LABORATORY SCALE STUDIES OF CYANOBACTERIA,
Synechococcus BG0011

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To my parents
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Microalgae are the photosynthetic prokaryotic microorganisms that are capable of utilizing carbon dioxide, water, and sunlight to produce biomass. The biomass could be used for the production of biogas, biodiesel, bioethanol etc. depending on the strain. Cyanobacteria used for the research work was *Synechococcus*, BG0011. This strain has the unique property of secreting highly viscous polysaccharide into the media, while the culture is in the stationary phase of the growth. Microalgae is capable of utilizing nutrients from the wastewater, for their growth. The biomass left after the extraction of oils, could be used as a valuable fertilizer.

In this study, batch and semi-continuous growth studies were done to determine the feasibility of the cultivating *Synechococcus* in laboratory scale photobioreactor. The cell growth was determined by measuring cell optical density on a daily basis. Dry cell weight analysis were also done to determine the biomass productivity. Studies were done to determine the effect of light on polysaccharide production. It was observed that light is necessary for the polysaccharide production to take place. Polysaccharide produced during the light will get consumed during the dark, for the cells to carry out
various cellular activities and also for cell maintenance. It was also observed that polysaccharide starts separating out from the biomass when the harvested culture is kept in the presence of light, without being disturbed. Polysaccharide produced could be anaerobically digested to produce methane.

Modeling of the batch and semi-continuous phase was also done to determine the biomass productivity when the growth rate reaches steady state.
CHAPTER 1
INTRODUCTION

With looming energy shortage, increasing environmental concerns and rapid industrial development, one of the biggest challenges facing us is to effectively produce viable alternatives to conventional energy systems. Biofuels, as they are derived from biodegradable resources, are believed to replace fossil fuels in the future. Carbon dioxide produced by industry and other sources could be utilized for biofuel production, thereby contributing to global warming minimization. Biofuels are being produced since a long time back. In the earlier days, biofuels were mainly derived from edible feedstock such as sugarcane, corn and soybean, causing increase in food prices (Maurycy et al., 2012). Later, waste material and lignocellulosic feedstocks such as switchgrass was used for producing biofuels, which showed better yields and lower land requirements. But, in order to meet the increasing energy requirements, there was a need for biofuel production system with much better yields and which doesn’t require arable land. Such potential was seen in the algal-biofuel system, as some of the algal species could be grown in saline, brackish and industrial wastewater.

Microalgae are the photosynthetic microorganisms that can convert carbon dioxide and water, by utilizing sun’s energy. They are mainly composed of 6% to 52% proteins, 7% to 23% lipids, and 5% to 23% carbohydrates, with actual composition varying from species to species (Anand, 2010). The protein content of most algal species is quite high with C/N ratio of 10.2 (Brown et al., 1997). There are many algal species in the world and they can be isolated from marine environments. The bioproduct of interest will determine the algal species to be used. Some species have high lipid content and they can be used for biodiesel production. Some may be good for
producing biomethane by the anaerobic digestion of algal biomass and carbohydrates produced by it. Some important algal species that are being used industrially as well as academically are *Synechococcus*, *Chlorella vulgaris*, *Nannochloropsis* and *Scenedesmus* etc. Metabolic engineering is playing a very important role in increasing the biofuel productivity. Through metabolic engineering, genes of the algal species can be modified to improve the species growth rate, lipid production, and carbohydrate production etc.

**Cultivation of Algae**

There are mainly three methods of cultivation of algae i.e. batch cultivation, fed-batch cultivation and continuous cultivation. For laboratory scale experimental purposes, batch method of cultivation is generally employed in order to determine the behavior of the culture before it could be used for large scale industrial applications. In the batch cultivation, the reactors are fed with nutrient media and inoculum and the algae is allowed to grow under controlled conditions of temperature, light intensity and pH etc. Initially, the growth curve shows a lag phase which is due to the cells acclimatizing to the environment. The lag phase could be reduced by using inoculum from the exponential phase of algal growth. Lag phase is followed by exponential phase, during which the cells grow exponentially, utilizing nutrients and light energy. By the end of exponential phase, the cells have shown the maximum growth, most of the nutrients have been consumed. The next phase is the stationary phase, during which, the rate at which cells are being produced becomes equal to the rate at which cells are dying thereby showing no net cell growth. Death phase follows the stationary phase, during which cells start dying due to nutrient inhibition. After the end of batch phase, the biomass is harvested and is used for analysis and characterization.
Parameters Affecting Algal Growth

The ideal growth conditions for algae and cyanobacteria are strain specific. Environmental factors such as light, temperature, pH, salinity, mixing can have considerable effect on algal growth and biofuel productivity. Optimizing these parameters, before carrying out large scale biofuel production is very important.

Light

Light is the main and only source of energy for autotrophic algal cultures. In the absence, or low intensity of light, algae won’t be able to grow and the cells would die. The growth rate will increase as the light intensity is increased and will reach the maximum at a certain light intensity. Increasing light intensity from that point onwards will retard the growth rate due to damaging of light receptors of algae (Anand, 2010). Algal biomass could cause self-shading, in which the cells prevents the light from penetrating into the layers beneath it. To prevent self-shading, it is very important to keep the culture layer shallow and also to have proper mixing of the culture. Ideally, the light should be uniformly distributed.

Temperature

Temperature is an important parameter for algal growth, as it can greatly influence the growth rate, cell composition, and gas transfer rates. Generally, algal growth rate increases with increasing temperature up to a certain levels, beyond which, cell growth decreases due to heat up. Within the optimum temperature range, growth rate doubles for every 10°C rise in temperature. In photobioreactors, temperature can be easily controlled in the optimum range, but it is not easy in case of open systems. In open systems, special attention is needed during early mornings and evenings because
during these times, there is lack of synchronization between light intensity and temperature which could lead to photo inhibition and biomass losses (Abayomi et al., 2009).

**pH, Carbon dioxide transfer, Nutrients, Mixing**

In order to achieve maximum growth rates, it is very important to prevent carbon limitation, as most of the biomass (45%-50%) is made up of carbon. Carbon dioxide is generally injected with air stream in required proportions. pH should remain in the optimum range to prevent any inhibition. Proper mixing of the culture is important as it allows uniform distribution of light, prevents top layer inhibition, and also helps in nutrient uptake by removing the boundary layer around the cells. Also, it is desirable to have nutrients such as nitrogen, phosphorous, vitamins, in excess.

**Methods of Algae Cultivation**

**Open Ponds**

For large scale algae cultivation, open pond system is the most beneficial as long as the culture can be maintained easily. For open cultivation, the strain should be capable of withstanding large temperature fluctuations, high salinity, and high or low pH, in order to prevent the foreign microorganisms. Raceway pond is the most commonly used open pond system as it has paddle wheels associated with it which gives high productivity and high biomass. It has been found that a dry weight of 1g/L and productivity of 60 to 100 mg/L/day is achieved when paddle wheel is installed at a depth of around 15 cm (Pulz, 2001). The drawbacks of open pond system is high water losses by evaporation, uncontrolled environmental conditions, and contamination by pathogens.
Photobioreactors

In order to overcome the limitations associated with open system, closed system technology was developed in order to have better control over environmental conditions, and to prevent sensitive strains from contamination. Photobioreactors are highly recommended for high value products such as pharmaceuticals and cosmetics. Though, high productivity of biomass could be attained using photobioreactors, high equipment cost makes it less favorable for bioenergy production. For better efficiencies, there should be a uniform light distribution to prevent photo inhibition. In photobioreactors, cells can face problem due to turbulence and shear stress caused by incoming air stream.

Fermenters

Fermenters can also be used for certain algal strains which are capable of growing heterotrophically utilizing single organic source for carbon and energy requirements. With fermenters, we can achieve high productivity, high yields and better downstreaming as we can have better control over the environmental conditions and the culture won’t face the problem of fluctuating temperature, light intensity, gas transfer rate etc. The only drawback of using fermenters is the high operating cost associated with it, due to high electricity consumption and expensive controllers.

Harvesting of Algae

Harvesting of algal biomass is very important as it can have considerable effect on the economics of the bioproduct being produced. Generally, harvesting accounts for 10%-30% of the total operating cost. There are various methods of algae harvesting, the choice of which depends on the strain and the bioproduct of interest. Generally, algae is not harvested in a single step, as it is very difficult and economically infeasible
to harvest algae from dilute solutions. First, the algal biomass is concentrated by flocculation, which is further concentrated by centrifuging and filtration. Flocculation is the method in which negative charges on the algal cell surfaces is neutralized causing agglomeration of algal biomass. Chemical flocculation is a method which employs chemicals such as aluminium sulphate and ferric chloride for charge neutralization. Though effective, chemical flocculation cannot be used for bioenergy production due to their high cost. Moreover, these chemicals can cause severe inhibition to methanogenic bacteria present in wastewater, which can reduce the biomethane potential.

Bioflocculation and electrofloculation are other flocculation methods employing settling tank and electric field respectively, to separate the biomass.

Centrifugation is a technique which employs gravitational force to separate the biomass. Cell size determines the rpm and time of centrifuging. Large size particles settles faster as compared to small size particles (Abayomi et al., 2009). It is the high operating cost of centrifuging, which limits its use. Filtration is another method of separating biomass from the liquid. In this, the broth is passed through membranes of different pore size to separate the cells. It is cheap and effective as compared to other methods but the membrane clogging limits its use to large cell size algal strains (Abayomi et al., 2009).

**Algae Applications**

**Bioenergy:** Cyanobacteria and microalgae are capable of growing because of their simple structure. They have high lipid content which makes them suitable for the biodiesel production. Moreover, producing biodiesel from oil is less energy-intensive process which makes the overall economics favorable. Some algal species are capable of producing sugars during their growth. These sugars can be separated from biomass
and could be fermented to produce ethanol, which is a high value product (Ueda et al., 1996). The biomass remained after extracting sugars can be utilized for biomethane production through anaerobic digestion of the biomass. Research is being carried out to genetically modify algal strain so as to secrete ethanol directly into the media in which algae is growing. But it will take lot of research before such technology could be applied on a large scale. Some algal strains also have the capability of producing polysaccharides during stationary phase. The polysaccharide produced could be separated and anaerobically digested to achieve high biomethane potential. Cyanobacteria can also be used for the biohydrogen production, which has several advantages over electrochemical hydrogen production, the most important of which is biohydrogen production is less energy-intensive as compared to electrochemical hydrogen production.

**Algal wastewater treatment:** Nowadays, algae play an important role in treating wastewater from various industries and municipalities. The reason behind such popularity is the ability of algae to utilize various nutrients (Nitrogen, Phosphorous, etc.) for its growth. The nutrient rich algal biomass can be used as an effective biofertilizer and the oxygen rich effluent is discharged into the river, without affecting the aquatic life. The widely used algal species for wastewater treatment are *Spirulina* (Olgun et al., 2003), *Chlorella* (Gonzales et al., 1997), *Scenedesmus* (Martinez et al., 2000) etc.

**Heavy metal remediation and CO₂ fixation:** Some algal species are capable of uptaking heavy metals such as Zn, Cd. These metals get adsorbed on algal cells through physical adsorption. Later on, these metals get into the cytoplasm of algal cells through chemisorption (Omar, 2002), thereby preventing the water bodies from
contamination. Metal sorption capability of algal cells depends on biomass concentration, metal concentration, pH, temperature, etc. It is very difficult to fit standard adsorption isotherms as the standard isotherm does not take into account the effect of above mentioned parameters. In order to apply algal strain for heavy metal removal, it is very important to understand the mechanism of metal uptake by algal cells, develop better adsorption models, genetically modify algal strains to improve sorption, and make the process economically feasible.

Algae contribute in fixing atmospheric carbon dioxide thereby mitigating GHG emissions. These days, algae is being used industrially to remove carbon dioxide from exhaust gases of the chimneys. Algae is capable of utilizing carbon dioxide, nickel, mercury, sulfur and nitrogen compounds, for biomass growth. The biomass so produced could be used for biofuel production, or burnt to produce heat.

**Research Objectives**

*Synechococcus sp*, cyanobacteria, a unicellular species was isolated from the lagoon in Florida. This species was found to have a unique property of producing polysaccharide during the stationary phase of algal growth. It is a prokaryotic organism which is capable of growing in salt water, thereby mitigating the effect of pathogens.

The main objectives of the research were:

- Develop a photobioreactor which can be used for algal cultivation in the laboratory.
- To determine the feasibility of algal growth without being contaminated by pathogens.
- Modify media preparation method to prevent precipitation of some of the media components, thereby improving the shelf-life of the media.
- Comparison of batch growth with exponential growth curve.
• Determining the effect of light and dark cycle on polysaccharide production.
• Determining the water losses by evaporation and carrying out semi-continuous phase so as to reach the steady state.
• Comparison of dry cell weight with optical density of biomass
• Effect of CO$_2$ enriched air, on the growth curve
• Modeling of the semi-continuous growth phase to determine the steady state
Figure 1-1. Open Pond System (picture taken from phys.org/news/2011-04-mere-pond-scum.html)

Figure 1-2. Photobioreactor (picture taken from Anand, 2010)
CHAPTER 2
EXPERIMENTAL PROCEDURE

Materials

The cyanobacteria strain used throughout the research work was supplied by Dr. Phlips EJ, Department of fisheries and aquaculture, University of Florida. Synechococcus sp. BG0011 strain was isolated from Florida keys (Phlips et al., 1989). It is a unicellular prokaryotic cyanobacteria with a unique property of producing polysaccharide during nitrogen starved stationary phase of the algal growth. This strain is of great interest among the researchers because the polymer produced by it could be anaerobically digested for biofuel production. Moreover, this strain can withstand high salinities thereby preventing any contamination by pathogens. The microscopic image of the cell is shown in the Figure 2-1.

The nutrient media containing N, P, Ca, salt, trace elements, vitamins etc. was used. The pH was kept in the optimum range of 7.5 to 8.5. The temperature was controlled at 28°C. The exact media composition can be found in the Appendix A.

Media Preparation

The cyanobacteria was grown in batch in the nutrient media. The media was autoclaved in an autoclave for 30 minutes, at 121°C. The pH was adjusted using HCl and NaOH. The optimum pH is 8.2 for this cyanobacteria. Care should be taken that media is not stored in the refrigerator, to prevent precipitation. Also, care is to be taken that all the components are not added together. Some components are autoclaved and added separately. The exact composition and method of media preparation can be found in the Appendix A.
Reactor Setup and Design

Four glass bottles of 250 ml capacity each were used for the growth of algae. The working volume of 200 ml nutrient media in each reactor was used for algae growth. Three reactors were inoculated with exponentially growing inoculum.
Exponentially growing inoculum was used in order to minimize the lag phase, so as to achieve a perfect exponentially growing curve. One of the reactors was used as a control to check contamination, so the inoculum was not added to it. All the reactors were closed with rubber corks having inlet for filtered air, an exit for exhaust, and a sampling port. Air pump was used as a source of air for sparging the reactors. Rotameters were used to adjust the air flowrate into each reactor. Air flowrate of 400ml/min (2 vvm) was used in each reactor. Air from the pump was first passed through the rotameter with set point of 1.6 L/min. From the rotameter, air was then passed through the humidifier in order to make up for the water losses by evaporation. Humidifier was followed by a vertical glass column in order to condense extra vapors. In the initial runs, vertical columns were not used due to which extra vapors started flowing through the rotameters making them saturated with water. This caused the fluctuations in flowrate. In order to have constant flow rate and to prevent rotameters from being saturated, vertical columns were used which solved that problem. Humidified air was then passed through the four rotameters, each with a set point of 400 ml/min. The outlet air from rotameters were passed through the filters in order to supply sterilized air and to prevent any contamination.

All the reactors, tubings, clamps, flasks etc. were thoroughly washed and autoclaved at 121°C for 20 min, before attaching it to the reactor system. The media was prepared in the laminar chamber and was transferred to the reactors aseptically in the laminar chamber. Light and dark cycle of 13 hours and 11 hours respectively, was used throughout the research work. Fluorescent lights were installed in the chamber and the temperature was kept track of by a thermocouple.
Figure 2-3. Schematic of Reactor setup
1.5 ml of culture sample, from each reactor, was withdrawn on a daily basis and pH and OD measurements were done. During the semi-continuous phase is going on, sample volume taken was 5 ml from each reactor. Before taking the sample, turn off the pump for 5 minutes so that there is no pressure build up in the reactors. In the meantime, switch on the OD spectrophotometer as it takes around 15 minutes to warm up. Vortex meter is used to properly mix the culture before taking the sample. Vortexing ensures that the sample is uniformly mixed and there are no dead zones in the reactor. Dry cell weight analysis were also done and compared with OD and fluorometry measurements.

The humidifier and exhaust flask were kept in the black box installed with UV light. Its purpose was to maintain sterility at all times. The black box was kept outside
the growth chamber. In order to prevent pressure build up inside the reactors, the exits from the reactors were not clamped, but were passed through a flask containing high pH (pH of 12) solution to prevent any back contamination.

The schematic diagram of the reactor setup can be found in the Figure 2-3

**Optical Density Measurements**

Optical density measurements were used as a mean to determine the cell growth rate. Milton Roy Spectronic 401 instrument was used to measure OD. Steps for measuring OD are as follows:

1. Switch on the OD device and leave it on for 15-20 min for the bulb to warm up.
2. Aseptically take 1.5 ml of sample from each reactor.
3. Once the device has been warmed up, set the wavelength to 540 nm. This wavelength is used for cell biomass.
4. Take 1ml of the growth media sample in the cuvette and set it as blank.
5. Take 1 ml of culture sample, vortex it for some time (generally 3-5 seconds). Care should be taken that it should not form bubbles while vortexing.
6. Repeat the step 5 for the same sample for five times. Final value of OD is determined by taking the average of the five readings.
7. Repeat step 4-6 for all the samples. Record all the readings in the excel sheet on a daily basis to determine the growth curve.

**Dry Cell Weight Analysis**

Dry cell weight analysis of the biomass was done to determine the amount of dry biomass that has been produced per unit reactor volume. As the cells grow, more biomass is produced and more dry biomass will be achieved. The graph between dry weight and OD values is then drawn to determine the linearity between them. Once the graph is plotted between dry weight and OD, same graph will be used as a reference to determine the dry weight from the OD values of the culture.
Methodology:

1. Take 0.45 micron Whatman filter paper of required size. Keep it in the oven at 105°C, for 24 hours.

2. Take aseptically, 1ml sample from each reactor.

3. Determine the salinity of the sample using salinity meter.

4. Centrifuge the samples at 8000 rpm for 10 minutes.

5. Decant the liquid. Pour 1ml of distilled water in the centrifuge tube. Vortex it, and again determine the salinity.

6. Step 5 is repeated till salinity value drops to near zero. Generally, it takes 6 washings per sample to bring the salinity to zero.

7. Take the filter paper out from the oven and determine the initial weight of the filter paper. Let it be a grams.

8. Filter 3ml sample (1 ml from each reactor) using vacuum filtration assembly. Care should be taken that the filter paper should not break due to pressure difference.

9. Filter equivalent amount of water as used for cell washing. This is done to prevent any error associated with the presence of impurities in the water.

10. Keep the filter paper in the oven for 24 hours, at 105°C.

11. After 24 hours, take the filter paper out and determine the final weight. Let it be b grams.

12. The difference of a and b will give the weight of biomass present in 3 ml of culture volume. The units could be converted to g/L.

13. The graph is then plotted between dry weight and OD and the linearity is determined using MS excel tools.

Although, dry weight analysis gives no idea whether the dry weight is associated with dead cells or living cells, but it is a very useful tool to determine the product yields.
Figure 2-5. Spectrophotometer for Measuring Optical Density, photo courtesy of Raghavendran Murali

Figure 2-6. pH meter, photo courtesy of Raghavendran Murali
CHAPTER 3
RESULTS AND DISCUSSION

Batch studies of *Synechococcus* were done in order to study the behavior of the culture and to determine the batch growth curve. The graphs were plotted between Optical Density (OD) and time to determine the growth parameters such as specific growth rate, maximum specific growth rate. In semi-continuous phase, harvesting of the reactors was done by withdrawing 5 ml of culture volume every day and replacing it with equal volume of growth media.

**Batch 1**

In the first batch cultivation, the main aim was to determine whether the culture could be grown in the reactor that was designed and developed in the lab. The reactor set up used in the first batch studies were without the vertical column. It was during this batch that we faced the problem of rotameters being saturated with water. The reactor set up was modified after the end of this batch studies. The reactors were inoculated so as to have an initial OD of 0.005 in each growth reactor. The other difference between the first batch and other was the use of air cylinder instead of air pump for supplying air to the reactors. Air flow rate of 1 L/min per reactor was used in each reactor. Air cylinder got emptied in 10-12 hours due to which we had to switch to vacuum pump for aeration.

Due to the oversaturation of rotameters, the filters also got clogged with water and the air supply to the reactors was stopped. The culture was harvested, rotameters were dried, and the filters were re-autoclaved before using them for the next batch. In order to solve the problem of oversaturation, vertical glass column (using pipette) was installed after the humidifier.
As can be seen in the Figure 3-1, two reactors showed almost the same growth rate, but the third didn’t show any growth. It could be due to the sticking of the culture to the reactors while inoculating. As far as contamination is concerned, there was no contamination as the control reactor didn’t show any change in OD. Batch 1 data and studies helped in making the reactor setup more sophisticated. Also, Batch 1 studies showed that the reactor setup is capable of growing the culture and temperature can be easily controlled.

**Batch 2**

Before the start of Batch 2, it was observed that the media was precipitating. Small experiments were done to determine the cause of precipitation and it was observed that due to the reaction between calcium chloride and potassium phosphate, calcium phosphate was being produced which was causing precipitation. In order to solve that problem, some changes were made in the media preparation method as can be seen in Appendix A. The OD of the media was found to be same as that of distilled water. In Batch 2, air flow rate was changed to 0.4 L/min as too much flowrate was causing froth.

During Batch 2, the batch lasted for 12 days. At the end of batch phase, the total volume lost by culture sampling and evaporation was found to be 75 ml. This volume loss was made up by adding media to the reactor. This caused a sharp decrease in the OD as can be seen in the Figure 3-3. At the end of batch phase, the reactors were not stopped, instead they were continued in semi-continuous mode. The aim was to withdraw 5ml of sample every day and make up the reactor volume to 200 ml. The volume lost by evaporation was also kept track of. It was observed that 3ml media per
Figure 3-1. Batch 1 Growth Curve

Figure 3-2. Batch 2 Growth Curve
reactor was being lost by evaporation on a daily basis. Moreover, salinity analysis showed that the loss was only of water, not the media.

The main purpose of doing this was to determine the time the reactor will take to reach steady state. The 5 ml sample collected on a daily basis was transferred to a flask to determine polysaccharide production. The reactors had to be stopped on 43rd day because sampling was missed on some days. So in order to achieve perfect semi-continuous growth, the reactors were harvested and were planned to start again.

**Batch 3 and Batch 4**

These batches didn’t last long and had to be stopped after the end of batch phase. No significant data were acquired from these batches because the nutrient media got precipitated. Fresh nutrient media was prepared for the next experiments.
Batch 5

Before starting Batch 5, many modifications were done in the reactor system, and all the problems and challenges faced during previous batches were removed. Batch 5 gave maximum number of data points and it lasted for 52 days. Dry cell weight and modeling was done based on Batch 5 samples and data points.

The exponential (batch) phase lasted for 18 days. On 18\textsuperscript{th} day, the media lost by sampling and evaporation was replaced with nutrient media and water. On 19\textsuperscript{th} day, semi-continuous phase sampling was started. The sampling was 5 ml/ reactor. The semi-continuous phase was continued till the stationary phase was reached, and we got some data points of stationary phase also. As can be seen in Figure 3-5, there is a dip on 18\textsuperscript{th} day, which is due to the dilution of the reactors. The samples collected during the batch phase were used for dry cell weight analysis. The samples of semi-continuous phase were kept on storing in the flask to determine the polysaccharide production analysis.

Average Optical Density

The optical density data obtained from all the batches was averaged and the graph was plotted between Average Optical Density versus Time. As shown in Figure 3-6, the average optical density change with time, showing standard deviation.

Modeling of Cyanobacteria Growth in a Reactor

Model 1

ASSUMPTIONS:

- No substrate inhibition
- No effect of light shading (due to algal growth)
- Temperature is constant
- pH is constant
- Effect of light/dark cycle not considered.
MODEL EQUATIONS

BATCH PHASE

\[ C = C_0 e^{\mu t} \quad \mu = 0.3 \text{ days}^{-1} \quad (3-1) \]

ON DILUTION

\[ C_{18}' = C_{18} * d_1 \quad (3-2) \]
\[ d_1 = 120/200 \]

SEMI-CONTINUOUS PHASE:

\[ C_{19} = C_0 e^{\mu t} \quad (C_0 = C_{18}') \quad (3-3) \]
\[ C_{19}' = C_{19} * d_2 \quad (3-4) \]
\[ d_2 = 192/200 \]

In the Model 1, the effect of light shading by biomass was not considered. The value of specific growth rate was assumed, and the parameter estimation was done using solver. \( C_{18} \) is the biomass concentration (or OD) on \( 18^{th} \) day. \( C_{18}' \) is the biomass concentration after dilution (when the reactor was made up to 200 ml). \( d_1 \) is the dilution factor. Similarly, using eq. (3-3) and eq. (3-4), biomass concentration was calculated. The model values were then compared with experimental data. Figure 3-7 gives the comparison between model and experimental values. This is the best curve fit that we obtained using Model 1. We can see that there is a lot of deviation of model curve from the experimental curve. It is due to the assumptions that there is no substrate inhibition and no light limitation. But actually, there are lots of factors that can have considerable effect on the growth rate. So the model was modified taking into account the effect of light limitation, which gave a better curve fitting as shown in Model 2.
Figure 3-4. Batch 5 Growth Curve

Figure 3-5. Dry Cell Weight vs Optical Density
Figure 3-6. Average Optical Density of batch phases (Batch 1 to Batch 5)

\[ y = 0.0113e^{0.313x} \]
\[ R^2 = 0.9503 \]

Figure 3-7. Model 1 (Without taking the effect of light shading)
Model 2

ASSUMPTIONS:

- All substrates are in excess No substrate inhibition.
- Effect light shading (due to algal growth)
- Temperature is constant
- Effect of light/dark cycle not considered.

MODEL EQUATIONS

$$\mu = \mu_{\text{max}} \times \varepsilon ; \quad 0 \leq \varepsilon \leq 1 \quad (3-5)$$

$$\varepsilon = 1 - I ; \quad I = \text{Light Limitation (varies with OD)} \quad (3-6)$$

$$I = f \times X ; \quad X = \text{OD (Optical Density)} \quad (3-7)$$

; \; f = \text{constant (value determined using Solver in Excel)}$

In order to reduce the error between model and experimental data, and to have better curve fitting, the effect of light shading was included in Model 2. In Model 2, eq. (3-1) to eq. (3-7) were used for curve fitting. Batch and semi-continuous phase were modeled separately and finally the data was combined and was compared with the experimental data. In this model, it is assumed that as the algae grows, the biomass causes hindrance to the light leading to light limitation. The light limitation factor I is dependent on optical density of biomass. The constant f is a factor used for calculating I, for changing OD values. The value of f was estimated using solver. $\varepsilon$ is calculated by subtracting I from 1. The value of $\mu_{\text{max}}$ was found to be 0.54 (days$^{-1}$) using solver. The value of f was found to be 0.59.

As can be seen in Figure 3-8, Model 2 gave a very good curve fitting for batch phase. Similarly, applying Model 2 for semi-continuous phase, the graph for both the batch and semi-continuous phase was obtained as shown in Figure 3-9. Though model didn’t give very good curve fitting for semi-continuous phase, still the error is in the...
acceptable range. To make the Model 2 more accurate, there is a need to consider the effect of other parameters also, like substrate inhibition, temperature, effect of light/dark cycle etc.

Figure 3-10 shows the graph between biomass concentration (g/L) and time. Biomass concentration is calculated by using the results obtained for dry cell weight analysis. It can be seen in Figure 3-10, that at the 60\(^{th}\) day of algal growth, we can have a continuous supply of biomass at a concentration of 0.5 g/L.
Figure 3-8. Model 2 (Taking into account the effect of light shading)

Figure 3-9. Model 2 (Taking into account the effect of light shading)
Figure 3-10. Model 2 (Biomass concentration change with time)
CHAPTER 4
CONCLUSIONS

Cyanobacteria, *Synechococcus* sp. BG0011, is capable of growing without being contaminated by unwanted microorganisms. It can be grown in water of high salinities. This strain produces a highly viscous polysaccharide during stationary phase. Light is an important factor for increasing the production of polysaccharide. Dry cell weight analysis gives linear relationship between biomass concentration and optical density. Biomass concentration can be increased by increased by sparging carbon dioxide into the growth reactor. It is very important to characterize the polysaccharide produced, and also to determine its biomethane potential through anaerobic digestion.

Through batch studies of *Synechococcus*, it was observed that on an average, exponential growth phase ceases at around 15th day of growth. Average maximum specific growth rate of 0.313 days\(^{-1}\) was observed. Maximum cell density of 0.5 g/L was obtained.

The effect of substrate inhibition, temperature, light/dark cycle should be included in the modeling of batch and semi-continuous growth phase, to better understand the growth kinetics of this cyanobacteria.
APPENDIX
_Synechococcus_ MEDIA PREPARATION

**Step 1:**

Prepare three appropriately sized containers. Container 1 should be an open bucket style container, able to hold the total amount of media you wish to create. Container 2 can be any shape (preferable with screw-on lid), and should be at least 10% of the size of the first container. Container 3 is for autoclaved deionized water only and should be at least 25% the size of the first container (preferably with screw-on lid). Containers 1 and 2 should be on a magnetic stirring plate, and should be stirred when mixing components.

**Step 2:**

Fill container 1 with the following components in the following order:

<table>
<thead>
<tr>
<th>Per Liter of Media Desired</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.</strong> Deionized H$_2$O</td>
</tr>
<tr>
<td><strong>2.</strong> Na$_2$EDTA</td>
</tr>
<tr>
<td><strong>3.</strong> KCl</td>
</tr>
<tr>
<td><strong>4.</strong> MgSO$_4$·7H$_2$O</td>
</tr>
<tr>
<td><strong>5.</strong> Stock Solution 1 (Trace Elements)</td>
</tr>
<tr>
<td><strong>6.</strong> Stock Solution 2 (Iron)</td>
</tr>
<tr>
<td><strong>7.</strong> Stock Solution 3 (Vitamins)</td>
</tr>
<tr>
<td><strong>8.</strong> Stock Solution 4 (Molybdenum)</td>
</tr>
<tr>
<td><strong>9.</strong> Heps</td>
</tr>
<tr>
<td><strong>10.</strong> NaCl</td>
</tr>
</tbody>
</table>

Note: It may be necessary to put the contents of this container into another container suitable for autoclaving (preferably with screw-on lid).
Step 3:

Fill container 2 with the following components:

<table>
<thead>
<tr>
<th>Per Liter of Media Desired</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Deionized H₂O</td>
</tr>
<tr>
<td>2. CaCl₂·2H₂O</td>
</tr>
<tr>
<td>3. K₂HPO₄</td>
</tr>
</tbody>
</table>

Note: The mixing of CaCl₂·2H₂O and K₂HPO₄ causes a precipitate of Calcium phosphate, this is normal and expected. This precipitate will form a single phase when later mixed with the contents of container 1.

Step 4:

Fill container 3 with the following components:

<table>
<thead>
<tr>
<th>Per Liter of Media Desired</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Deionized H₂O</td>
</tr>
</tbody>
</table>

Step 5:

Autoclave all three containers for 30min, and allow them to cool to room temperature. Before autoclaving, make sure that all three solutions are in autoclave rated containers with screw-on lids. Also make sure that these lids are screwed on loosely (about 1/3 to 1/2 of the way tight) and covered with aluminum foil.

Note: If the contents of container 1 were put into a new container, make sure to also autoclave the original open bucket-style container for future use.

Note: Steps 6 thru 11 should be done in a laminar hood.
Step 6:

In a laminar hood, pour the contents of container 1 (which has most likely been put into a new autoclave rated container with screw on lid) into the same open bucket-style container used previously that you have just autoclaved. This container should be on a magnetic stirring plate.

Step 7:

Now slowly mix in the contents of container 2. It is very important here that there is smooth, consistent, and ample mixing at this point. Once all the contents from container 2 are added to the solution, allow the solution to mix for about 5min.

Step 8:

Re-measure the new solution to see how much autoclaved deionized water from container 3 needs to be added to make your solution the desired amount. Once you have calculated how much from container 3 you need to add, subtract the following amounts to make space for the NaOH.

- 30ml for 1.0M NaOH
- 120ml-140ml for 0.2M NaOH

Step 9:

Add the necessary amount from container 3 to make the solution exactly the desired amount, minus the amount of NaOH you calculated earlier.

Step 10:

Add approx. 30ml of 1.0M NaOH or about 120ml-140ml of 0.2M NaOH to the solution in small increments, testing for pH with a sterile probe, until solution
reaches 8.2 pH. It is extremely important that the solution is being mixed well as you add the NaOH. Failure to do so can and usually does cause a precipitate to form.

Note: CaCl$_2$·2H$_2$O and K$_2$HPO$_4$ are soluble in acidic solution, so when adding a base to this mixture extreme care must be taken so the solution doesn’t precipitate. It is also important to note that heat can cause this single-phase mixture to precipitate.

**Step 11:**

Transfer your final solution into an appropriate sealable container.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Yatin Behl was born in 1986 in Jalandhar in the northern state of Punjab in India. He did his primary and secondary education in a convent school in Jalandhar. He completed his Bachelor of Technology in chemical and bio engineering from National Institute of Technology Jalandhar, India. He received his MS from the University of Florida in Spring 2013.