	366	3.0730
		Average	**6.7**	**325.0**	**1.83**

Table 16. Raw data on *C. prototheco*ides. Flask B was exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 2×10^5 cells mL^{-1} and grown for 10 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.03	96	2.40	290	1.8005
2	6.04	58	1.16	331	0.6194
4	6.34	477	9.54	321	1.0493
6	6.37	552	11.04	346	1.3651
8	6.39	529	10.58	364	1.7635
10	6.38	514	10.28	326	3.5320
		Average	**7.5**	**329.7**	**1.69**

Table 17. Raw data on *C. protothecoides*. Flask C was exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 2×10^5 cells mL^{-1} and grown for 10 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.03	96	2.40	290	1.8005
2	6.10	59	1.18	314	0.8456
4	6.37	491	9.82	353	3.6410
6	6.30	568	11.36	370	2.5365
8	6.42	559	11.18	400	2.8548
10	6.32	562	11.24	411	3.0780
		Average	**7.9**	**356.3**	**2.46**

Table 18. Raw data on *C. protothecoides*. Flask D was exposed to light intensity of 140 µmol $m^{-2}s^{-1}$, 15% CO_2 concentration and cultured in the continuous loop system. The culture were inoculated with 2×10^5 cells mL^{-1} and grown for 10 days.

Time (days)	pH Reading	Total Cell Counted	Cell Concentration (cells/mL x 10^5)	Total Relative Fluorescence Intensity	Average cells size (µm)
0	7.03	96	2.40	290	1.8005
2	6.04	44	0.88	295	1.8154
4	6.39	334	6.68	318	3.2950
6	6.47	403	8.06	316	1.5165
8	6.48	413	8.26	359	3.4705
10	6.45	496	9.92	374	2.7310
		Average	**6.0**	**325.3**	**2.44**