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Effect of Glucose Supplementation on Nighttime Biomass Loss and Productivity of Microalgae Chlorella

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EFFECT OF GLUCOSE SUPPLEMENTATION ON NIGHTTIME BIOMASS LOSS AND PRODUCTIVITY OF MICROALGAE *CHLORELLA*

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This thesis is dedicated to my parents and my family for their continuous support throughout my life.

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ABSTRACT

Microalgae have been proven to be a promising source for the production of biofuel. It has higher oil yield than any other food crop. Oil yield from microalgae is 7 to 13 times higher than the best food crop. There are several issues to be addressed for economic production of biofuel from microalgae. One such issue is cultivation of microalgae. Algae can be cultivated photoautotrophically or heterotrophically. Algae grown photoautotrophically lose up to 35% of the biomass during nighttime. Heterotrophic cultivation of microalgae has been proven to be resulting in faster microalgae production compared to photoautotrophic growth, but requires use of expensive external carbon sources as a supplement. Cultivation of microalgae with cyclic combination of photoautotrophic and heterotrophic growth may be an effective and economical method of micro-algae cultivation combining the advantages of photoautotrophic and heterotrophic growth. Cyclic cultivation of *Chlorella* was performed to study the effect of nighttime supplementation on nighttime biomass loss and productivity. Results showed increased biomass productivity compared to pure photoautotrophic productivity. As high as 33 g/m2-day, productivity values were reported with 0.5 g/L of glucose supplement concentration during nighttime compared to the control cycle with the productivity value of 4g/m2-day. Statistical analysis suggested

productivity values increased with glucose concentration. Bacterial concentration was an order of magnitude lesser than the biomass concentration. Glucose concentration data were collected to calculate the yield coefficient for different supplement concentration. Yield coefficient values up to 0.28gm biomass/gm glucose were achieved with supplement glucose concentration of 0.5 g/L.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

NOMENCLATURE

OD	Optical Density
DCW	Dry cell weight
C	Concentration, (g/L)
V	Volume, (L)
t	time
F_1	Dry Weight of Filter after drying at time t_1, (g)
F_o	Dry Weight of Filter before filtration at time t_0, (g)
V_f	Filtration volume of culture, (L)
A	Surface area of the open top bucket, (m^2)
P	Areal Productivity of the culture, (g/m2-day)
X	Biomass concentration, (g/L)
S	Substrate concentration, (g/L)
X_1	Biomass concentration at time t_1, (g/L)
X_0	Biomass concentration at time t_0, (g/L)
S_0	Sugar concentration in the culture at the start of the dark cycle at time t_0, (g/L)
S_1	Sugar concentration in the culture at the end of the dark cycle at time t_1, (g/L)
$Y_{x/s}$	Yield coefficient, (g biomass/g substrate)
$Y_{x/s}^{app}$	Apparent yield coefficient (g biomass/g substrate)
$Y_{x/s}^{th}$	Theoretical yield coefficient (g biomass/g substrate)
R_s	Rate of substrate consumption (g/ liter/time)
R_x	Rate of biomass production (g/liter/time)
μ	Specific growth rate (day^{-1})
M_s	Maintenance coefficient, the rate of consumption of substrate due to maintenance processes, (gm glucose/gm biomass/time)

CHAPTER I

INTRODUCTION

Fossil fuels have been used as a main source of energy due to their high energy density. Commercial exploitation of petroleum largely came in practice in the early 19thcentury bringing industrial revolution. With time, the unrestrained use of fossil fuel has increased exponentially, affecting both the availability of fuel and the environment. There are several reasons behind inventing alternate sources of energy.

Energy security is the number one driving force for a need to find out an alternate source of energy for the United States. It is predicted that, global demand for petroleum will increase 40% by 2025[14]. The United States imports almost half the quantity of transportation fuel that it uses. In 2010, the United States imported net 9.4 million barrel of crude oil. 49% of the imported fuel came from western hemisphere; while gulf countries contributed 19% of the total imports[7](Fig. 1.1).Importing a large quantity of fuel develops concerns about both security and economy.

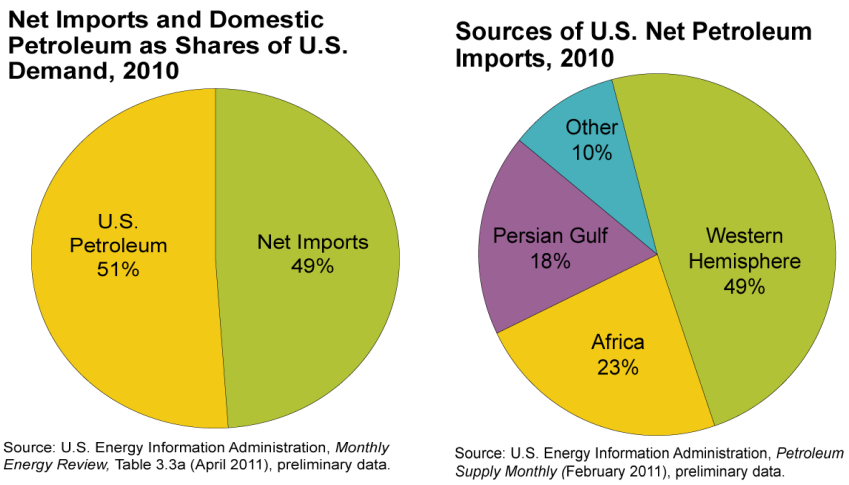

Figure 1.1 Net imports versus domestic petroleum production and percentage of net import from different nations[7].

Sources of fossil fuels are depleting very quickly due to unrestrained usage of fossil fuels. Several studies have indicated that world crude oil reserve will near an end between 2050 and 2075[17]. Prices of crude oil have been increasing (Fig. 1.2). With this fact in mind, it is required to find out an alternate source of energy well before an end of fossil fuel is reached.

Fig 1.2 Price Trend for crude, Dollars/Barrel[17].

Burning fossil fuels generates several greenhouse gases including the main greenhouse gas CO_2.It pours external carbon into the environment which had been out of

the environmental carbon cycle since millions of years, hence accelerating the issue of global warming. In past 150 years, use of fossil fuel has resulted in a 25% increase in environmental carbon dioxide[18].

Due to the serious concerns listed above it is important to identify alternate sources of energy which are both renewable and environmentally friendly. One such source for producing fuel is using biomass, and microalgae has proven to be the best available biomass source due to high oil yield and CO_2 utilization efficiency. Per unit area the yield of oil from microalgae is estimated to be 5,000 to 21,000gallons per acre per year, which is 7 to 31 times higher than the next best food crop, palm oil[5]. Moreover, microalgae derived biodiesel seems to have the potential to replace petroleum-derived transport fuels[14].

Several species of microalgae have been examined since the early 1970s as the potential source of fuel replacement. Later research proved microalgae as an efficient source to produce not only the fuel but also fertilizer, chemicals and health food supplements. There are several advantages from using microalgae as a source of fuel production as compared with any other source. Higher growth rate of microalgae compared with other food crops makes it possible to produce higher amount of biofuel. Cultivation of microalgae requires less water and can be done even with marine water. Hence, it does not compete for land with other food crops. It has higher tolerance to CO_2[10]. The "look back at the U.S. Department of Energy's Aquatic Species Program" provides significant research on microalgae to establish it as a potential renewable source of energy. The focus of the program (1978-1996) was to produce biodiesel from high lipid-containing algae in ponds, utilizing waste CO_2 from coal fired power plants[15].

Research was concerned with finding a species of algae that produces high amount of oil and can also survive in extreme environment of temperature, pH or salinity. Out of the many areas they studied about microalgae, one of the areas on which they focused was the algae growth system, which is one of the most expensive steps of the overall process of conversion of microalgae biomass to biofuel. It was concluded from the study that the factors that affect the cost of production of microalgae are mostly biological and not the engineering[15]. That means we need to produce highly productive organisms which can convert maximum sunlight to biomass, can survive extreme conditions and have maximum lipid yield along with biomass yield.

This research involves the study of a method to increase the productivity of biomass and avoid nighttime biomass loss. It is necessary to understand the growth cycle of microalgae if we want to maximize the productivity of algae biomass. Algae are phytoplankton and hence they grow using the process of photosynthesis. During the process of photosynthesis, the energy is provided by light to form glucose and ATP[16].

Photosynthesis:

$6CO_2 + 6H_2O + \text{Light energy} \rightarrow C_6H_{12}O_6 + 6O_2$

During the process of respiration glucose produced from photosynthesis is consumed to produce water and 36 mole of ATP.

Respiration:

$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O + 36ATP$

Thus, the glucose produced during photosynthesis is broken down during the process of respiration. It has been observed that algae culture loses biomass during night time. Up to 25% of the biomass produced during the daylight may be lost during dark

cycle[4]. Based on the investigation by other researchers, the percentage of biomass lost during night time may be even higher. The provision of an external source of carbon during the night time can be used to either maintain biomass or increase the biomass content during the dark cycle.

The most common type of growth that we see in nature is photoautotrophic. It is the ability of the green plant to grow in the presence of sunlight using CO_2.When an organism uses energy from some external carbon source except sunlight for the same purpose, it is called heterotrophic growth. Results from previous research have shown higher biomass growth rates for heterotrophic growth. However, purely heterotrophic culture is limited by cost of external carbon source. There is a possibility that combining two growth mechanisms may combine their advantages and increase the algae biomass production while keeping the biomass production cost at some optimum level. Ogbonna and Tanaka studied the night time biomass loss and the effect of different parameters such as daytime light intensity, temperature, and rate of mixing on night time biomass loss of *Chlorella pyrenoidosa*[12]. They also studied the effect of some external carbon source on night time biomass loss of microalgae. Results from their research proved that microalgae cells breakdown intracellular carbohydrate during the process of respiration to obtain energy in the absence of light energy. They showed that some external carbon source can be used to decrease the night time biomass loss. They also concluded that the external carbon source had negligible effects biochemical composition of microalgae[12].

Assuming the technique of cyclic growth system would be more economical and would increase the biomass growth at the same time, it was implemented for this experiment. I implemented photoautotrophic growth during the day (referred to as light

cycle from here) and heterotrophic growth during the night (referred to as dark cycle from here) with an external carbon source. Phycal, Inc. had done base line experiments at a 50 mL shaker flask level. The goal of this experimental work was to study the effect of external carbon sources on biomass concentration during dark cycles with a sterile environment. Phycal used different sources of carbon such as glucose, sucrose, glycerol, and formic acid. The goal of my research was to scale the same process for higher operating volume and to a non-sterile environment. The main objectives during the research were:

1) Scale up the process of nighttime supplementation at higher scale with non-sterile environment and optimize the set up for the process

2) Find out the minimum supplement concentration required to stop night-time biomass loss and study the effect of different supplement concentration on night-time biomass loss and biomass productivity

3) Determine the yield coefficient of heterotrophic growth of *Chlorella sp.*

CHAPTER II

BACKGROUND

2.1 Microalgae Species

Microalgae is a photosynthetic organism capable of converting solar energy into usable forms of energy, e.g. oil. The Algal Collection of the US National Herbarium consists of approximately 320,500 dried specimens. It is necessary to know which algae species are feasible for oil production. Microalgae, organisms less than 0.4 mm diameter, are preferred for biofuel production. Microalgae have high growth rates, less complex structure, and some species of microalgae have high oil content. The Aquatic Species Program identified algae species which have higher oil yield and which can withstand severe conditions. They screened out 300 species, which they recognized as species having high oil yield and biomass production[15]. Table 2.1 shows several algae species and their oil yield[14].

Table 2.1 Microalgae species and their respective oil content[14], (measured as dry cell weight %).

Microalgae species	**Oil content(% dw)**
Botryococcusbraunii	25-75
Chlorella sp.	28-32
Crypthecodiniumcohnii	20
Chlorella protothecoides (autotrophic/ heterotrophic)	15-55
Cylindrotheca sp.	16-37
Dunaliellaprimolecta	23
Isochrysissp	25-33
Monallanthussalina	>20
Nannochloris sp.	20-35
Nannochloropsissp	31-68
Neochlorisoleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylumtricornutum	20-30
Schizochytriumsp	50-77
Tetraselmissueica	15-23

Among all the available oil producing microalgae, *Chlorella* has been recognized as one of the most promising algae for production of biofuel due to its high oil yield and biomass content. It is one of the genuses being studied worldwide as a mass oil producing crop. It is unicellular green algae, round shaped with a diameter of 6 microns in the class *Trebouxiophyceae*(Fig. 2.1).

Figure 2.1 *Chlorella* genus (http://www.sciencephoto.com/media/15631/enlarge).

Chlorella vulgaris and *Chlorella protothecoides* are two widely available strains of chlorella species. They have shown great potential as the future industrial bio energy producers due to their robustness, high growth rate, high oil content and they can be cultured under heterotrophic and autotrophic conditions[9].

2.2 Algae Metabolism

Algae can grow autotrophically or it can grow heterotrophically. It depends on which type of carbon source the algae utilize. It is necessary to understand the effect of different types of carbon sources on algae metabolism as well as metabolic pathways.

a) Photosynthesis & Respiration

Autotrophs obtain their carbon from CO_2. Energy for autotrophic growth is supplied by light. Photosynthesis takes place in two phases (Fig. 2.2). The first phase of photosynthesis is called light phase as the reaction of this phase happens in light. During the light phase, light energy is captured by chlorophyll in chloroplasts and converted into the biochemical energy in the form of ATP (adenosine triphosphate). Light absorption by chlorophyll molecule results an electronic excitation and the excited chlorophyll molecule transfers the energy to series of enzymes and ATP is produced as the end product[16].

$H_2O + NADP^+ + P_i + ADP + LIGHT \rightarrow Oxygen + NADPH + H^+ + ATP$

NADPH (nicotinamide adenine dinucleotide phosphate) serves as electron transporters. The second phase of photoautotrophic metabolism is known as dark phase, as the reaction of this phase does not require light energy to occur. However, they do require the products of light phase. In the second phase, the energy rich products of first phase such as ATP and NADPH are used as the energy to reduce the CO_2 captured during the process to glucose[16]. Figure 2.2 explains the light reaction and the dark reaction.

$CO_2 + NADPH + H^+ + ATP \rightarrow 1/6C_6H_{12}O_6 + NADP^+ + ADP + P_i$

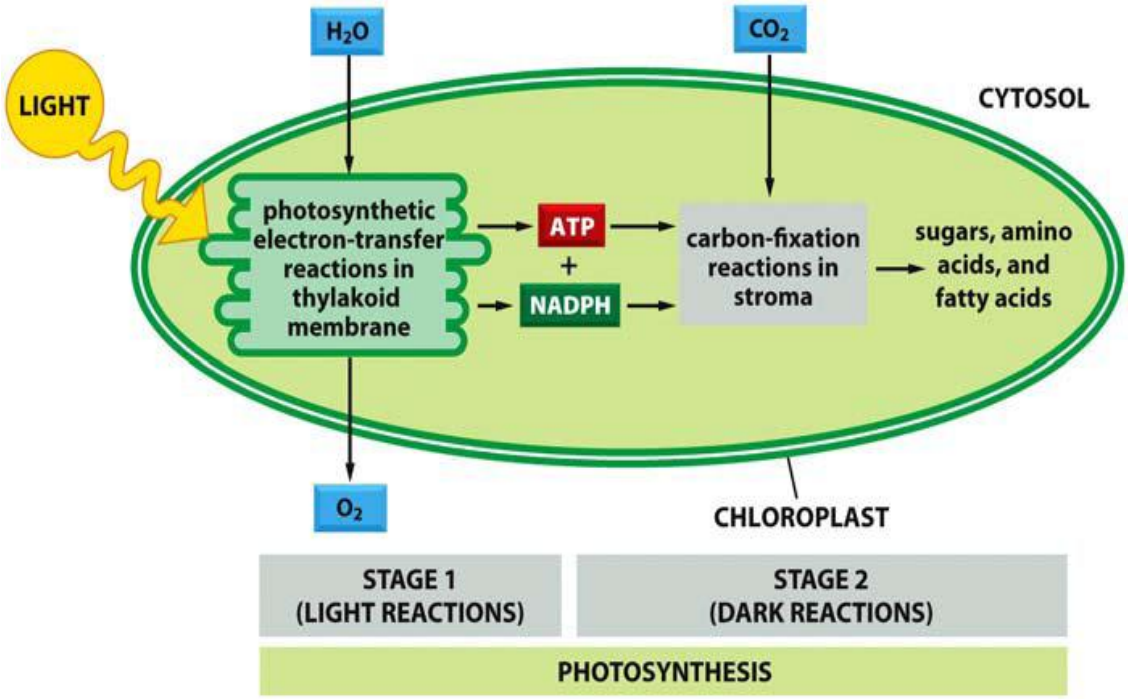

Figure 2.2 Process of Photosynthesis, light and dark reactions.[3]

b) Glucose Metabolism

There are several kinds of metabolic pathways used by different organisms, including algae, for the catabolism of glucose. Catabolism by glycolysis, or the Embden-

Meyerhof-Parnas (EMP) pathway, is the primary pathway. Glucose is catabolised aerobically in three different phases: (1) EMP pathway (ferments glucose to pyruvate), (2) Krebs tricarboxylic acid (TCA) cycle for conversion of pyruvate to CO_2 and NADH and (3) Electron transport for formation of ATP. The final phase is the production of energy in terms of ATP. EMP pathway breaks down glucose to two pyruvate molecules. Pyruvate is a very important metabolite which can be converted to different end products based on the type of metabolism. Under anaerobic conditions, pyruvate may be converted to products such as ethanol, acetic acid etc. Under aerobic conditions, pyruvate is converted to CO_2 and NADH through TCA cycle[16].

Overall reaction for EMP pathway (glycolysis) is:

$$\text{Glucose} + 2\ ADP + 2\ NAD^+ + 2\ Pi \rightarrow 2\ \text{pyruvate} + 2\ ATP + 2(NADH + H^+)$$

During Krebs cycle, pyruvate produced during EMP pathway transfers its reducing power to NAD^+. For each pyruvate molecule entering the Krebs cycle, three CO_2, four $NADH+H^+$ and one $FADH_2$ are produced. $NADH+H^+$ and $FADH_2$ are used for biosynthetic pathways or for ATP generation through respiration. The last phase is the respiration reaction which is also known as electron transport chain. Electrons from $NADH+H^+$ and $FADH_2$ are transferred to oxygen via series of electron carriers, and ATP is formed.

Research has proven that glucose promotes physiological changes in C*hlorella vulguris* which affects the metabolic pathways for carbon assimilation, and size of cell[13]. Algae use either EMP pathway or Phosphate pathway (PPP) for glucose metabolism under aerobic condtions[13]. Only 1% of the glucose remains as free glucose and more than

85% of the glucose is converted to either oligo or polysaccharides. It has been observed that, under darkness glucose is metabolized mainly by the PPP pathway, and the EMP pathway is used during light conditions[13].

2.3 Growth Processes

Growth rate is a very important parameter while studying algae growth. It is desirable to maximize the growth. Algae can be grown using mainly two mechanisms: 1) photoautotrophic and 2) heterotrophic growth. Algae grows photoautotrophically when grown under natural sun light energy, using energy from sunlight to perform photosynthesis, and using CO_2 as carbon source. Heterotrophic growth occurs when algae are grown in dark with some external carbon source which provides energy for the metabolic activities. Growing algae in an open pond under natural sunlight conditions is widely accepted method to produce microalgae photoautotrophically due to its economic feasibility. Algae can be grown in closed photo-bioreactors aseptically with light that mimics natural sunlight conditions. There also has been extensive research done with the heterotrophic cultivation.

A study was conducted to merge the positive aspects of autotrophs and heterotrophs. *Chlorella protothecoides* were grown autotrophically[19]. At the end of the autotrophic cycle, cells were left to sediment, supernatant was discarded and cells were re-suspended in heterotrophic medium without light. Researchers named this growth method as photosynthesis-fermentation model (PFM) and the results were compared with photosynthesis model (PM) and fermentation model (FM), autotrophic and heterotrophic growth models respectively. Results showed increased biomass productivity during photosynthetic growth and biomass concentration was further maximized during

heterotrophic fermentation[19]. The advantage of adding carbon source is increased in the later growth phase as well, when the cell density is much higher and light cannot penetrate enough to achieve higher growth rate. Heterotrophic growth is not light dependent and hence higher growth rates can be achieved. Study of metabolic pathways suggested CO_2re-fixationoccurred during heterotrophic fermentation which resulted in higher lipid yield indicating the advantage of PFM associated with lipid production as well. 61.5% less CO_2 was liberated in the fermentation stage of the PFM than control group of pure heterotrophic growth for the same yield of oil. They also checked the lipid yield and the data showed 69% higher lipid yield in fermentation stage during PFM compared to the FM[19]. Green color of the broth was observed to be faded during the fermentation stage of PFM. Chlorophyll content was monitored and changes in the pigment concentrations were compared (Fig. 2.3).

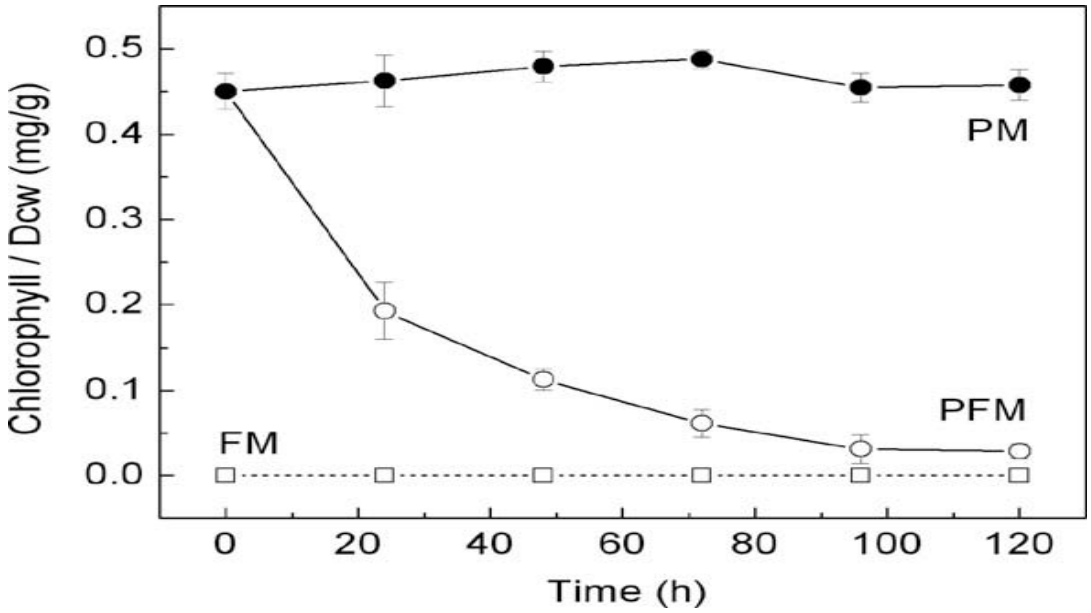

Figure 2.3 Chlorophyll content in three cell growth modes of *C. Protothecoides*[19], mean±standard deviation.

During PFM, chlorophyll content was decreased from 0.45 to 0.029 mg/g DCW (Dry cell weight) during 120 hour incubation period. Chlorophyll content of the cell in the PM remained unchanged at about 0.45 mg/g DCW. Electron microscopy results showed decrease in chlorophyll content during fermentation stage occurred because of gradually biodegrading chloroplasts[19]. They also observed the generation of lipid droplets inside cytoplasm after undergoing heterotrophic metabolism[19].

Other research on photoautotrophic, heterotrophic and mixotrophic cultures demonstrated the advantage of cyclic growth systems[11]. The main idea behind this research was to develop culture system with efficient utilization of light as light is the most important limiting factor during photosynthetic growth of the culture. They concluded that, with the light provision in photoautotrophic cultures, it is practically impossible to achieve the productivities as high as heterotrophic or mixotrophic cultures[11]. The simultaneous existence of completely dark, light limited, light saturated and light inhibition zone inside the photo-bioreactor is commonly observed. Due to the factors involved, it is difficult to produce cheap and efficient photo-bioreactors with high growth rates. To achieve the productivities as high as heterotrophic cultures, 0.1 mm thick cultures would be required which is not a practical solution. Mixotrophic culture or sequential heterotrophic/photoautotrophic culture system was suggested as a cultivation system to achieve higher productivities. Another way to cultivate the algae with high productivities is by employing sequential heterotrophic/photoautotrophic culture system. One can cultivate the cells heterotrophically to high concentrations and then transfer it to photoautotrophic conditions for accumulation of photosynthetic products. It is important to make sure that the organic carbon source is completely utilized before the start of light

cycle. One can also try to supply exhaust gas from heterotrophic phase for aeration of the photoautotrophic phase. This process can be economical and can help reduce environmental CO_2[11].

2.4 Nighttime Biomass Loss

It has been reported that, 35% of the biomass produced during the light cycle may be lost during the night time through respiration[12]. During the day time, cells use sun light to photosynthesize and produce biomass. But during night time, in the absence of sunlight, cells do not show any growth in biomass. Respiration is the process of breaking down organic carbon. Cells respire during night time to maintain themselves. In the absence of sunlight or any other carbon source, cells metabolize themselves to obtain the energy required for maintenance[12]. There are several factors that may affect the biomass loss during night time such as temperature, pH, and light intensity during the day time and rate of mixing.

A study was conducted on *Chlorella pyrenoidosa* to investigate the effect of all the factors listed above and interesting results were obtained. It was observed that nighttime biomass loss was increased with decrease in daytime temperature if night time temperature was kept constant at 30^0C[12]. They also studied the changes in biochemical composition of the cell and it was concluded that as much as 49% of the cell's carbohydrate was lost during night time[12]. That supports the fact that in the absence of sunlight and other carbon sources, cells derive their required energy by metabolizing intracellular carbohydrate. Based on their research on effect of light intensity and rate of mixing they concluded that higher light intensities during the day or higher mixing during the night lead to higher biomass loss during night[12].This is because of the reason that,

higher the carbohydrate content, higher the biomass loss during night time. And, when the light intensity during the day time was controlled at 100µmol/m^2-sec then the cell growth rate, protein content, and carbohydrate content were lower than the values obtained at 250µmol/m^2sec. This supported the hypothesis that the higher light intensity during the day time increased daytime carbohydrate storage, which was readily broken down at night[12].Decreasing the night temperature lead to reduction in biomass loss, which may be the result of reduced respiration rate at lower temperatures. All the parameters investigated above affected the biochemical composition of the cell i.e. mainly carbohydrates and protein which affects the biomass loss. Experiments with cyclic growth of C*hlorella pyrenoidosa* with nighttime supplementation resulted in reduced biomass loss and negligible effects on carbohydrate-protein content[12].

It is concluded from the above results that, one can try to reduce the night time biomass loss by optimizing the parameters listed above. Lower day time temperatures, lower light intensity during the day time, avoiding mixing during the nighttime, cyclic autotrophic-heterotrophic cultivation are some of the ways to reduce nighttime biomass loss. The manipulation of such parameters during the day affects the biomass loss during nighttime due to their influence on protein/carbohydrate balance in the cells. Manipulating the protein and carbohydrate composition of the cell can help to reduce the nighttime biomass loss but it is impossible to prevent the biomass loss during the night. As *Chlorella* can be grown both ways either as a heterotroph or an autotroph, it is good to investigate the effect of such growth conditions on night time biomass loss.

2.5 Nighttime Supplements

There are several supplements available which can be used as nighttime supplements, such as: glucose, glycerol, cassava, formic acid, ethanol, and sodium acetate. Glucose seems to be the most promising carbon source for maximizing biomass growth. Very high rates of growth and respiration are obtained with glucose as substrate compared to other types of substrate[12]. Glucose possesses more energy content per mole compared with most other substrates. Glucose produces approximately 2.8 kJ/mole energy compared to acetate which produces 0.8 kJ/mole of energy[13]. At the same time there are some disadvantages associated with it. Glucose is a comparatively expensive source of carbon, means it is hard to commercialize the process. Also, supplementing with glucose can lead to contamination of the culture with bacteria or fungi. Effects of other carbon sources have been studied. Ethanol and sodium acetate were employed as a carbon source in order to avoid contamination problems, but the concentrations of biomass achieved during dark cycle while the supplementation of ethanol or sodium acetate were lower than that achieved during glucose supplementation[12]. However, results suggest that ethanol and sodium acetate can be successfully employed as a carbon source for the process of cyclic photoautotrophic and heterotrophic cultivation.

CHAPTER III

MATERIALS & METHODS

The *Chlorella* strain was obtained from Phycal, St. Louis Lab facilities. Sterilized seed culture plates were maintained to start the sterile inoculum cycle. The inoculum cycle consists of a series of scale-up steps, where the cells are transferred from one stage to another, increasing the size of each culture. One such stage is cultivation in sterile 20 - Liter carboys. After the cells were grown in the carboys, they were used as inoculums for this work.

3.1 Medium Recipe

3.1.1 Growth Medium

Growth of microalgae depends on immediate availability of nutrients. The Modified high salt Medium IV (MHS IV) with Vitamin B1 was used as a growth medium for all experiments. The recipe for original modified high salt medium was obtained from Phycal, St. Louis lab facilities. This growth medium has all necessary nutrients for growth of algae cells and their survival. The compositions of stock solutions used to make MHS IV are shown in Table 3.1. Following volumes of stock solutions were used to make 1 liter of growth medium: 5mL of solution B, 5mL of phosphate solution, 1mL

of trace-metal solution, and 1mL of vitamin B1 solution. The rest of the volume was filled with de-ionized (DI) water to the 1L final volume.

Table 3.1 Modified high salt medium - IV Recipe.

Chemical	**Weight**
Solution B , 1 L Stock Solution	
NH_4CL	100 g
$MgSO_4.7H_2O$	4 g
$CaCl_2.2H_2O$	2 g
Phosphate Solution	
K_2HPO_4	288 g
KH_2PO_4	144 g
Trace Metal Solution, 1 L Stock Solution	
$C_6H_5Na_3O_7.2H_2O$	150 g
$FeCl_3.6H_2O$	10 g
$CuSO_4.5H_2O$	80 mg
$ZnSO_4.7H_2O$	1.25 g
$MnSO_4.H_2O$	380 mg
$CoCl_2.6H_2O$	250 mg
$Na_2MoO_4.2H_2O$	250 mg
H_3BO_3	5 g
Vitamin B1, 1 L Stock Solution	
Thiamine Hydrochloride (Vitamin B1)	100 mg

3.1.2 Carbon Supplements

Glucose was used as external carbon source for nighttime supplementation. A stock solution of 260 gm glucose/L was prepared.

$$C_1V_1 = C_2V_2 \quad (3.1)$$

The volume of the stock solution to be added to each bioreactor at the beginning of the dark cycle, to achieve the specified glucose concentration was calculated using Eq. 3.1 and shown in Table 3.2.

Table 3.2 Desired supplement concentration and volume of stock solution to be added to achieve that concentration.

Desired supplement concentration inside the culture, C_2(g/L)	**Volume of the stock solution required, V_1 (L)**
0.5	0.01
0.375	0.0075
0.250	0.005
0.125	0.0025

3.2 Data Collection

Samples were collected three times a day: morning samples before the dilution, morning samples after the dilution, and evening samples before the start of the dark cycle. Biomass concentrations were measured from each sample via absorbance at 750 nm, dry weight, and cell counts. Bacterial counts were collected randomly and glucose concentrations were measured from samples before the start of dark cycle and in the morning before the dilution.

3.2.1 Biomass Measurements

Three different techniques were used to determine the biomass concentration. Samples were diluted when required before taking readings if the cell density of culture was very high. Samples were diluted using 2390ppm ocean salt solution. Normally, 1 L stock of ocean salt solution was prepared by dissolving 2.39 gram of ocean salt in 1 L DI water.

A) Absorbance

Cell growth was monitored by optical density measurements at 750 nm (referred to as OD_{750}) using spectrophotometer (Thermo Scientific – Genesys 10Vis). Absorbance is the quickest, simplest and most economical way to measure the cell growth. As shown by Fig. 3.1,at the wavelength of 750 nm, chlorophyll does not have any effect on light utilization and hence reading gives only the measure of light which is absorbed by the size and quantity of cells[8], independent of chlorophyll content.

Around 2-3 ml of sample volume was added to a plastic cuvette. Samples were diluted to appropriate concentration to keep the OD_{750} value between 0.0 – 0.5. Absorbance readings are erroneous if the culture is contaminated, flocked or if the culture settled inside the cuvette.

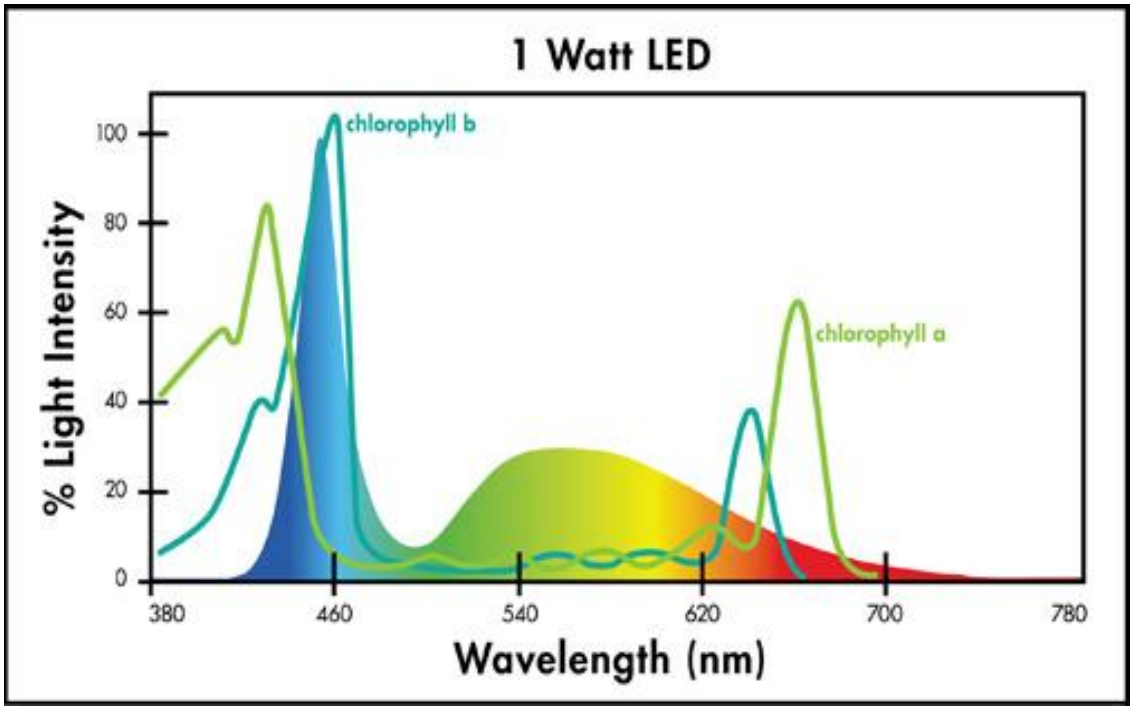

Figure 3.1 Light intensity absorbed by chlorophyll as a function of wave length (source: http://www.marineland.com/LEDp2.aspx).

B) Dry Weights

A predetermined volume of culture was passed through glass fiber filters using a setup connected to vacuum pump (Fig. 3.2). Filter diameter was 47 mm with pore size of 0.7 micron (Millipore Ireland Ltd.).Total volume of the liquid to be filtered was decided based on the optical density, determined before the dry weight measurement of the culture.

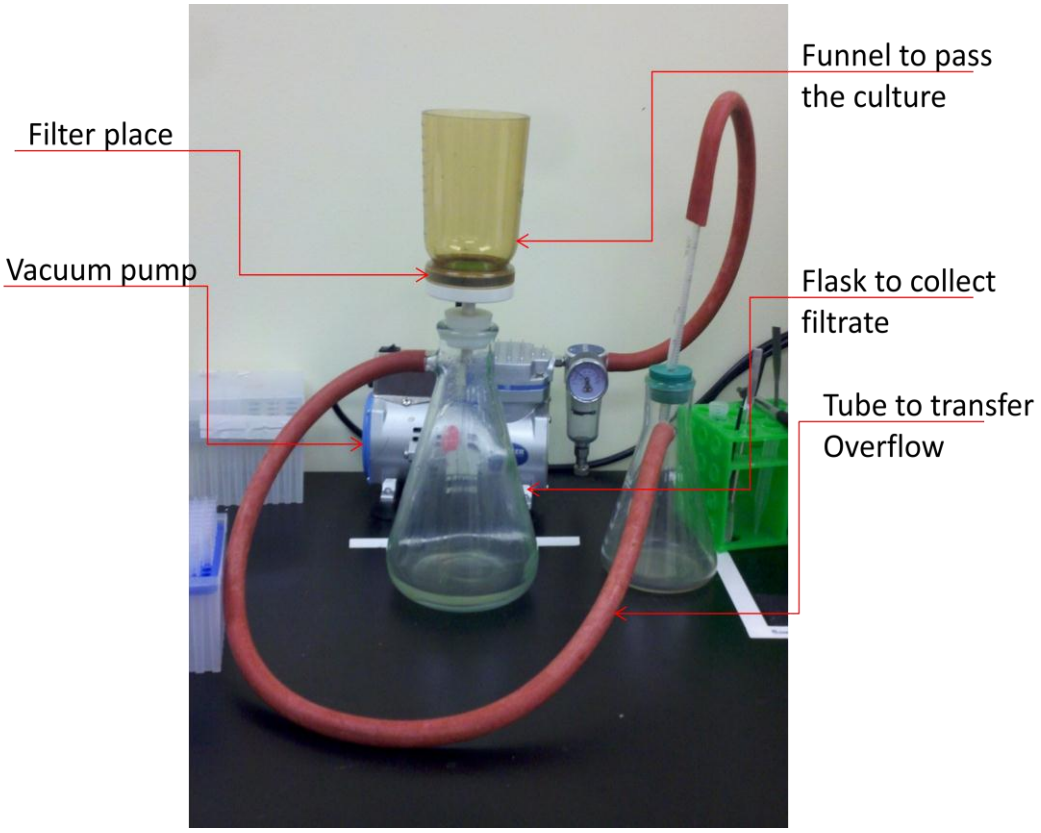

Figure 3.2 Dry weight manifold set up.

On the filter it is necessary to get a minimum retention of 10 mg biomass after filtration to get precise biomass concentration measurements. A correlation developed at Phycal between optical density and biomass concentration was used to find the culture volume to be filtered to get the required retention of biomass weight on the filter. The sample volume to be processed was usually about 50 mL. After the culture was filtered, the side of the funnels and the surface of the filter were washed with 5 mL 0.125 M ammonium bicarbonate solution to remove precipitated medium salts and any other inorganic substances. It is necessary to wash biomass samples to avoid errors in biomass measurements; unwashed biomass samples have dry weights 1.2 times higher than washed sample[20].

The vacuum pump in the setup helped to speed the filtering process. Gloves were used while labeling the filters to avoid errors in biomass measurement. Once the process was completed, filters were carefully moved from the manifold to the bio-drier (Fig.3.3) using forceps. Filters were dried at 145^0F for 24 hours and weighed. Difference in filter weight yielded total biomass retained on filters. The biomass concentration was calculated using known volume of the culture passed through the filter. A control filter was used for correction in dry weight measurements. A pre-weighed labeled filter was used as a control filter. It was dried in the bio drier along with the processed filters and weighed. The weight difference of the control filter was accounted as correction for the processed filters, which helps account for the analytical errors.

$$Concentration = \frac{F_1 - F_0}{V_f} \tag{3.2}$$

where, F_1= Dry weight of the filter after drying, F_0= Dry weight of the filter before filtration, and V_f= Filtration volume of the culture.

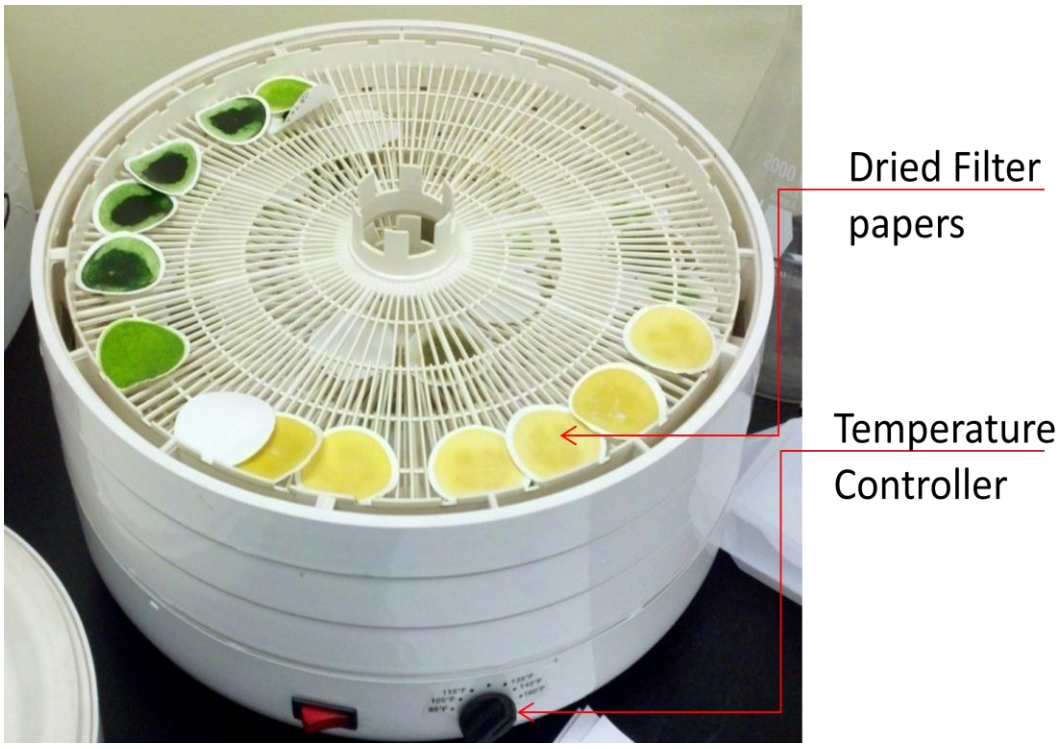

Figure 3.3 Bio-drier.

C) Cell Counts

Cell counts can be used to quantify the number of cells and understand the effect of contamination in the culture. Cell count samples were preserved with Lugol's solution for approximately 3 weeks before counting. Lugol's solution helps preserve phytoplankton samples[1]. 2 ml culture samples were stored at 4^0C.

Nikon Eclipse E200 series microscope was used for cell counting and contamination checks. Cell counts were done at the 20X objective. Cell counting was done manually using a Hemocytometer. As shown in figure 3.4, large square in the Hemocytometer has an area of 0.04 mm^2 and with a depth of 0.1 mm between slide and cover slip. Each large square in Hemocytometer has volume of 0.004mm^3 i.e. 4E-03 µL. Total volume for 5 such large squares would be 2E-02 µL. Cells present in the five large

squares on one side of chamber of a Hemocytometer were counted. To calculate cells present in 1 mL of sample,

$$\text{Cells/mL} = \text{Total number of cells counted /2E-05 mL} \quad (3.3)$$

10 µL of a well-mixed sample was injected into the Hemocytometer. Cells were allowed to settle before counting. Samples were diluted using 2ppt salt solution as required based on the concentration of the culture and the dilution factor was accounted for the later calculation. Cell counts were also used to observe the extent of contamination, if any. Cell counts using Hemocytometer is a time consuming process.

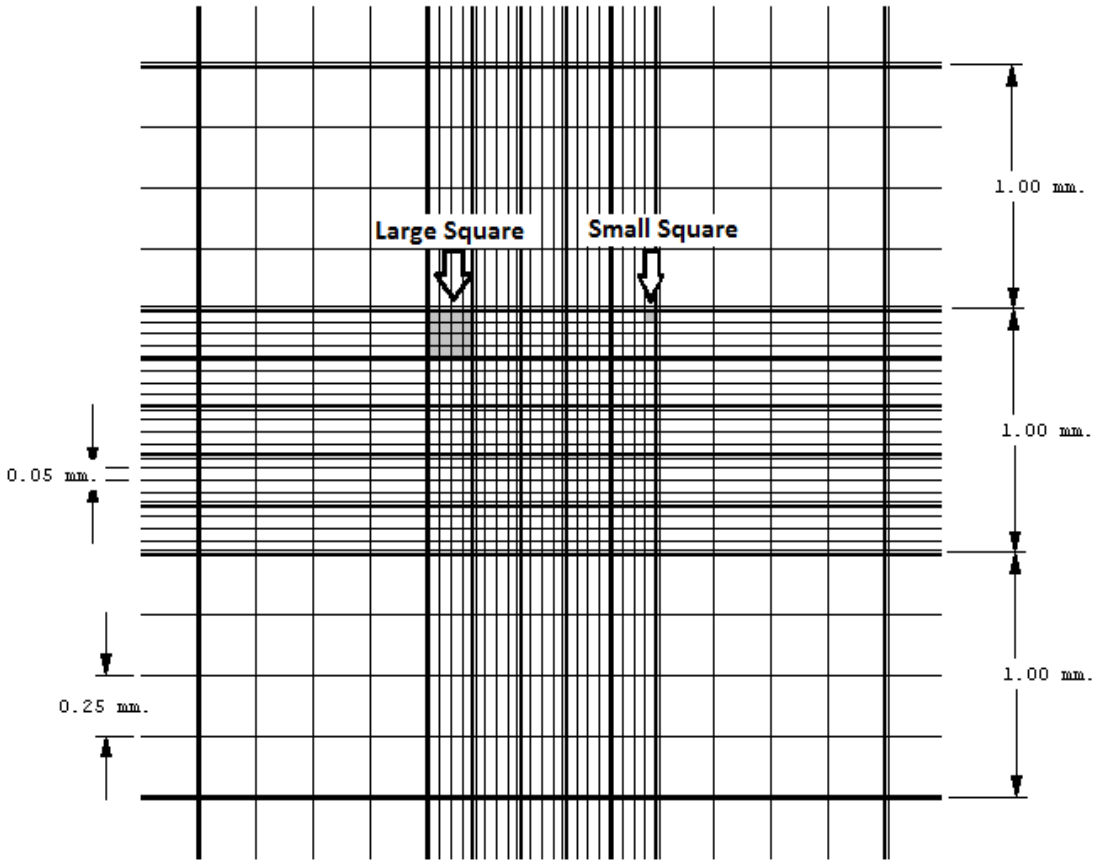

Figure 3.4 Counting chambers of Hemocytometer (source: http://www.emsdiasum.com/microscopy/products/magnifier/counting.aspx#63510).

3.2.2 pH & Temperature

pH and temperature were randomly checked to determine the consistency of the process. pH is one of the most important parameter for proper cell growth. pH around 6.8 was maintained using the gas mixture of air and CO_2(5% v/v). There was also a buffer medium inside MHS – IV which helped to prevent pH to fluctuate. pH was measured using a pH meter(Oakton Instruments). Temperature was also measured using the same meter with attached temperature probe. Temperature was maintained at 28 ± 2^0C using chillers with temperature sensor.

3.2.3 Glucose Samples

Glucose concentrations were measured from the samples collected at the start and end of the dark cycle. 1 mL of well-mixed cell suspension was filtered through a 0.2 micron filter. A dedicated syringe was used for each treatment to avoid cross contaminations. Filtered samples were stored at 4^0C and later processed through HPLC. Samples were analyzed by co-workers at Phycal for glucose concentration to calculate an important parameter for the process, yield coefficient.

Theoretically, there should be a linear relationship between the amount of biomass produced and the amount of substrate consumed. This relationship can be expressed quantitatively using the biomass yield coefficient, Y_{XS}. The yield coefficient equation can be derived through the mass balance of substrate for a simple batch reactor. Yield coefficient is classified in two terms; 1) Theoretical yield coefficient, $Y_{x/s}^{th}$, and 2) apparent yield coefficient, $Y_{x/s}^{app}$. The theoretical yield can be defined as the maximum yield possible. It is derived mainly from reaction stoichiometry. If all the substrate

provided was consumed for production of biomass only, the theoretical yield coefficient as high as 0.68 could be obtained by providing glucose as a substrate assuming average algal biomass composition as $CO_{0.48}H_{1.83}N_{0.11}P_{0.01.}$[4] It indicates that a substrate is used only to synthesize the biomass in the stoichiometric reaction. However, that is not the case with real cultures. Part of a substrate is consumed for maintenance demands such as maintenance of membrane potential and internal pH, turnover of cellular components and cell motility[6]. For a carbon source such as glucose which is carbon and energy source, substrate may be consumed as[16];

$\Delta S = \Delta S$ (assimilation into biomass) + ΔS (assimilated into extra cellular product) + ΔS (growth energy) + ΔS (maintenance energy)

A substrate consumed by such kinds of metabolic functions would not necessarily produce biomass. Hence, observed biomass yield coefficient would not match with the theoretical biomass yield coefficient. There are large numbers of factors which can influence the biomass yield as well, such as medium composition, type of carbon source, pH, temperature, and health of the culture. Observed yield ($Y_{x/s}^{app}$) is the ratio of weight difference of biomass to the weight difference of substrate.

3.3 Growth System

3.3.1 Carboy Inoculum

20 L clear plastic carboys (Fig. 3.5) (manufactured by Nalgene) were used to prepare inoculums with a total operating volume of 62.4 liter. The absorbance of the culture in the carboy was around 1.0 OD_{750}at time of inoculation into the bioreactors. Total volume of the inoculums can be calculated using the equation 3.4.

$$C_1V_1 = C_2V_2 \quad (3.4)$$

where, C_1= absorbance of the culture inside the inoculum, V_1= volume of the inoculum, C_2= desired absorbance of the culture required in bioreactors, V_2= total volume of the culture = 62.4 L. The inoculum volume was distributed evenly in 12 bioreactors. Rest of the volume inside each bucket was filled with MHS IV media to the final volume of 5.2L per bioreactor. After inoculation, the starting absorbance in each bioreactor was approximately 0.3 OD_{750}.

Figure3.5 Cultivation of microalgae in 20 L carboy.

3.3.2 Open Top Buckets as a Batch Bio-reactor

As shown in figure 3.6, an open top polypropylene bucket was used as a bioreactor for each experiment. Experiments were conducted as a batch process. The surface area of the bucket at the top was 0.0379 m^2. The operating volume of the reactor was 5.2 L and the operating depth was 15 cm. Due to opaque sides of the bucket, only the

top surface of the culture was exposed to light. The buckets were easy to clean and use again. Standard cleaning procedure was followed. Buckets containing cell suspension were treated with bleach, (around 30 mL) to kill the algae cells and other micro-organisms. After 30 minutes of bleach treatment, the bleached culture was thrown away. Later, the buckets were cleaned with soapy water and thoroughly rinsed with distilled water three times to remove any bleach/soap residue inside the bucket. The buckets shall be henceforth referred to as bioreactors.

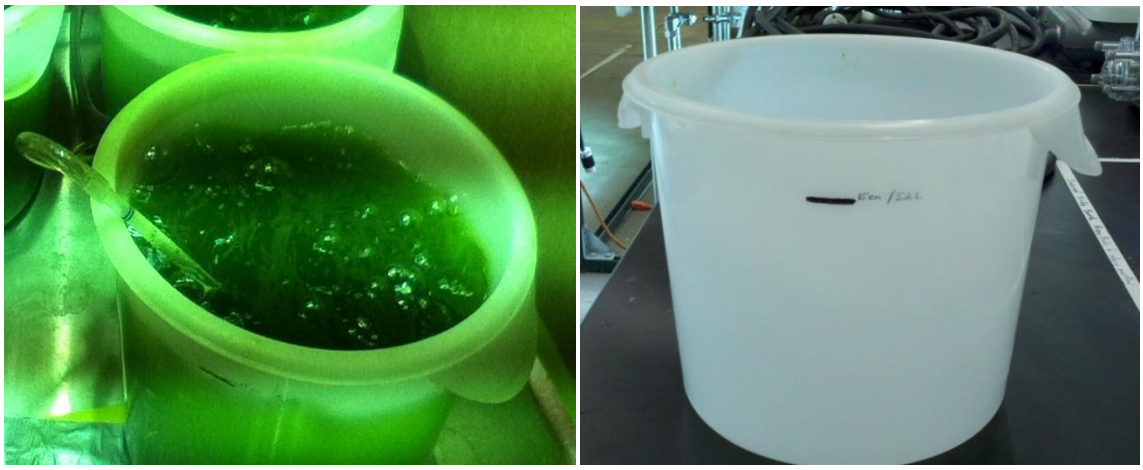

Figure 3.6 Open top buckets (bioreactor).

3.3.3 Gas Delivery and Mixing System

The mixture of air and industrial grade CO_2(5% v/v) was supplied to the culture to ensure proper gas requirements and mixing. Two dedicated flow meters were used for individual gas flow control. A main manifold setup as shown in figure 3.7 was used to mix the gas in proper proportions. Gas to the main manifold was supplied from air and CO_2(supplied from the utility area) compressors at a pressure of around 10 psi through ¾" braded tubing. The gas mixture from the main manifold was distributed to four small ¾" x ¼" silicon manifold dedicated to each compartment through ¾" braded tubing. Each

manifold had 4 outlets. The gas mixture from the small manifold was distributed further to individual bioreactors using ¼" silicon tubing. The end of the silicon tubing was attached with a 10 mL pipette for gas delivery to each bioreactor (Fig. 3.8). The pipettes were long enough to reach the bottom of the bioreactor to allow more contact time between gas mixture and culture liquid. Each compartment in the set up contained 3-4 bioreactors. Gas flow to each bioreactor was controlled by a dedicated flow meter. The gas mixture was delivered at a constant flow rate of 3.5 SCFH (0.317 vvm) to each bioreactor.

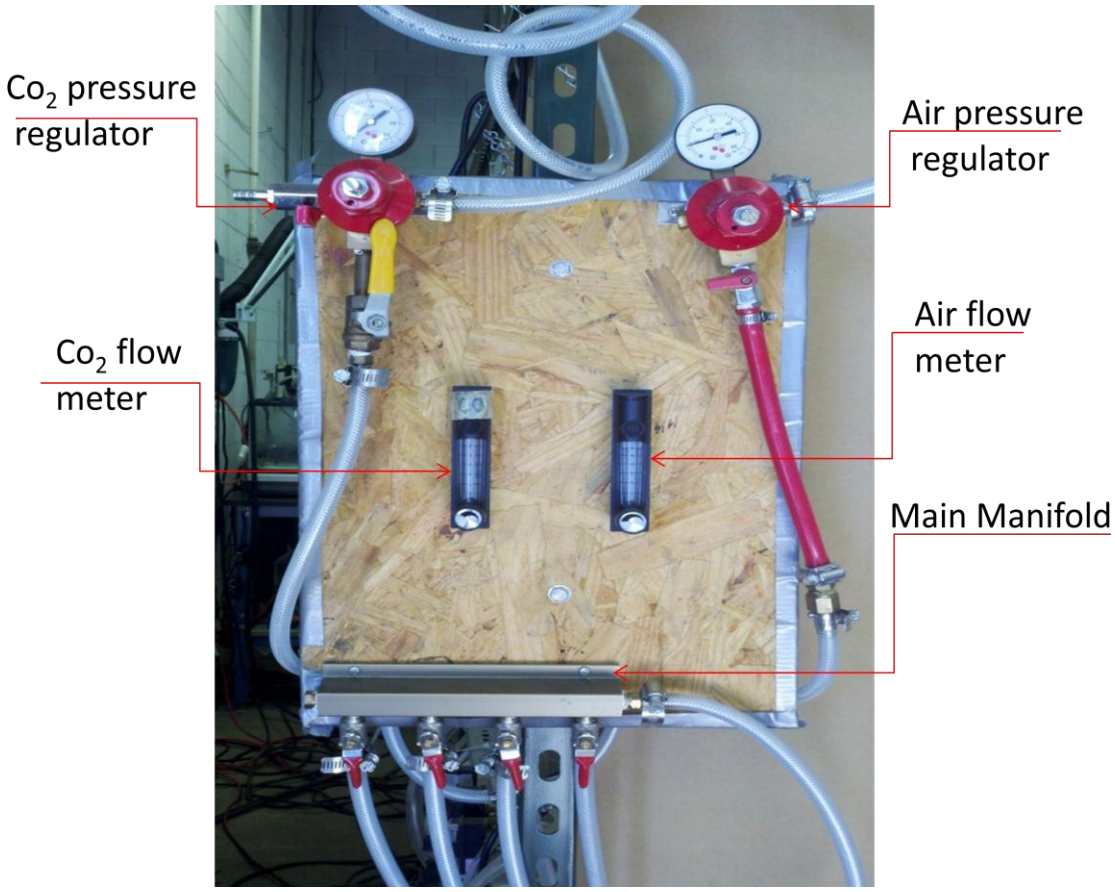

Figure 3.7 Gas delivering system.

Air lift agitation was employed for mixing the culture. Gas flow to the reactor was calibrated by naked eye observation to avoid the loss of culture due to overflow at the

same time ensuring proper mixing. An external pipette was used for mixing every time before taking the sample, to ensure well-mixed sample.

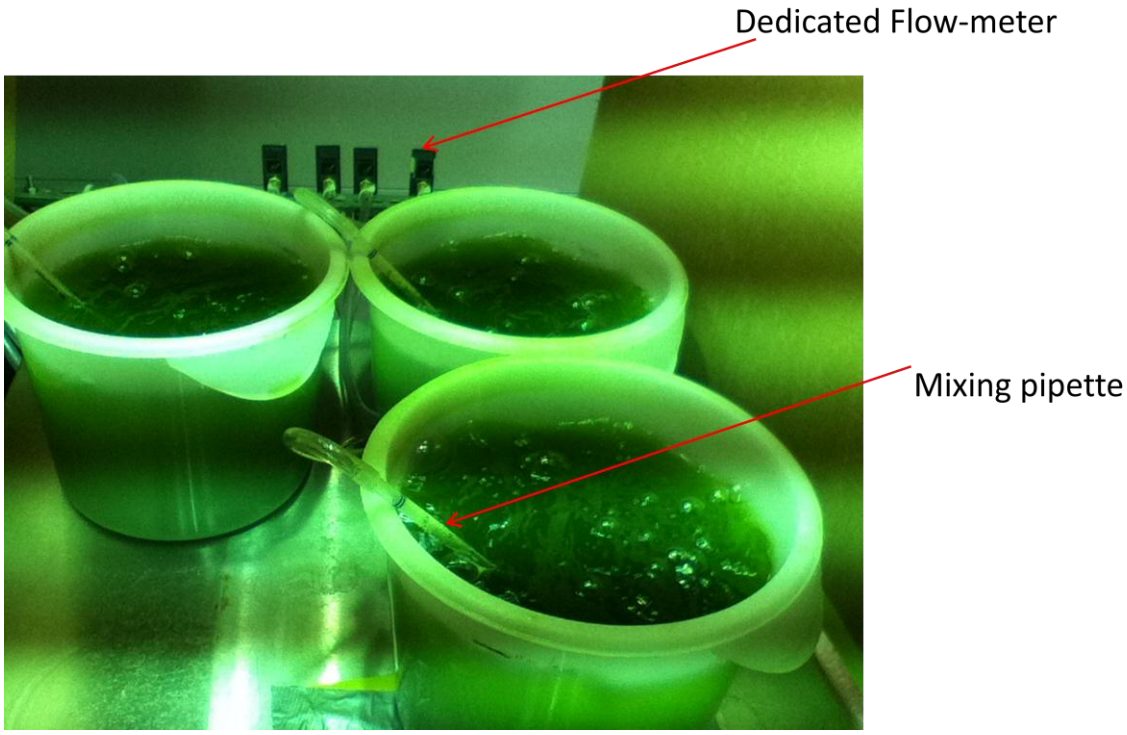

Figure 3.8 Mixing system.

3.3.4 Light

Light intensity was maintained approximately around 400 µmole/ m^2-sec for each bioreactor. Light intensity to the surface of culture was measured using LI-250A light meter (LI-COR Environmental). Four- 1000 watt metal halide lights (Fig. 3.9) (6500K, Eye lightening Int.), one dedicated to each compartment, (one light dedicated to 3-4 bioreactors) were used to mimic the natural sun light conditions.

Figure 3.9 Metal halide bulb used to mimic the sunlight conditions.

3.4 Growth Conditions

Growth of microalgae depends on many parameters such as temperature, light intensity, pH etc. To ensure the proper growth, consistency in the above parameters is important.

Temperature was controlled by a cooling water system at 28 ± 2^{0}C. The cooling water system was designed to be a big rectangular glass tank which could hold up to four bioreactors. Small impeller pumps as shown in figure 3.10 a) were used for mixing the water inside the tank. Two big high capacity chillers (Manufactured by Trade Wind Chillers Inc.) were employed for temperature control inside four cooling tanks. Each chiller had two chiller arms (cooling electrode) and one temperature probe/sensor. Assuming the temperature inside all the compartments was same, one chiller was dedicated to two adjacent cooling tanks. One temperature probe provided the controlling signals to chiller via temperature sensor, which helped to control the temperature inside the water tank. Temperature was maintained at 28 ± 2^{o}C in the water inside the tank. Out of four compartments, one compartment was dedicated to 24 hour control and it

contained the temperature probe. Refer figure 3.10 b) for schematics of the culture system with cooling tank and chiller arm.

Figure 3.10 a) Impeller pump for water circulation inside water tank.

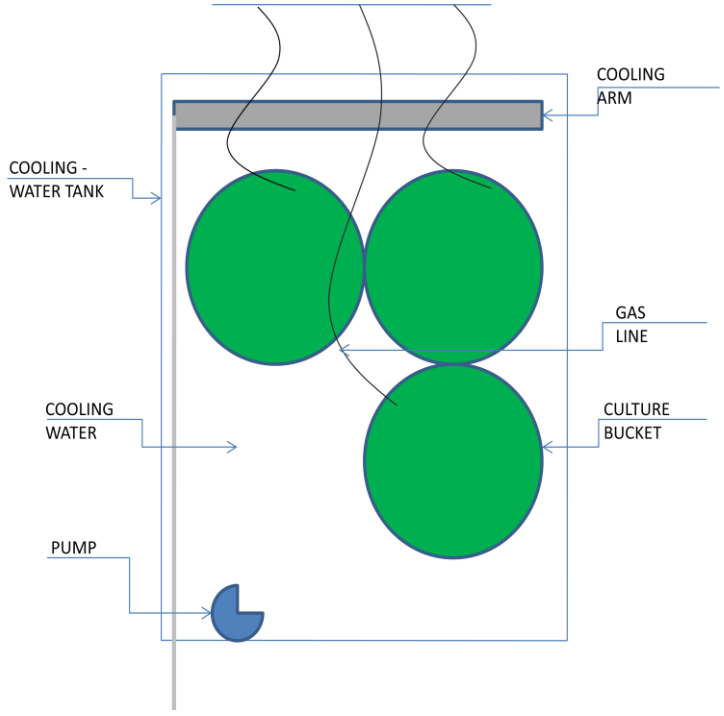

Figure 3.10 b) Schematic of the culture systems with cooling water tank.

3.5 Operating Procedure

After inoculating the bioreactors, cultures were allowed to grow before supplementation. During this period, all the cultures were grown on a 24 hour light cycle with no supplementation. Baseline experiments were done at Phycal to find out the minimum biomass concentration required for supplementation. Results from those experiments suggested the minimum absorbance of the culture to be at least 0.5 OD_{750}before one can start supplementing the culture. The idea behind this procedure was to develop enough algae cell density in the culture so that bacteria would not out-compete the algae cells after supplementation. Absorbance of 1.0 was selected as the baseline to start the supplementation experiment. It normally took around 3-4 days for the cells to grow up to absorbance of 1.0. The set up contained 12 bioreactors operated as batch reactors. Each cooling water tank contained 3 bioreactors. The experiment was performed with four treatments of supplements and each treatment had 3 replicates. Treatments and number of replicates are cited in table 3.3. Tests were divided in 2 separate experiments, performed sequentially.

Table 3.3 List of Supplement treatments and respective replicates.

Treatment	No. of Replicates
0.5 g/L glucose supplements	3
0.375 g/L glucose supplements	3
0.250 g/L glucose supplements	3
0.125 g/L glucose supplements	3
12 hour light/12 hour dark control	3
24 hour light control	3

The first experiment consisted of the 0.375g/L and 0.5 g/L glucose concentration treatments and the second experiment consisted of the 0.125 g/L and 0.250 g/L glucose concentration treatments. Both experiment consisted 12 hour light/dark and 24 hour light control treatments.

The experimental setup is shown in figure 3.11 with an enlarged view of bioreactor in figure 3.12. The supplementation treatments were conducted with 12h light/12h dark cycle (12 h L/D). There were two control treatments, one with 12 hour light/12 hour dark cycle and another with 24 hour light cycle. Control treatments did not receive any supplements. All the cultured bioreactors were diluted to absorbance 1.0 to have the same initial absorbance conditions. The dilution step was performed every morning and dilution volume was calculated based on the morning absorbance value recorded. Fresh MHS IV was used to dilute the culture. Samples were collected following the dilution process for the measurement of parameters such as: absorbance, dry weights, and cell counts as discussed earlier.

12 hour light cycle was observed from 8:00 a.m.to 8:00 p.m. Once diluted, cultures were allowed to grow throughout the day without any treatments. Cultures were supplemented before the start of dark cycle at 8:00 p.m. Cultures received supplements with predefined glucose concentrations for respective treatments. Samples were collected immediately following the supplementation to measure the actual glucose concentration, absorbance, dry weights, and cell counts. Bioreactors in dark cycle were covered with black cotton net cloth to avoid trespassing of surrounding lights and hence better experimental conditions. The cultures were allowed to grow without any further interruption until morning.

Figure 3.11 Experimental Setup.

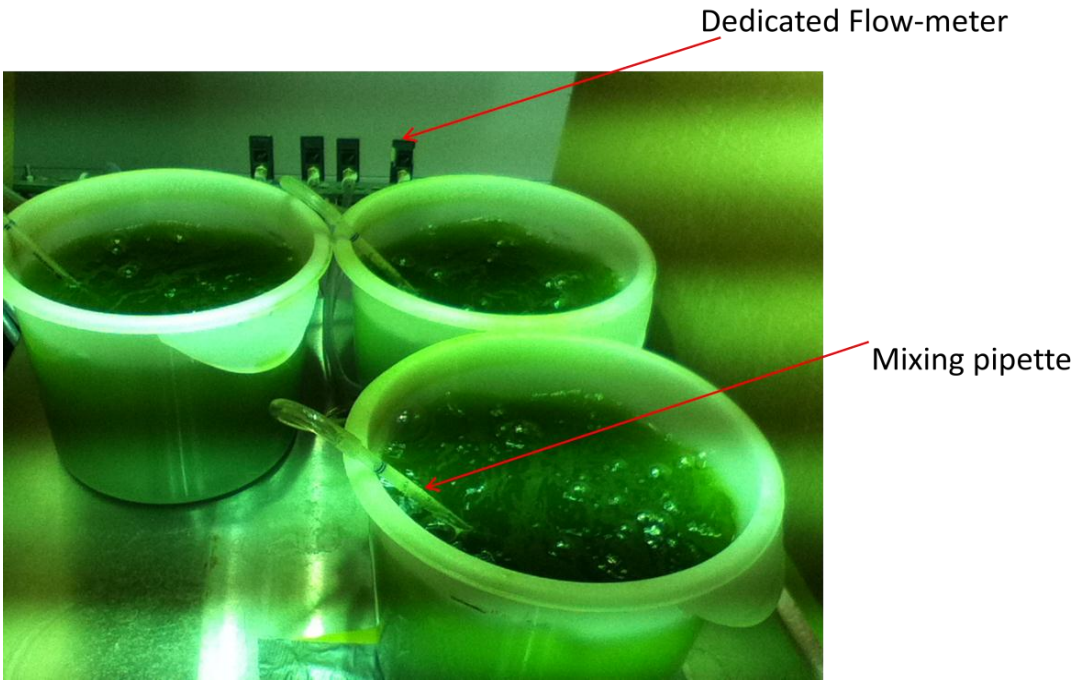

Figure 3.12 Cultured buckets with air lift agitation arrangement.

In the morning, before end of dark cycle at around 7.30 a.m., samples were collected in a way similar to the start of dark cycle to measure glucose concentration, absorbance, dry weight, and cell counts. Glucose samples in the morning were used to determine the glucose consumed during the dark cycle. Glucose concentration measurement was the essential part for understanding the extent of sugar utilization, the effect of supplementation on growth of micro algae, and contamination. Morning absorbance readings were used to calculate the dilution volume required for each bucket. Morning samples were collected following the dilution. A similar procedure was followed for two consecutive weeks.

During the first week, the test cultures were treated with 0.5 g/L and 0.375 g/L glucose concentration and for the second week, cultures with 0.5 g/L treatment were treated with 0.250 g/L and cultures with 0.375 g/L treatment were treated with 0.125 g/L glucose concentration. It was important to have as precise value as possible for absorbance in the morning as it was used to calculate the dilution volume required for each bucket. Following formula was used to calculate the dilution volume.

$$C_1V_1 = C_2V_2$$

where, C_1 = Absorbance of the culture, V_1 = Required volume that remains in the bioreactor for desired dilution, C_2 = Required Absorbance, 1.0 OD_{750},andV_2 = Operating volume of the culture. Once we calculate the V_1, $V = 5.2 – V_1$ L of culture was removed from the bioreactor and it was replaced with same amount of fresh MHS IV media.

CHAPTER IV

RESULTS& DISCUSSIONS

4.1 Effect of Glucose Supplementation on Biomass Growth

This experiment was conducted to study the effect of glucose supplementation on nighttime biomass loss. The cultures were supplemented with a predefined glucose concentration at the start of the dark cycle and were left to grow over night without any further interruptions. In the morning all the cultures were diluted to the optical density of 1.0 OD_{750} and were left to grow in the light for 12 hours without any interruptions. Samples were collected three times a day to retrieve absorbance, dry weight, cell counts, and glucose concentration data. Absorbance, dry weights, and cell count data were used to quantify biomass growth. Glucose samples were collected at the start and at the end of dark cycle to study the effect of glucose concentration on biomass loss during nighttime.

Results for absorbance and dry weights data are shown in Figures 4.1-4.4. The results for the 12 h L/D control treatment showed decrease in biomass concentration during the dark cycle and the lowest overall growth, as measured by dry weight (Fig. 4.2). The 24 hour control showed growth during both cycles measured as OD_{750} and dry weight (Figs. 4.1 and 4.2).

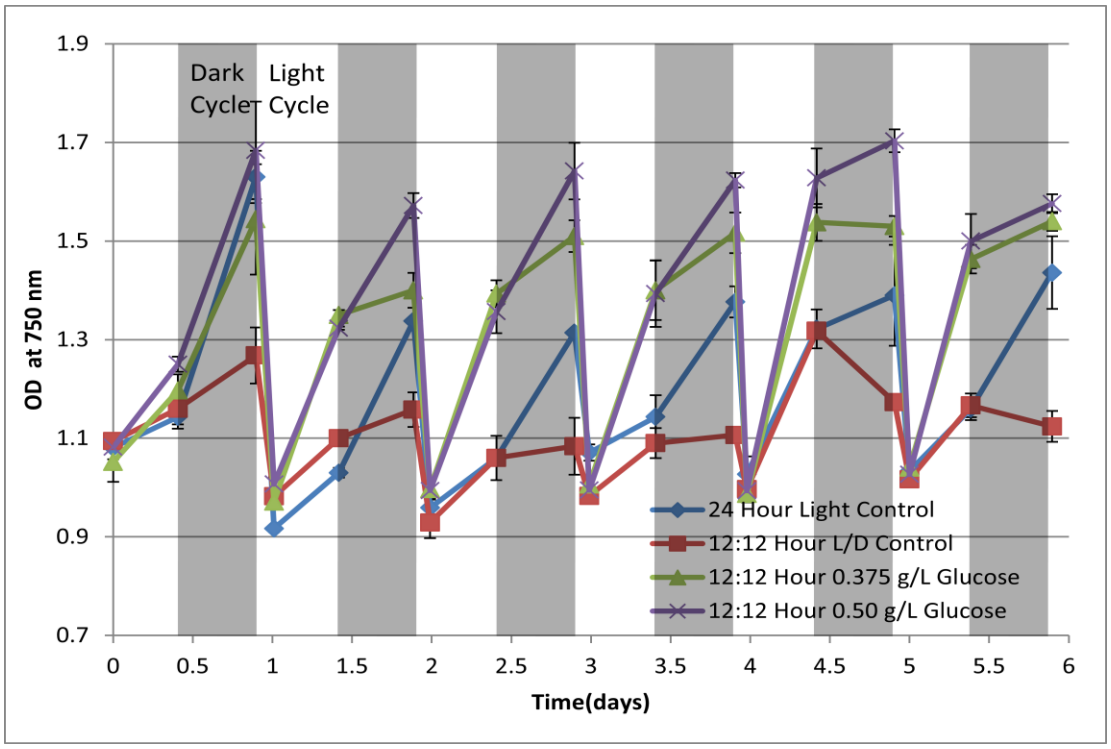

Figure 4.1 Optical density of the cultures measured as OD_{750} nm versus time, mean ± standard error, n=3 (supplement concentration 0.375 g/L & 0.5 g/L).

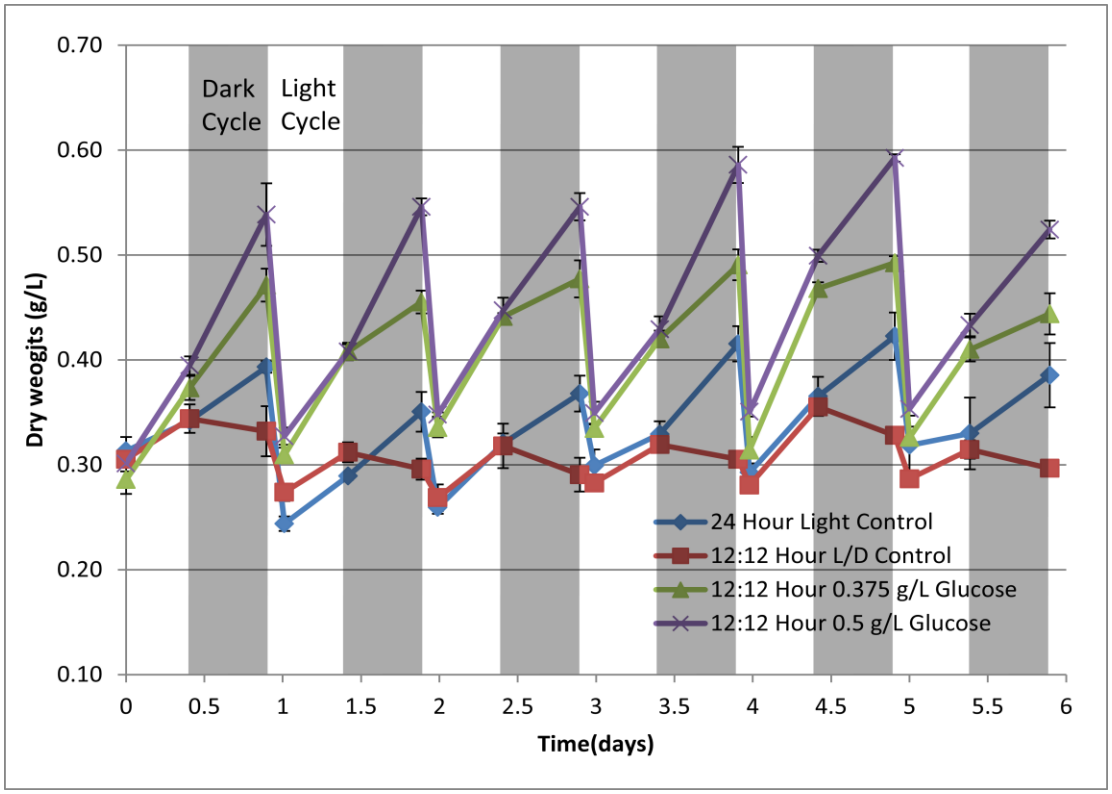

Figure 4.2 Dry weights (g/L) of the cultures versus time, mean ± standard error, n=3 (supplement concentration 0.375 g//L & 0.5 g/L).

The culture supplemented with glucose concentration of 0.5 g/L achieved maximum growth (measured as OD_{750}) during the dark cycle followed by the culture with 0.375 g/L supplement concentration and the 24 hour light control (Fig. 4.1). Similar results were obtained for dry weight (Fig. 4.2). Results were reproducible with everyday dilution during the course of the experiment.

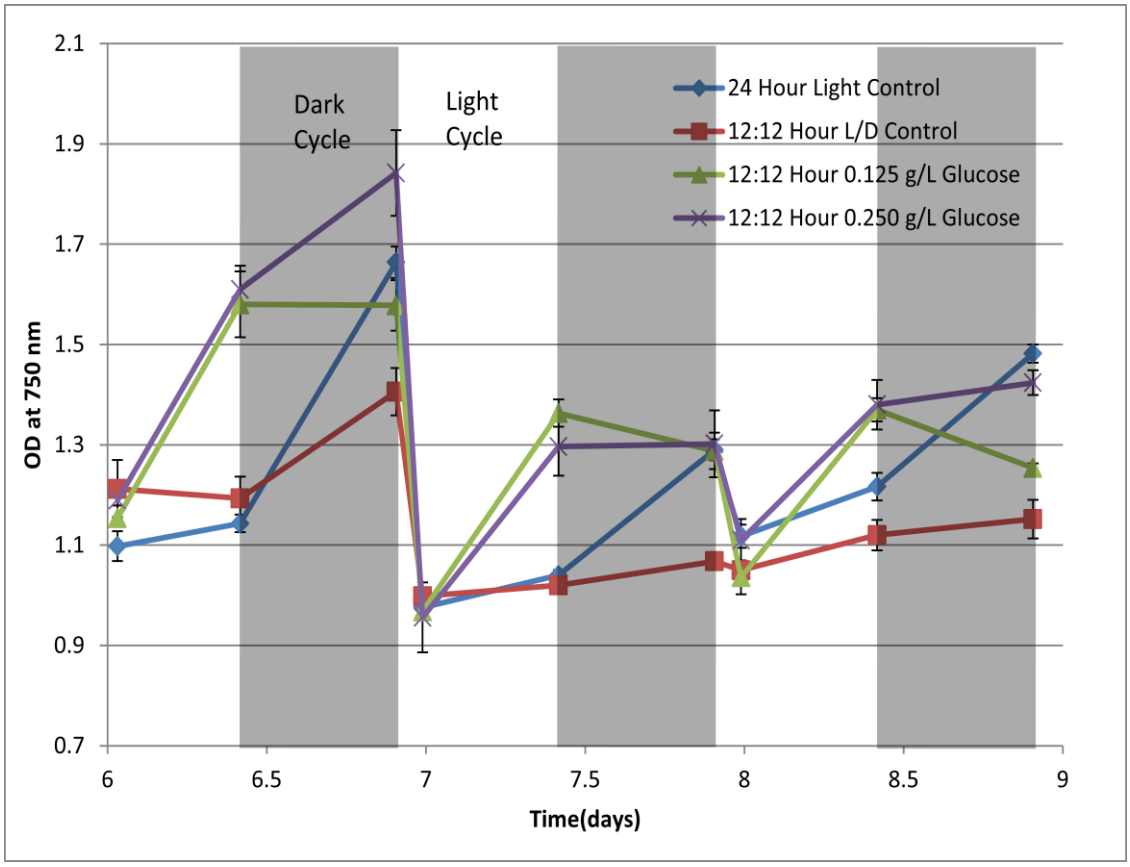

Figure 4.3 Optical density of the cultures measured as OD_{750} nm versus time, mean ± standard error, n=3 (supplement concentration 0.125 g/L & 0.250 g/L).

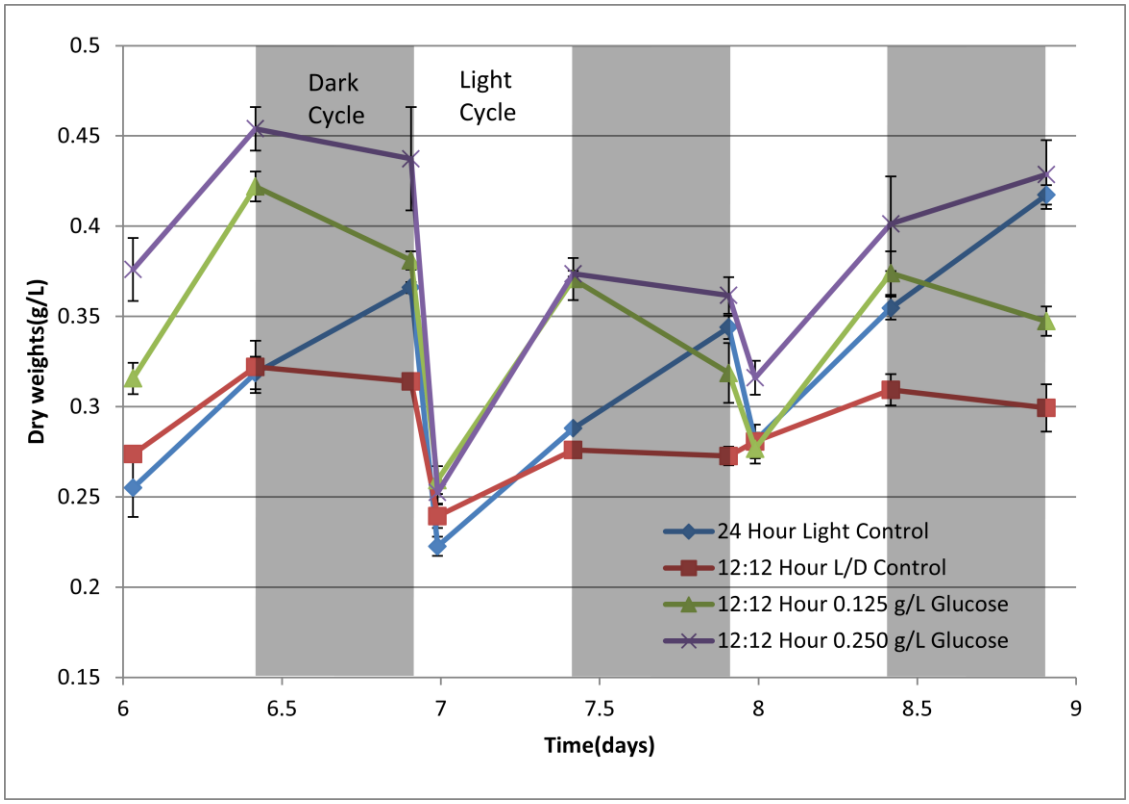

Figure 4.4 Dry weights (g/L) of the culture versus time, mean ± standard error, n=3 (supplement concentration 0.125 g/L and 0.250 g/L).

Absorbance and dry weights data from Figures 4.3 and 4.4 suggest that, the cultures supplemented with 0.250 g/L glucose compensated for biomass loss during the dark cycle. The cultures with 0.125 g/L glucose supplementation showed a loss in biomass concentration during the dark cycle, and the cultures with 24 hour light control showed growth during both cycles (Fig. 4.3 & 4.4). Similar trends between the results for absorbance and dry weight were observed during this sequential experiment as well with reproducibility. It was observed from the previous experiment at Phycal that supplementation with 0.250 g/L concentration helps to maintain the biomass concentration during dark cycle without any significant biomass loss. Results from this experiment confirmed those observations.

Absorbance and biomass data were also used to calculate the biomass to absorbance ratio. The biomass to absorbance ratios provides us with a quick reference to understand the progress of the experiment. Values for biomass to absorbance ratio from the previous experiments at Phycal were used as a reference. Deviation from the reference value helped us to understand the effect of different treatments on biomass concentration. It also helped us to understand the effect of other parameters such as contaminations with bacteria or fungi, change in cell size, or human error, while collecting the sample or analyzing them. Previous experimental data from Phycal suggested that biomass to absorbance ratio was approximately0.28 g/L/OD_{750}. The values for this experiment are shown in Table 4.1. They are comparable with previous results for control treatments.

Table 4.1 Calculated average biomass to absorbance ratio for different treatments.

Supplement Concentration (g/L)	Average Biomass to O.D Ratio ± std. error, (g/L/OD_{750})
0.500	0.33±0.01, n =54
0.375	0.31±0.01, n=54
0.250	0.28±0.01, n=27
0.125	0.27±0.01, n=27
12 hour L/D control	0.28±0.01, n=54
24 hour light control	0.28±0.01, n=54

The cultures supplemented with 0.375 g/L and 0.5 g/L of glucose showed deviation from the other treatments. The data for supplementation treatments were compared with the control treatment using two-sample, unequal variances t-test. The results for the t-test are shown in Table 4.2. Based on the p-value from the test, it was concluded that the cultures with higher concentration of supplementation treatment showed greater Biomass/$O.D_{750}$ ratio compared to the control cultures. This might be due

to change in physiology of cells due to the glucose supplementation/heterotrophic growth[13]. Previously, researchers have shown accumulation of lipid and other metabolites during the heterotrophic cultivation[19].

Table 4.2 Two sample t-test for comparison of differences in Biomass/OD_{750} ratio between control treatment and supplementation treatments.

T-test –Treatments	Results, p-value
24 h-control versus 0.125 g/L glucose supplementation	<0.01
24 h-control versus0.250 g/L glucose supplementation	0.50
24 h-control versus 0.375 g/L glucose supplementation	<0.01
24 h-control versus 0.5 g/L glucose supplementation	<0.01

To obtain the correlation between dry weight and absorbance for different supplementation treatments, biomass dry weight values for different treatments were plotted against the absorbance values (Fig. 4.5). Slopes for different treatments were retrieved from the plot to determine the correlation. Table 4.3 shows the value of correlation (slope) for different treatments.

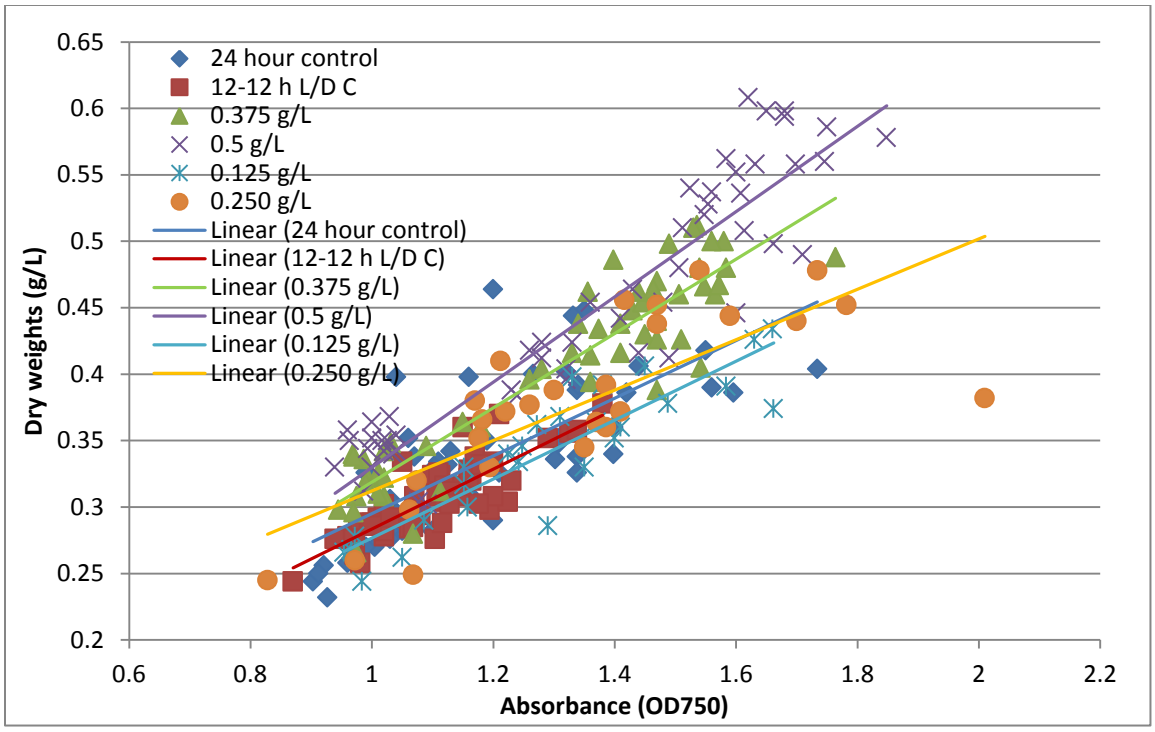

Figure 4.5 Dry weight (g/L) versus absorbance measured as $OD_{750}$for different treatments.

Table 4.3 Retrieved correlation between dry weight and OD_{750} for different treatments.

Treatment	Correlation(slope)	Intercept
24 hour control	0.21±0.03	Not significantly different from zero at 5% risk
12 hour L-12 hour D control	0.23±0.02	
0.125 g/L	0.22±0.02	
0.250 g/L	0.19±0.03	
0.375 g/L	0.28±0.02	
0.5 g/L	0.32±0.02	

Cell count results were generally consistent with previous results of absorbance and dry weight for all treatments (Fig. 4.6, 4.7).However, there is a large amount of scatter in the data. One of the reasons could be the human error involved while counting

the cells. Accuracy of the cell count depends on many factors such as sampling, diluting, and filling of the counting chamber, as well as the choice of the right type of counting chamber and range of cell concentration. Cell counts sampled were stored for almost three weeks before being analyzed which might also have affected the results.

Cell count helps us understand the effect of contamination/bacteria. Cell count results for 0.250 g/L supplement concentration followed the same trend followed by one of the previous experiments at Phycal, Inc. during which the biomass concentration remained constant while supplementing with 0.250 g/L glucose.

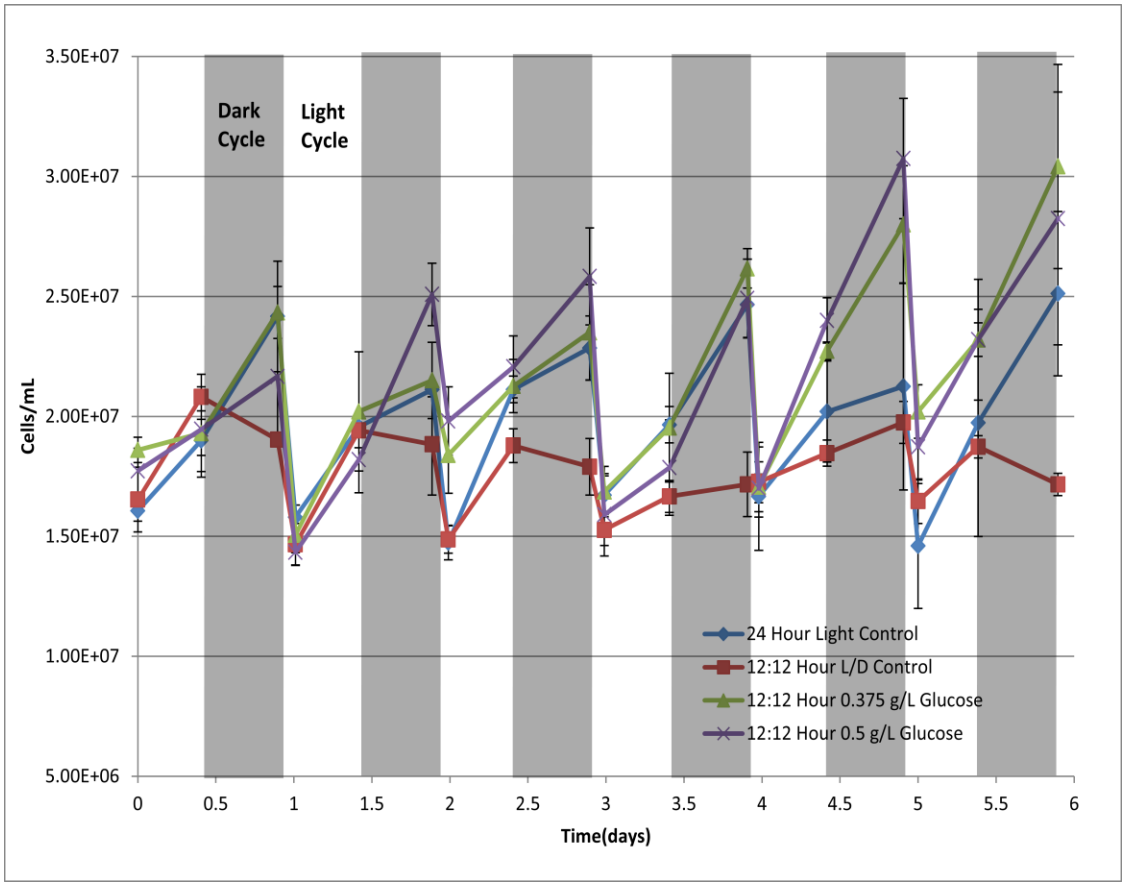

Figure 4.6Effect of 0.5 & 0.375 g/L supplementation on biomass, as measured using cell-count cells/mL, mean ± standard error, n=3.

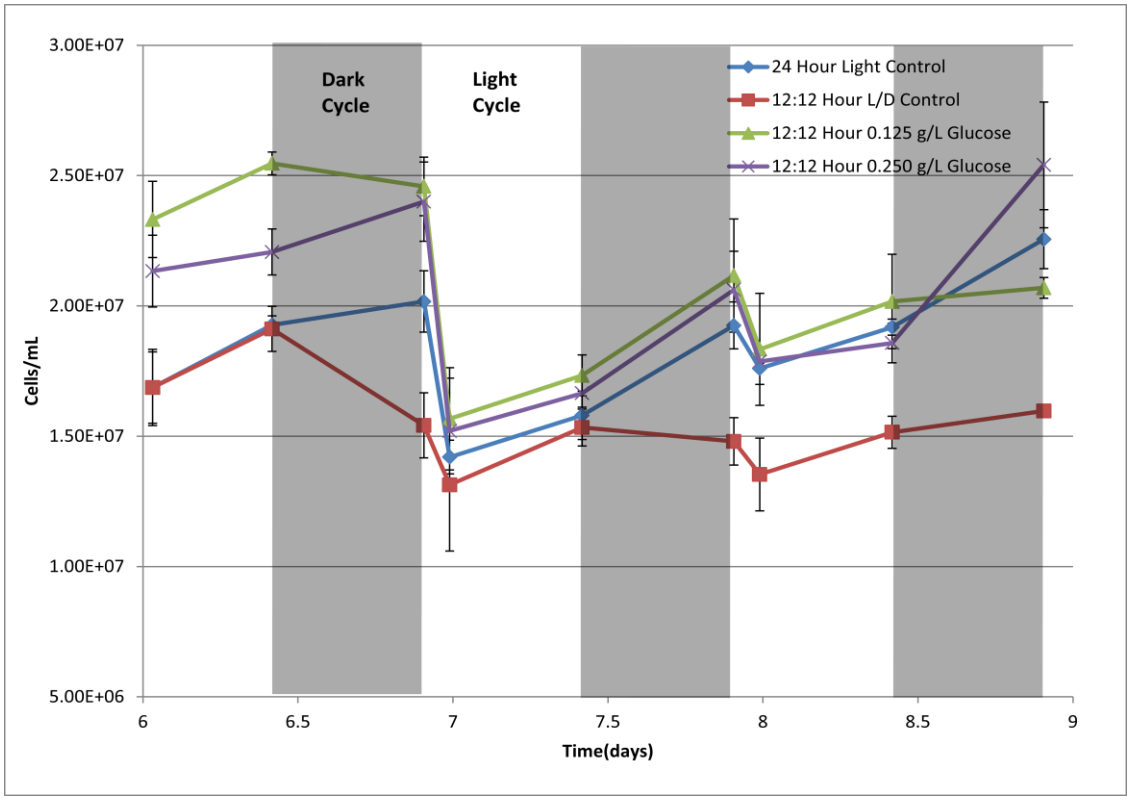

Figure 4.7Effect of 0.250 & 0.125 g/L supplementation on biomass, as measured using cell-count cells/mL, mean ± standard error, n=3.

4.2 Contamination/Bacterial Counts

Bacterial counts were performed by co-workers at Phycal, Inc. Figure 4.8 shows that the bacterial counts were approximately 10^6 CFU (colony forming units) per ml. At the concentration 10^6 cells/ml, bacterial concentration was about one order of magnitude lower than the algae concentration (Fig. 4.6, 4.7).Assuming average values for the algae and bacteria cell diameters as 6 micron and 1 micron, respectively; the percentage volume of bacteria in the algae culture can be calculated for geometrically spherical cells. Using cell count data for bacteria and microalgae, the percentage volume of bacteria cells in the algae cell culture was then estimated to be 0.05 %. The result suggested that the bacterial concentration was very low compared to the algae cell concentration. At this concentration, we can hypothesize that all the glucose being supplied was consumed by

algae cells. So while improvements can be made to reduce the contamination, it is unlikely that at this concentration bacteria can significantly affect the algae. There was no culture crash (culture loss due to contamination) during any time of the experiment. Algae concentration results were consistent and reproducible as shown in figure 4.8. Results from figure 4.8 do not support the hypothesis that supplementation would promote contamination since supplemented cultures had less bacteria compared to control cultures.

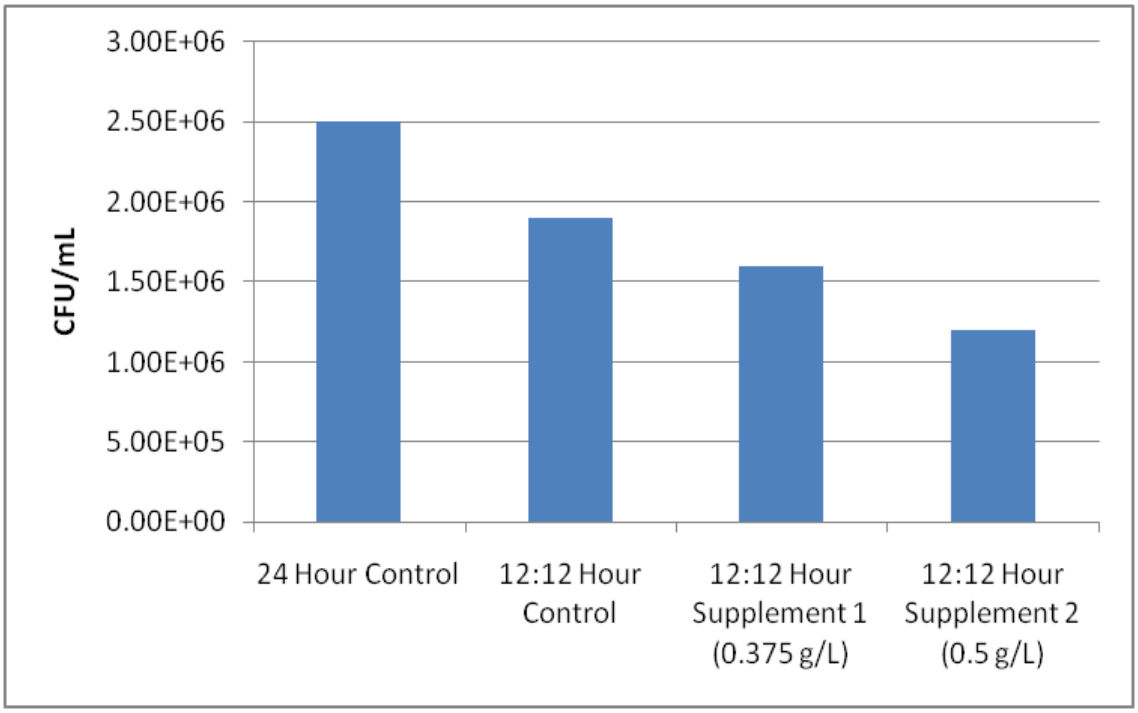

Figure 4.8 CFU (colony forming units per ml) versus treatments (Actual colony generation treatments were performed by co-workers at Phycal, Inc.).

4.3 Biomass Productivity

Biomass productivity is defined as the amount of biomass produced per unit volume or area per unit time. Areal productivity was calculated to understand and compare the production of biomass between supplementation treatments and control treatments. Biomass dry weights (X), working volume of the bioreactor (V), and the top

surface area of the open top bioreactor buckets (A) were used to calculate the areal productivities.

$$P = \frac{(X_1 - X_0)}{A * \Delta t} * V \quad (4.1)$$

Two types of productivities were calculated: 1) Overall productivity, and 2) Daytime productivity. Daytime productivity was calculated to quantify the growth during light cycle only. It was calculated to compare the photoautotrophic biomass growth between the control cultures and supplemented cultures. Overall productivity (Δt = 24 hour) was calculated to quantify the overall growth during a 24 hour cycle. That helped us understand the effect of supplementation not only during the dark cycle but also during the light cycle. It is very important to know the productivity data for both types of growth to understand the effect of photoautotrophic and heterotrophic growth on biomass production.

Dry-weight data from Figures 4.2 and 4.4 were used to calculate the productivity values. Productivity values for all days were averaged and are shown in Figures 4.9, and 4.10, for overall and daytime productivities, respectively. The results show that supplementation increased both the daytime and overall biomass productivities compared to the control cultures. Result from Figure 4.9 show that the supplemented cultures obtained higher overall productivities compared to those of the 12-12 hour L/D control cycle. Productivities increased with increased amount of supplementation. Overall productivity for 0.5 g/L supplementation treatment was 8-foldthe productivity for 12-12 L/D control treatment. Overall productivity for 0.250 g/L supplementation treatment was 4-fold the productivity for the 12-12 hour L/D control treatment. Results for daytime

productivity from Figure 4.10show that supplemented cultures obtained higher daytime productivity compared to the control cultures at any point during the experiment. Daytime productivity for the 0.5 g/L supplementation treatment was 2-fold the productivity for control treatments. Daytime productivity for 0.250 g/L supplementation was also 2-fold that of the control treatments.

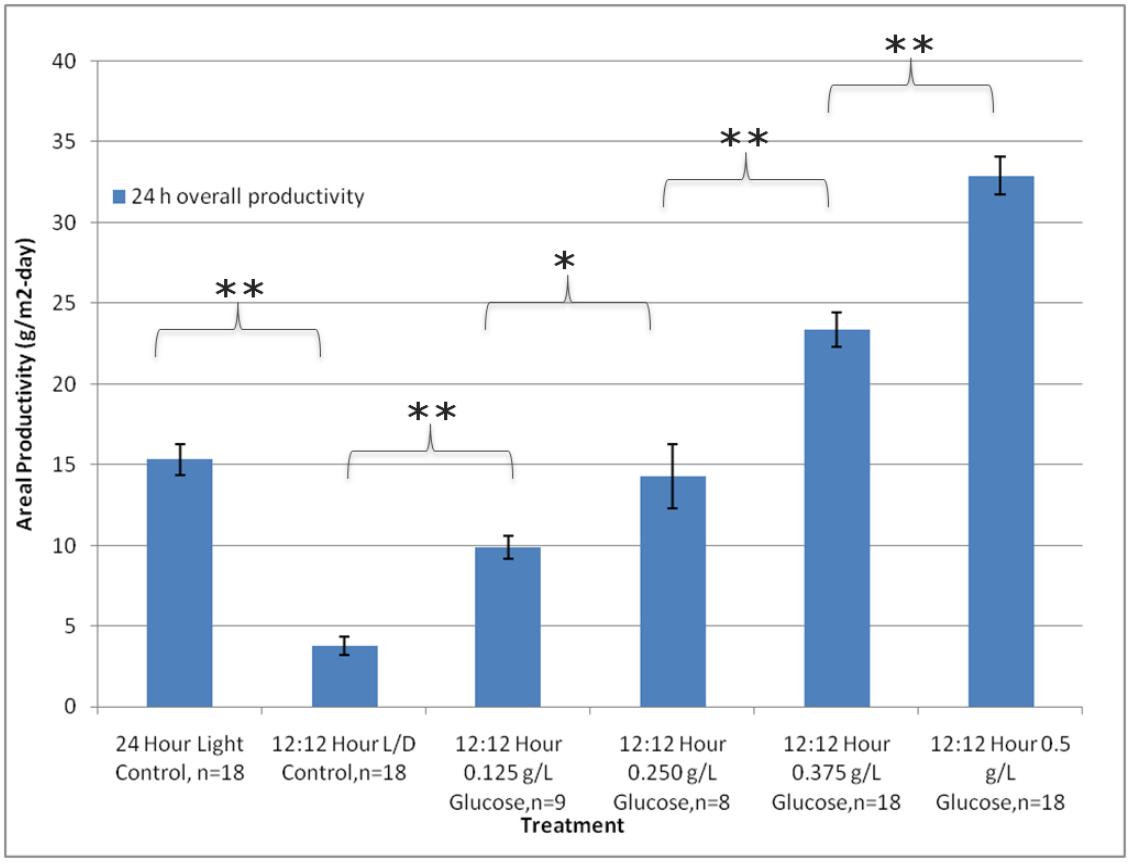

Figure 4.9 Summary of average overall productivity for different treatments, mean ± standard error, (T-test outcomes: **p<0.01, *p<0.05).

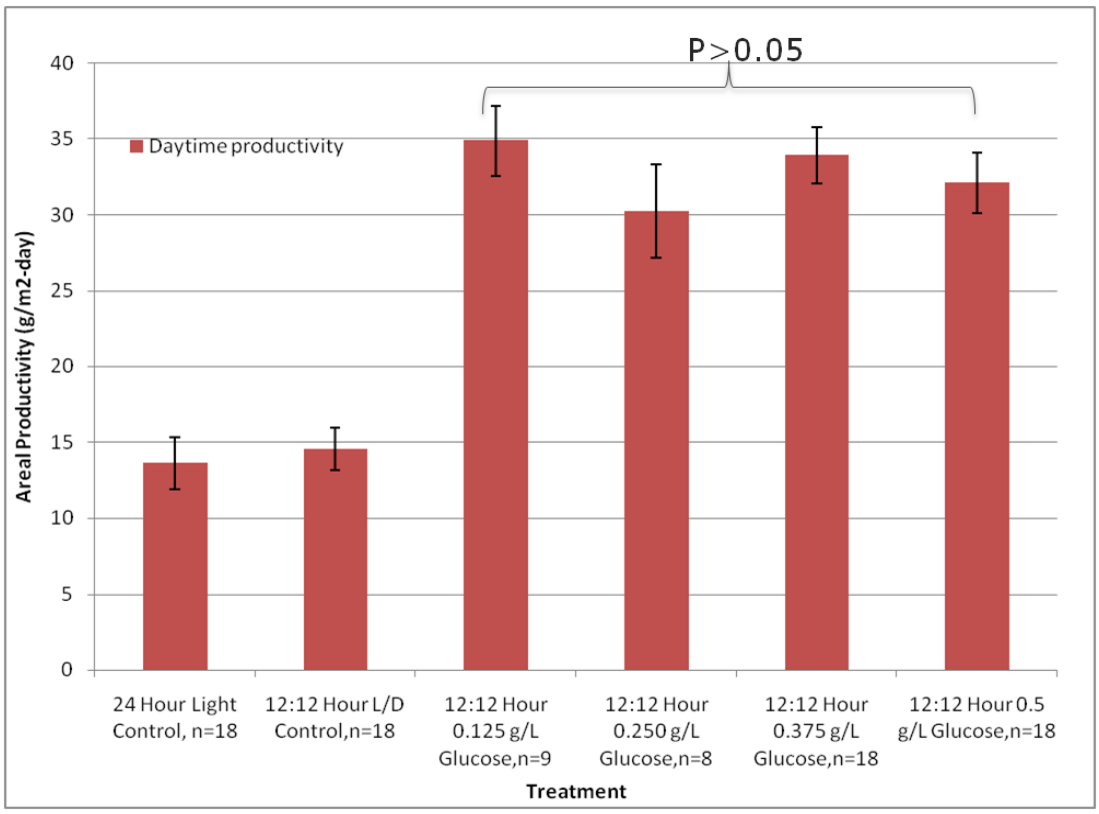

Figure 4.10 Summary of average daytime productivity for different treatments, mean ± standard error, (T-test outcomes>0.05).

The results shown in Figures 4.9 and 4.10 clearly show the difference between the cultures which received supplementation treatments at night and the control cultures which did not receive any treatments. Cultures with adequate amounts of supplementation (0.5 & 0.375 g/L) achieved higher growth during dark cycles (Fig. 4.2) signifying the advantage of supplementation during dark cycles. Their biomass concentration increased during night time leading to higher productivities. The cultures supplemented with 0.250 g/L and 0.125 g/L had lower overall biomass productivity compared with other supplemented treatments and the 24 hour control treatment. In these cultures, no sign of growth was observed during nighttime (Fig. 4.4). Results showed loss in biomass concentration during nighttime when supplemented with 0.125 g/L glucose concentration leading to lower overall productivity, decreasing from daytime productivity

value of 35 g/m2-day to an overall productivity value of 10 g/m2-day. Similarly, 12-12 light/dark control also showed decrease in productivity value from 15 g/m2-day for daytime to 4 g/m2-day for overall productivity, signifying night time biomass loss in control cultures with the dark cycle.

Figure 4.10 shows that, surprisingly, supplementation treatments had higher day time productivity compared to that of the 12-12 hour L/D control treatment. There might be three reasons for this trend of biomass growth: 1) Glucose might have been carried over during the light cycle, which might have led to the mixotrophic growth of the culture. Higher growth with mixotrophic culture has been achieved. 2) As discussed in the background section, heterotrophic growth of the culture leads to degradation of chlorophyll in chloroplasts[19]. Decrease in chlorophyll content may lead to higher light transmitting inside the culture, causing higher photosynthetic rates, providing there is still sufficient chlorophyll for photosynthesis. It may lead to higher growth during the light cycle. 3) Additional starch production at night, which when used for energy during the daytime with excess energy from light and CO_2 might increase the growth during daytime.

Cultures supplemented with 0.375 g/L and 0.500 g/L glucose concentration showed higher overall productivity than control treatments at any point during the experiment due to higher biomass growth during both cycles. Figure 4.9& 4.10 shows, culture with 24 hour light control showed consistent productivity values signifying the effect of 24 hour light on the growth of the culture. The culture with 12-12 L/D control showed less overall biomass productivity due to the loss in biomass concentration during night time. But daytime productivity results for that culture signifies the effect of

photoautotrophic growth on the culture. Control treatments showed purely photoautotrophic growth during daytime because they were never supplemented during the night cycle.

Two sample t-tests between the overall productivity values for different treatments were conducted to determine if the difference between the productivity data for different treatment was statistically significant. Results for the t-tests between different treatments are shown in Table 4.3. The results indicate that the differences in productivity values for each treatment were statistically significant. In other words, it indicates that each treatment had a different effect on biomass loss during night time and, hence, different overall productivity values.

Table 4.4Two-sample t-test for comparison of differences in average overall productivity values for different treatments.

T-test, treatments	p-values
24 hour light control & 12-12 hour L/D control	<0.01
12-12 hour L/D control & 0.125 g/L supplementation	<0.01
0.125 g/L & 0.250 g/L supplementation	<0.05
0.250 g/L & 0.375 g/L supplementation	<0.01
0.375 g/L & 0.5 g/L supplementation	<0.01

Another t-test was conducted between the results for daytime productivity between supplementation treatments to study whether the daytime productivity values for the supplemented cultures are significantly different from each other or not. Results are shown in Table 4.4. The p-values suggested that the difference between daytime productivity values for the supplemented treatments was not statistically significant,

indicating all the supplemented cultures behaved in a similar way during the daytime. A possible way to interpret this is that when cells are supplemented with glucose, they first stored some of it as starch. Once the fixed amount of starch is produced and stored, the excess glucose is used for respiration and biomass production.

Table 4.5Two sample t-test for comparison of differences in average daytime productivity values for different supplementation treatments.

T-test, treatments	p-values
0.125 g/L & 0.250 g/L supplementation	0.12
0.250 g/L & 0.375 g/L supplementation	0.16
0.375 g/L & 0.5 g/L supplementation	0.25

Figures 4.11 to 4.14show the detailed results for overall and daytime productivity for all treatments during the course of experiment. It can be observed from Figure 4.12 that daytime productivity for the date of 06/25 showed a sudden increase compared to the previous results in the same figure. The samples for that day were collected by co-workers at Phycal, Inc. Inconsistency in the method of collecting samples may have led to changes in results during that day.

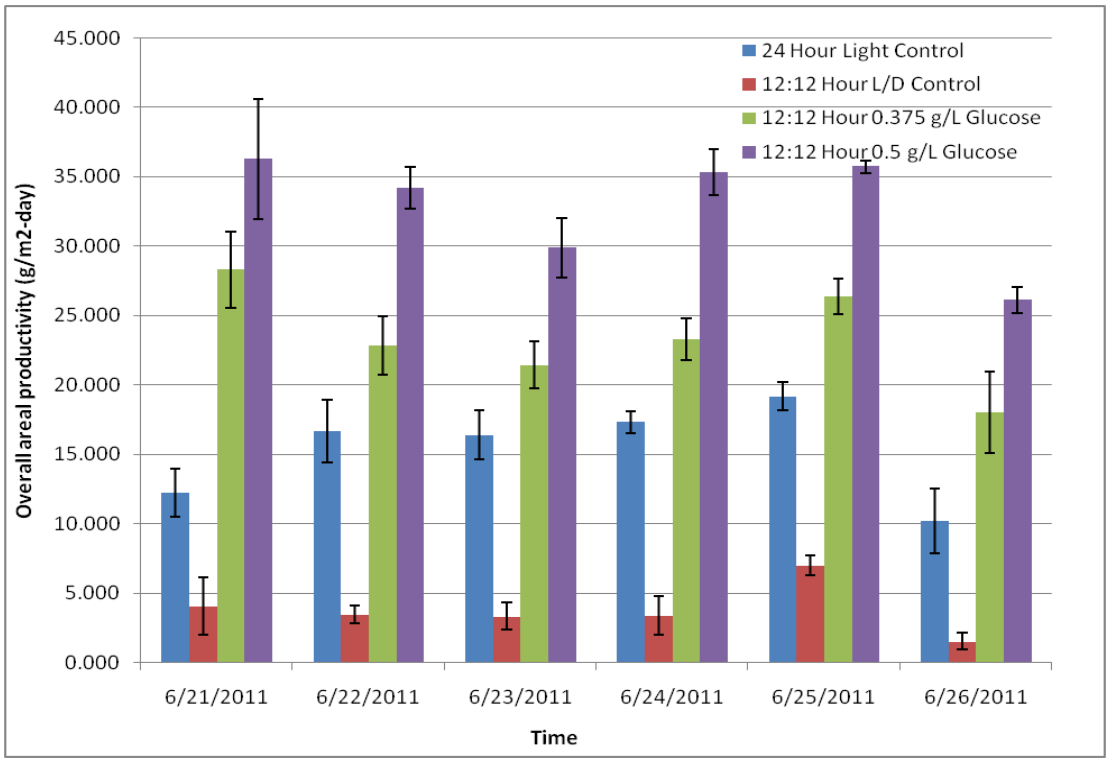

Figure 4.11 Overall biomass productivity (for the period of 24 hours), mean ± standard error, n=3 (supplement concentration 0.375 g/L and 0.5 g/L).

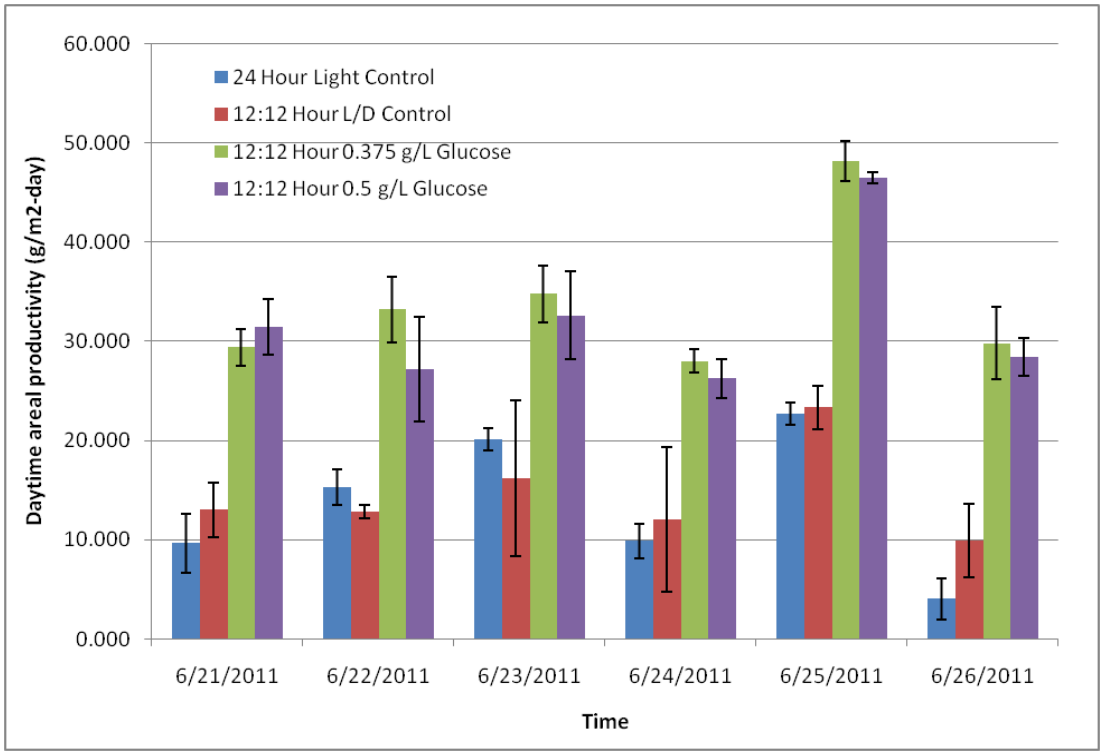

Figure 4.12Daytime biomass productivity (for the period of 12 hour light cycle), mean ± standard error, n=3 (supplement concentration 0.375 g/L and 0.5 g/L).

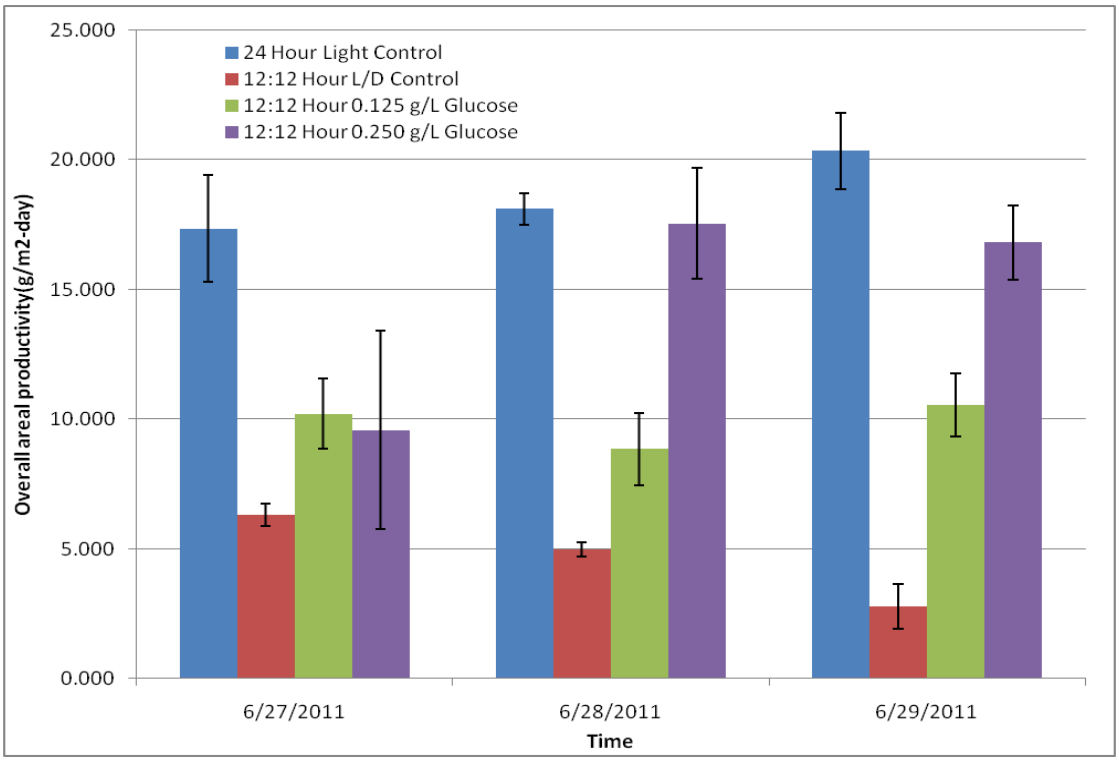

Figure 4.13 Overall biomass productivity (for the period of 24 hour), mean ± standard error, n=3 (Supplement concentration 0.125 g/L and 0.250 g/L).

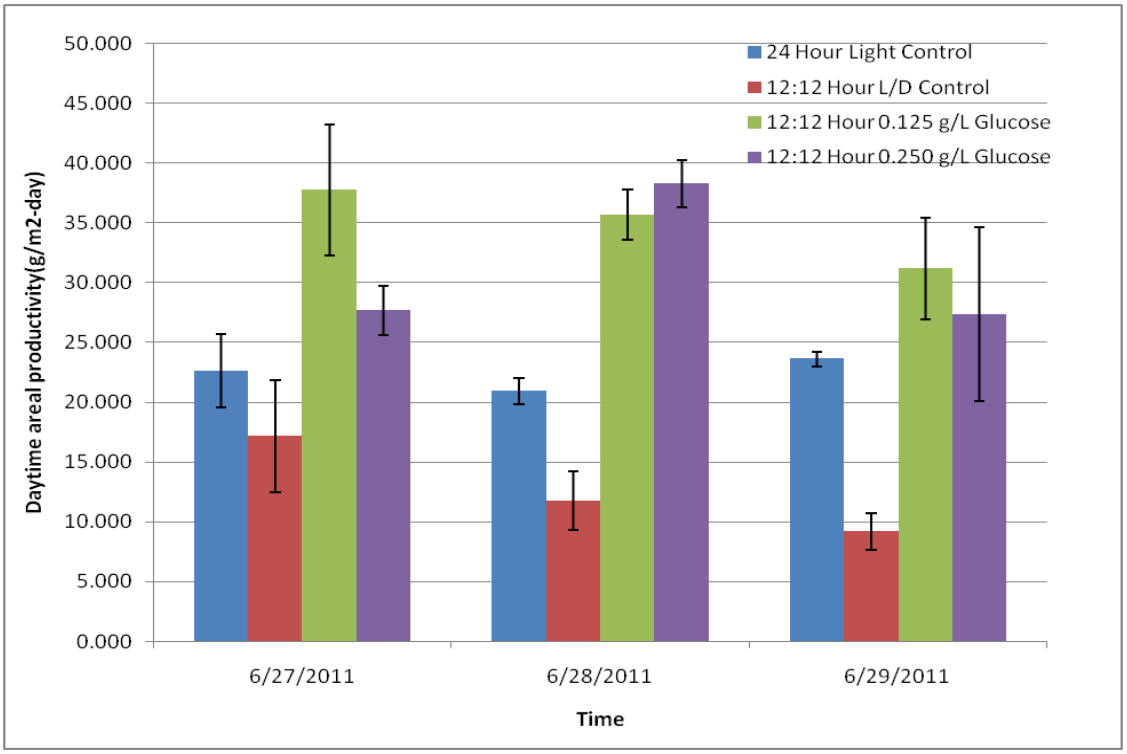

Figure 4.14 Daytime biomass productivity (for the period of 12 hour light cycle), mean ± standard error, n=3 (Supplement concentration 0.125 g/L and 0.250 g/L).

4.4 Biomass Yield

The yield coefficient helps us understand biomass production with sugar consumption, assuming growth due to other factors during dark cycle is negligible. The yield coefficient was calculated using biomass dry weights and glucose concentration measured for the same time frame. Samples were collected every time at the start of dark cycle and at the end of the cycle, and analyzed for glucose concentration. (Supplements were injected at the start of dark cycle every day).

The apparent biomass yield coefficient was calculated from the difference in biomass concentration at the end and start of the dark cycle(X_1 - X_o) divided by the difference in glucose concentration (S_o-S_1), i.e.

$$Y_{x/s}^{app} = \frac{(X_1 - X_0)}{(S_0 - S_1)} \quad (4.2)$$

Where, X= biomass concentration and S= glucose concentration.

Glucose concentrations were determined through HPLC with the help of coworkers at Phycal, Inc. Figures 4.15 & 4.16 show the glucose concentration data collected at the start of dark cycle and at the end of dark cycle for different treatments. As the result show, there was negligible glucose left after the end of the dark cycle.

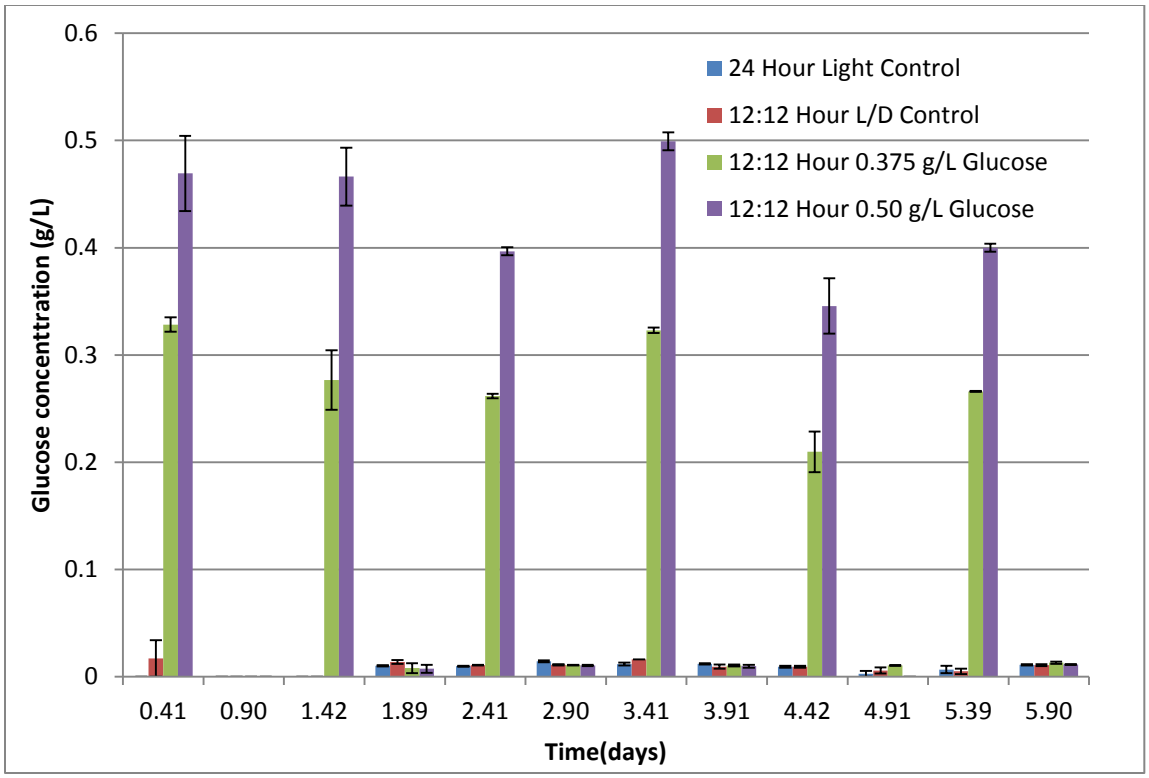

Fig 4.15 Glucose concentration (g/L) measured through HPLC versus time, mean ± standard error, n=3 (supplement concentration 0.375 g/L & 0.5 g/L).

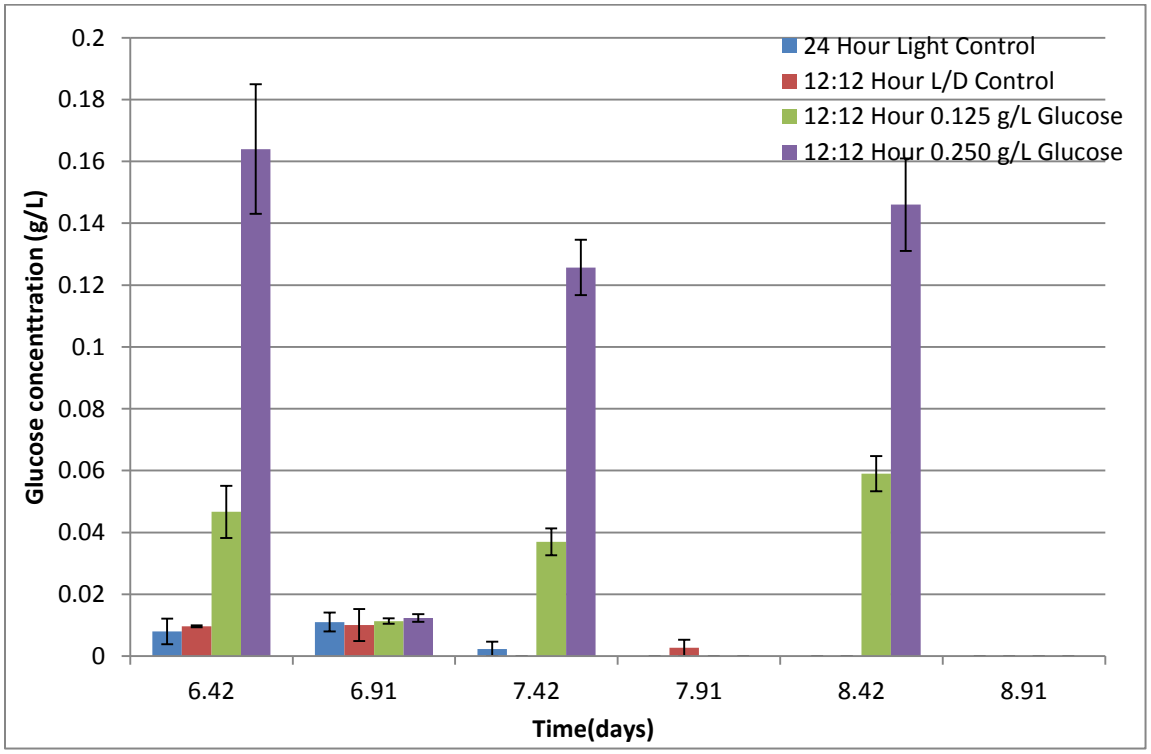

Fig 4.16Glucose concentration (g/L) measured through HPLC versus time, mean ± standard error, n=3 (supplement concentration 0.125 g/L & 0.250 g/L).

Glucose concentration data shows discrepancies in the measurements at the start of the dark cycle compared to the concentration expected based on the amount added. Glucose samples were stored at 4^0C for the period of approximately two weeks before being analyzed through HPLC. That might be the reason for low glucose concentration at the start of the dark cycle. HPLC data for sugar samples showed that all supplements being supplied were consumed by the end of the dark cycle, and hence we can say that the light cycle had only autotrophic growth. Based on the sugar concentration data, average results for yield coefficients are shown in Table 4.2.

Table 4.6Apparent yield Coefficients$Y_{x/s}$, mean ± standard error.

Supplement Concentration (g/L)	**Yield Coefficient(gm biomass/gm glucose)**
0.5	0.28±0.03, n=18
0.375	0.20±0.10, n=18
0.250	Not significantly different from zero at 5% risk
0.125	-0.10±0.8, n=6

A Model for substrate consumption with maintenance demand was used, (assuming no product formation by substrate) [2];

$$R_s = \frac{\mu X}{Y_{x/s}^{th}} + M_s X \tag{4.3}$$

where, μ = specific growth rate (day^{-1}), X = biomass concentration, and M_s=maintenance coefficient (gm substrate/gm biomass/time).The observed yield coefficient can be calculated using,

$$Y_{x/s}^{app} = \frac{R_x}{R_s} \tag{4.4}$$

where, the rate of biomass production is given by,

$$R_x = \mu X \tag{4.5}$$

Combining equation 4.4 and 4.5 yields;

$$R_s = \frac{\mu X}{Y_{x/s}^{app}} \tag{4.6}$$

Combining equation 4.3 and 4.6 yields;

$$\frac{\mu X}{Y_{x/s}^{app}} = \frac{\mu X}{Y_{x/s}^{th}} + M_s X \tag{4.7}$$

Specific growth rate values were calculated using the biomass concentration at the start and at the end of dark cycle. The rate of biomass production can be given by,

$$\frac{dX}{dt} = \mu X \tag{4.8}$$

Integrating the equation over time, we can derive;

$$\mu = \frac{\ln X_2 - lnX_1}{\Delta t} \tag{4.9}$$

Equation 4.7 can be rearranged in three different ways to obtain the value of theoretical yield and maintenance coefficients.

1. Eq. 4.7 can be rearranged to[2],

$$\frac{1}{Y_{x/s}^{app}} = \frac{1}{Y_{x/s}^{th}} + \frac{M_s}{\mu} \tag{4.10}$$

Plotting $\frac{1}{Y_{x/s}^{app}}$ against $\frac{1}{\mu}$ (Lineweaver-Burk plot[16]), we can obtain the theoretical yield and maintenance coefficient from the intercept and slope of the graph (Fig. 4.17) respectively.

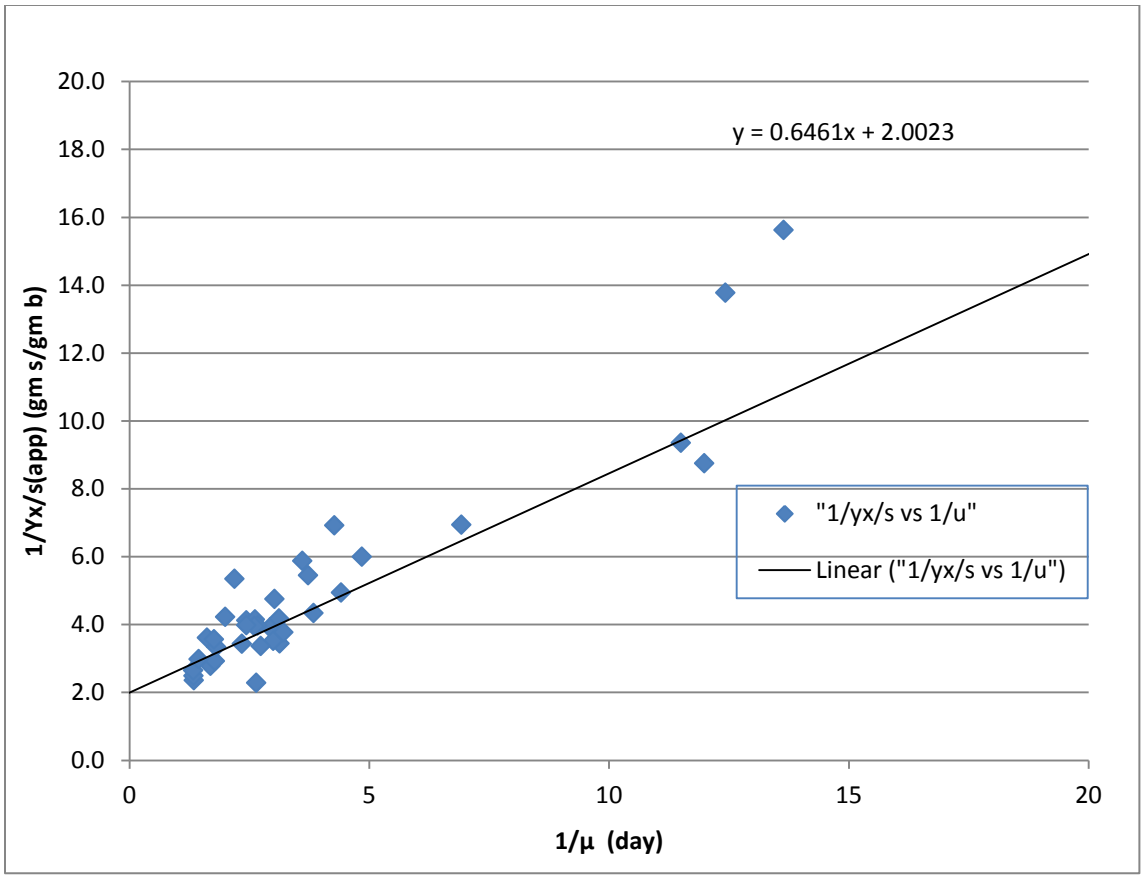

Figure 4.17 Plot of ($1/Y_{x/s}^{app}$) versus ($1/\mu$) for the calculation of theoretical yield coefficient.

The theoretical yield coefficient value of 0.5±0.2gm biomass/gm glucose and maintenance coefficient value of 0.65±0.03gm substrate/gm biomass/day was calculated from the graph.

2. Another rearrangement of Eq. 4.7 leads to the equation,

$$Y_{x/s}^{app} = Y_{x/s}^{th} - M_s Y_{x/s}^{th} \left(\frac{Y_{x/s}^{app}}{\mu}\right) \qquad (4.11)$$

Plotting $Y_{x/s}^{app}$ against ($Y_{x/s}^{app}$ / μ) (Eadie-Hofstee plot[16]), we can obtain theoretical yield coefficient and maintenance coefficient from the intercept and slope (Fig. 4.18) respectively. The theoretical yield coefficient value of 0.42±0.05gm biomass/gm glucose

and maintenance coefficient value of 0.63±0.14gm substrate/gm biomass/day was calculated from the graph.

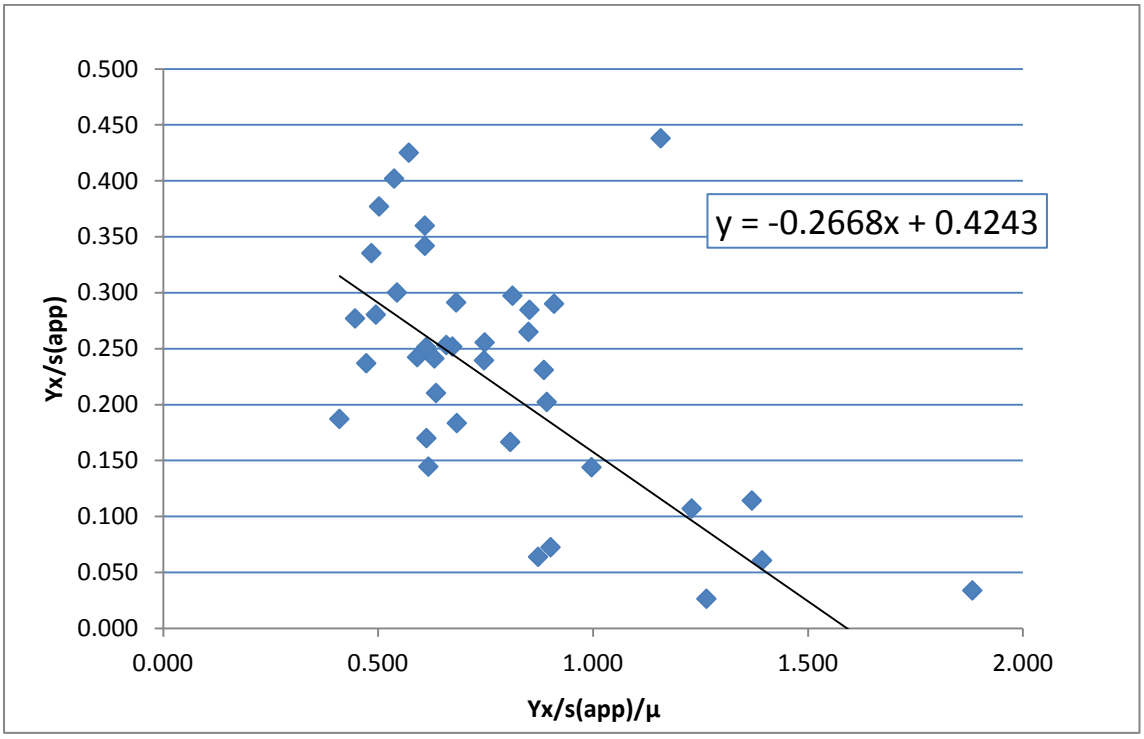

Figure 4.18Plot of ($Y_{x/s}^{app}$) versus ($Y_{x/s}^{app}$ /μ) for the calculation of theoretical yield coefficient.

3. Another arrangement of Eq. 4.7 leads to the equation,

$$\frac{\mu}{Y_{x.s}^{app}} = M_s + \frac{1}{Y_{x/s}^{th}}\mu \qquad (4.12)$$

Plotting (μ / $Y_{x/s}^{app}$) against μ (Hanes-Woolf plot[16]), we can obtain theoretical yield coefficient and maintenance coefficient from the slope and intercept (Fig.4.19) respectively. The theoretical yield coefficient value of 0.62±0.07gm biomass/gm glucose and maintenance coefficient value of 0.87±0.07 gm substrate/gm biomass/day was calculated from the graph.

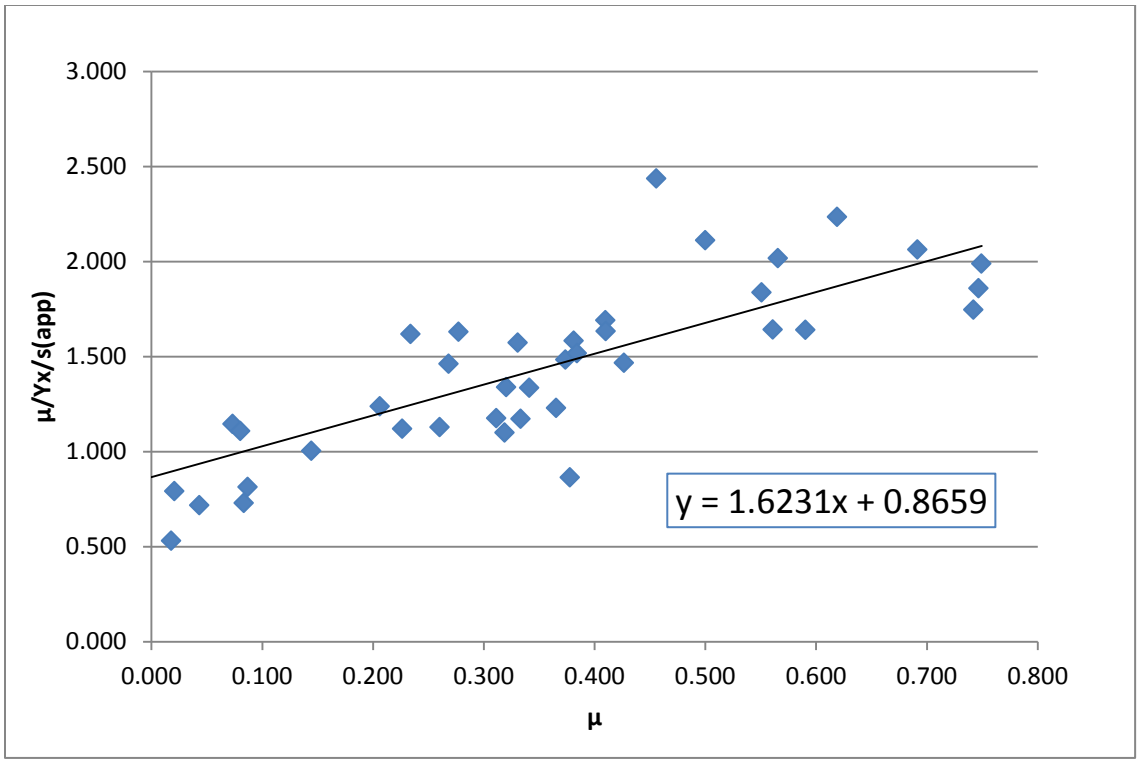

Figure 4.19Plot of ($\mu/Y_{x/s}^{app}$) versus (μ) for the calculation of theoretical yield coefficient.

It can be concluded from the above calculations that, Hanes-woolf plot arrangement leads to more precise results with less error. Using the equation 4.12 and Figure 4.19, the theoretical yield coefficient value of 0.62±0.07gm biomass/gm glucose and maintenance coefficient value of 0.87±0.07 gm substrate/gm biomass/day was calculated. Theoretical yield coefficients as high as 0.68 gm biomass/gm glucose can be obtained if substrate was consumed only for the biomass production, as described in section 3.2.3.It can be concluded from our result that the rest of the glucose might have been consumed for the other metabolic activities and maintenance requirements. Our measured theoretical yield was calculated using the average algae composition.

CHAPTERV

CONCLUSIONS & RECOMMENDATIONS

5.1 Conclusions

Results clearly showed the effect of supplementation on compensating for night time biomass loss. Depending on the requirement for the experiment, one can decide the supplement concentration to be used. The results concluded that, with 0.250 g/L glucose supplementation, one can stop nighttime biomass loss. Biomass productivities increased with the increased amount of supplementation. Productivity values up to 33 g/m2-day could be achieved with 0.5 g/L supplement concentration, which was 8 times the productivity of the 12 hour L/D control treatment. Statistical analysis between overall productivity values for each treatment proved that differences between them were statistically significant. Glucose concentration results showed that almost all the glucose supplied at the beginning of dark cycle was consumed at the end of dark cycle. Supplemented cultures showed higher growth both during dark cycles and light cycles. It supported the hypothesis that *Chlorella sp*. can grow either as a heterotroph or an autotroph. Bacterial counts results showed that the supplemented cultures had less amounts of contamination compared to the control treatments, which does not support the hypothesis that supplementation leads to contamination. Theoretical yield calculated from

the experimental results was different from the stoichiometric yield, suggesting that glucose was consumed for other maintenance activities and instead for the biomass production only. Flocculation of the algae cells is another problem during cultivation. The initial experiment at Phycal, Inc. showed flocculation. One of the hypotheses behind the observed flocculation is culture stress. Under any kind of culture stress, algae cells tend to flocculate. This stress may be induced on the culture due to several reasons such as: temperature fluctuation, pH fluctuations or bacterial contamination. Culture flocculation did not occur during the course of this experiment, indicating no adverse effect of any of the above issues on algae culture.

5.2 Recommendations

Study of metabolic pathways is a major research field. We did not study the effect of glucose on metabolic pathways of microalgae. A better understanding of the effect of glucose assimilation on metabolic pathways can help to understand the role of nighttime glucose supplementation on growth of microalgae. That can lead to even more precisely designed experiments and hence better results.

While supplementation increased biomass production, we did not investigate the effect of supplementation on lipid content. This should be investigated.

One can design the experiment to determine the glucose concentration continuously during nighttime. This can yield a more precise value of yield coefficient and can lead to better design of the process.

Scaling up the process at higher operating volume of the culture is necessary to determine the consistency of the results.

Study of cost analysis is required to understand the economic feasibility of the process of night time supplementation and to decide if the process can be scaled commercially or not.

Theoretical yield coefficient was calculated assuming error associated with each data point was equal. To get a more accurate value of the theoretical yield coefficient and the maintenance coefficient, one can do extensive error analysis.

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