DYNAMIC MODELING AND VALIDATION OF GROWTH OF Synechococcus BG0011 USING LABORATORY SCALE STUDIES

By

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
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To my Mom and Dad

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I would like to first thank my parents for their unwavering support of my education and for encouraging me to achieve more and set higher goals. I wouldn't be the same without them and I owe them everything I have accomplished until now.

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Major: Chemical Engineering

There is no doubt that mankind's continued reliance on fossil fuels has had significant effects on almost everything from our quality of life to, most importantly, the global climate scenario. Fossil fuels, being non-renewable, are expected to run out in a matter of years, 40 in case of oil if we are conservative. Most studies report that the world has reached its peak in oil production and is entering a terminal decline. Furthermore, the use of fossil fuels has resulted in an enormous increase in anthropogenic carbon dioxide emissions, which in turn worsens the situation by contributing to climate change. The need for a shift of focus to renewable sources of fuel has been higher than ever before. This project is aimed at taking those first few steps towards the same. The algal strain being studied in this work can (1) fix its own nitrogen (2) produce a highly viscous polymer that can be downstream processed into a useful derivative (3) grow in a range of environmental conditions. Synechococcus BG0011 is a cyanobacteria isolated from the Florida Keys that can fix its own nitrogen. This project focuses on the growth of the strain, effect of change in conditions on growth and primarily on modeling the growth based on observed experimental data.

Additionally, the model is used to validate a few characteristic parameters and finally a brief sensitivity analysis is carried out to study the various influences of the parameters calculated.

CHAPTER 1
INTRODUCTION

1.1 Cyanobacteria

Some say that the cyanobacteria are one of the oldest inhabitants of Earth stretching beyond even scientific study. Many still believe that this is an open question. Geologists and geochemists all agree that cyanobacteria have had a long evolutionary history. There have been findings of ancient rocks dating back 1.5 billion years that have some tracks of coupled objects which scientists think may be the “ancestors” of the modern day cyanobacteria (Benson *et al*., 2007). Some even go so far as to suggest that these were the first to introduce Oxygen into the Earth’s atmosphere through photosynthesis (Haselkorn, 2009). There might be a grain of truth in that as recent genomic analysis, in particular the analysis of sequences of proteins that make up the photosynthetic apparatus, has established that a cyanobacterium provided the ancestor of the chloroplast.

> Cyanobacteria are arguably the most successful group of microorganisms on earth. They are the most genetically diverse; they occupy a broad range of habitats across all latitudes, widespread in freshwater, marine and terrestrial ecosystems, and they are found in the most extreme niches such as hot springs, salt works, and hypersaline bays. Photoautotrophic, oxygen-producing cyanobacteria created the conditions in the planet's early atmosphere that directed the evolution of aerobic metabolism and eukarotic photosynthesis. Cyanobacteria fulfill vital ecological functions in the world's oceans, being important contributors to global carbon and nitrogen budgets.
>
> – Stewart and Falconer (1990)

Cyanobacteria can be found in almost every terrestrial and aquatic habitat; oceans, freshwater bodies, rocks, soil – they all have cyanobacteria in some form or the

other. Most commonly seen cyanobacteria are the aquatic ones, known for their highly visible and extensive blue green blooms in both freshwater and marine environments.

1.1.1 What's in a Name?

Cyanobacteria are also called blue green bacteria, blue green algae and Cyanophyta. In the olden days they were classified along with green algae, red algae and brown algae as photosynthetic microbes. It was generally agreed upon that all of these carried out the process of green plant photosynthesis, fixing Carbon Dioxide and generating Oxygen from water. It was only in the 1960s that people started taking a second look at the classification of cyanobacteria. As Robert Haselkorn puts it:

> But in the 1960s it became apparent, from new biochemical evidence, that the blue-green algae, unlike the other algae, are really bacteria: they are sensitive to penicillin because of their peptidoglycan cell walls; they have bacterial-sized ribosomes sensitive to the usual antibiotics; and they do not contain organelles such as chloroplasts and mitochondria. (Haselkorn, 2009)

The major outcome of this redefinition was that cyanobacteria entered the world of microbiology from the realm of botany.

1.1.2 What are Cyanobacteria?

Cyanobacteria are photosynthetic prokaryotes that have the ability to synthesize chlorophyll.*a*. Typically water is the electron donor during photosynthesis, resulting in the evolution of Oxygen. Until recently cyanobacteria have also been characterized by their ability to form the phycobilin pigment, phycocyanin. It is the high concentration of this pigment under certain conditions which leads to the bluish color of the organisms giving rise to the name blue green algae. However, increasing scientific evidence suggests that some prokaryotes that do not possess phycobilins form some type of chlorophyll and are closely related to organisms with phycocyanin (Whitton and Potts,

2012). Due to some shortcomings in molecular evidence these organisms are also classified as members of cyanobacteria in a broad sense.

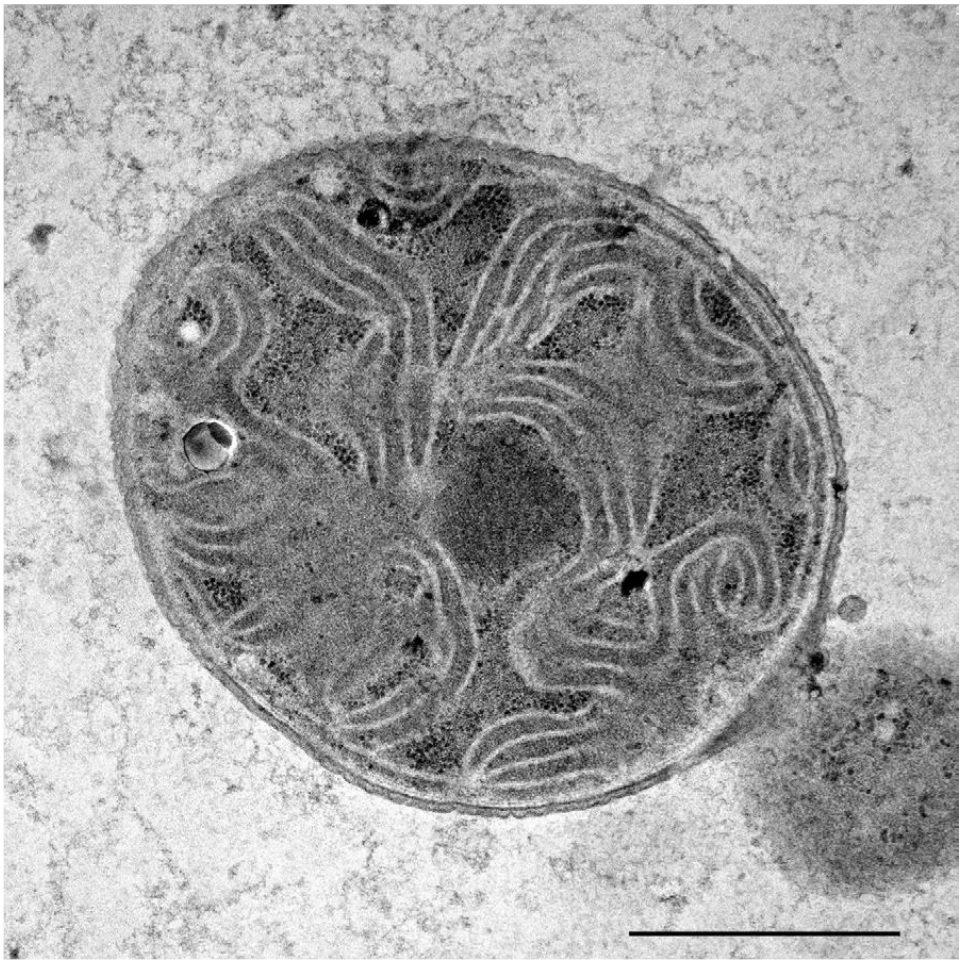

Figure 1-1. Electron micrograph of a section through Anabaena revealing the photosynthetic membranes and numerous ribosomes. (Photo courtesy of Haselkorn, 2009)

1.1.3 Diversity of Cyanobacteria

As briefly touched upon before, cyanobacteria are found in almost all types of habitat, both terrestrial and aquatic, showing in part their resilient nature. It is no surprise then that scientists have been able to recover over 35 strains of cyanobacteria from the coldest continent on earth, the Antarctica. The physiological characteristics of

the isolated cyanobacteria strains showed that nitrogen fixation, nitrate uptake, nitrate reduction, ammonium uptake and photosynthesis was unaffected at low temperature (5 degrees C) which indicated low temperature adaptation for Antarctic cyanobacteria (Singh and Elster, 2007). Interestingly this phenomenon was not evident in different strains of tropical origin. Reports suggest that the optimum temperature for nitrogen fixation for the different Antarctic cyanobacterial strains was in the range of 15-25 degrees C. The low endergonic activation energy exhibited overall reinforced the notion that cyanobacteria were very much adapted to the Antarctic ecosystem. It is interesting to take a look at the different terrains and climatic conditions where cyanobacteria thrive all around the world.

1.1.3.1 Geothermal springs

Geothermal springs are locations where groundwater heated by the earth's inner heat rise up to the ground. Many geothermal springs emit water near the boiling point. Reports of microorganisms positioning themselves on the outflow channels of geothermal springs according to temperature gradients have established that 73–74°C is the maximum temperature enabling development of cyanobacteria (Ward *et al*., 2012). Cyanobacteria belonging to the genus Synechococcus (Thermosynechococcus), are the most thermophilic.

A striking anomaly suggesting geographically restricted distributions of thermophilic cyanobacteria is that all forms of thermophilic Synechococcus appear to be absent from Icelandic hot springs, although numerous springs exist there that appear to be chemically suitable. Thermophilic Synechococcus have been found to occur in New Zealand and European springs, but these include only forms that grow up to a maximum temperature of 58 – 62 degrees C. Forms of Synechococcus that grow in

nature up to limits of 73–74 degrees C in the western contiguous United States and south into Central and South America, are absent in the geographic regions mentioned above, although they extend into eastern Asia and China. (Ward *et al*., 2012)

It is fascinating to note that two factors that do not play a significant role in the distribution of thermophilic cyanobacteria are latitude and day length, since many cyanobacteria resembling those of lower latitude springs have been found to occur in the hot springs of the east coast of Greenland at 70–71°N (Ward *et al*., 2012). In winter, these springs experience complete darkness while summer sees constant daylight for the entire region.

1.1.3.2 Cyanobacteria in water bodies

A great and sometimes dominant portion of phototrophic biomass and primary biomass production in ponds, streams, lakes at high latitudes and wetlands is comprised of cyanobacteria. A striking dissimilarity is observed in the polar seas, where the cyanobacteria are generally sparse in population or completely absent with the exception of some regions. Mat-forming cyanobacteria are found to be pervasive in polar aquatic ecosystems including lakes, ponds, streams and seeps. Tolerance to continuous low temperature, freeze-thaw cycles, high and low irradiances, desiccation and UV exposure are characteristic feature that make them suitable for life in the extreme polar environment (Vincent and Quesada, 2012). Explorers who noticed unusual biological communities growing on or underneath the ice in Polar Regions provided the very first reports of cyanobacteria in those areas (Vincent and Quesada, 2012). During an expedition to explore the McMurdo Dry Valleys, Antarctica in 1909, Griffith Taylor wrote:

> We came across a lake two miles long. It was of course frozen, but beneath the ice the water was very deep and we could see extensive water plants

Biologically, most aquatic cyanobacteria can be put into three functional groups: picocyanobacteria, bloom-formers, and mat-formers. Picocyanobacteria are prevalent in the oligotrophic freshwaters in high latitude regions, and there have been reports of extremely high concentrations in certain locations. Intriguingly, the adjacent polar oceans have little or no population of picocyanobacteria.

The second of the cyanobacterial groups, the bloom-forming cyanobacteria, are as of now not to be found in most polar aquatic environments, but they have been observed in subarctic waters and the changes in environment around the Polar Regions might be able to increase their numbers (Vincent and Quesada, 2012).

Mat-forming species are the most successful cyanobacteria in terms of biomass levels at high latitudes. They are more commonly present as mm-thick mats, films and aggregates, and can also be associated with aquatic mosses.

1.1.3.3 Cyanobacteria in deserts

Cyanobacteria inhabiting hot deserts experience a variety of environmental challenges. In addition to aridity and temperature extremes, they are also subject to high light intensity and, in some cases, high salinity. Studies of soil microalgae and cyanobacteria from hot deserts have revealed that they have several mechanisms to deal with extreme conditions (Hu *et al*., 2012). Unsurprisingly, water availability, which includes precipitation, condensation, and water vapor, is the most important factor in limiting the growth of desert microorganisms.

The study of cyanobacterial growth in such extreme conditions as the world's deserts, even though, deserts being devoid of moisture, they are restricted to situations

where sufficient moisture is retained for occasional growth to occur, point out one particular aspect of these organisms that has made them omnipresent – their outstanding ability to adapt and survive in some of the harshest environments. One prime example is the use of polysaccharide by the cyanobacteria. The production of exocellular polysaccharide in most cases is thought to be a form of nutrient storage. However, in arid conditions the polysaccharide actually helps the organisms withstand desiccation (Flechtner, 2007; Hu *et al*., 2012). They are composed of compounds which tend to be hygroscopic and are often pigmented.

In arid and semiarid areas where soil cover is sparse and patchy, open soil supports the development of microbiotic crusts. Microbiotic crusts are mixed communities of organisms which may include various combinations of bacteria, cyanobacteria, eukaryotic microalgae, lichens, fungi, mosses, and leafy liverworts. The relative proportion of these components is influenced by temperature, water availability, and soil type. Cyanobacteria can withstand the harshest conditions and generally dominate in poor sandy soils with neutral or slightly alkaline pH. They are less prevalent in acid soils. (Flechtner, 2007)

1.1.3.4 Cyanobacteria in extreme cold

It is a common misconception to think that cyanobacteria are prevalent only in those regions that are blessed with sunlight and heat, things that one might think are absolutely essential for growth of these organisms. But as briefly mentioned before, cyanobacteria have the ability to survive in extreme environments – environments with high salinity, environments with almost no water and even oil polluted environments. One such extreme environment is the cryosphere which is characterized by harsh freezing temperatures and scarcity of liquid water. Cryosphere is defined here as

regions that have temperatures below 0 degrees centigrade for most of the year, most notably the alpine regions and the Arctic and Antarctic.

As with other seemingly inhospitable surroundings, cyanobacteria have found some way or the other to grow and achieve remarkable biomass concentrations. The really interesting part is that research has shown that the strategy to microbial success in these harsh environments is not adaptation towards optimal growth at low temperatures but tolerance to environmental extremes. This complete non-existence of adaptive regulation to a low temperature regime may prove to be an ideal strategy in these ecosystems where a few hours are enough to witness widely fluctuating temperatures (Quesada and Vincent, 2012), reaching high values at which organisms fully adapted for growth in the cold could suffer severe physiological stress and mortality. In such subpar growth conditions cyanobacterial growth rate is decent to say the least. But in spite of this, it is possible achieve conspicuously large standing crops and colonization of most habitats. (Quesada and Vincent, 2012)

Another important feature is the ability to survive prolonged dormancy, accounting for widespread occurrence of cyanobacteria in such environments. Some of the strategies used by the cyanobacteria include living inside rocks where humidity can be higher and thermal variations can be buffered and forming dark colored mats on or within ice that absorbs sunlight, which increases temperatures enough to melt the ice in summer providing liquid water conditions.

Figure 1-2 shows endolithic cyanobacteria growing inside a sandstone rock recovered from the McMurdo Dry Valleys, a row of ice laden valleys located in Antarctica. The region is claimed to be one of the world’s most extreme deserts.

Figure 1-2. Endolithic cyanobacteria in a sandstone rock from the McMurdo Dry Valleys (Photo courtesy of Quesada and Vincent, 2012)

In order to decrease the damage produced by both osmotic shock and physical disruption, Polar Regional cyanobacteria have come up with some interesting strategies. Production of exocellular polysaccharides is thought to be a primary mechanism of protection. This reportedly helps by reducing the water loss and by restricting ice crystal formation to exterior cell sites. Some cyanobacteria also produce intracellular proteins which regulate the osmotic stress imposed by desiccation and antifreeze compounds which are thought to act as cryoprotectants.

1.1.3.5 Cyanobacteria in urban environments

We have looked at the ability of cyanobacteria to dwell in almost every imaginable natural habitat, from the various water bodies to the water starved deserts and from the thermal springs to the interior of rocks in cryogenic surroundings. However, there is one particular environment that is receiving increasing attention of late and that is the urban metropolises of the world. The beginning of the Nineteenth century marked the first recorded growth of microalgae and cyanobacteria on walls, masonry and other man-made materials (such as concrete, asphalt, glass and metal).

Since the surfaces of urban buildings provide the maximum area for the growth of cyanobacteria, one can argue that the trials faced by the microbes can be surmised by investigating what factors the surfaces are exposed to, though it is not entirely unlikely to find some microbial growth in underground systems. The surfaces of most urban buildings are exposed to full sunlight incident on that particular location thereby subject to high irradiance, high exposure to UV rays and possibly extreme dehydration. In addition to these, urban environments are characterized by high level of pollutants (gases such as CO, SO_2 and NO_x, hydrocarbons, ozone apart from aerosols, dust and heavy metals) which contributes to negative growth conditions for cyanobacteria. (Rindi, 2007)

Broadly speaking the knowledge available about the diversity and ecology of these microbial communities is still in its infant stage mainly because most studies focus on the biodeterioration caused by these organisms on man-made surfaces rather than their biology. Very little or nothing is known about the dispersal patterns of these organisms in such environments. Even though some study has been carried out concerning the distribution of some species and assemblages, more is needed as this aspect of cyanobacterial populations in urban settings depends on an intricate interaction of various factors and properties like (Rindi, 2007):

- Climate (temperature, light intensity, precipitation, wind, pollutants)
- Surface effects (chemistry, porosity, water holding capacity)
- Proximity of adjacent surfaces
- Internal properties of the wall or surface

1.2 Emissions and Fossil Fuels

1.2.1 A Little Bit of History

As early as late 19th century, people were debating about the effect of emissions into the atmosphere by humans and some were even brave enough to suggest that fossil fuel usage will cause a degradation of the atmosphere and thereby in turn the climate.

In the late 1890s American scientist Samuel Langley decided to measure the surface temperature of the moon by measuring the infrared radiation emanating from it. The angle of the Moon in the sky when a scientist took a measurement determined how much CO2 and water vapor the Moon's radiation had to pass through to reach the Earth's surface, resulting in weaker measurements when the Moon was low in the sky. This result was unsurprising given that scientists had known about infrared radiation absorption for decades.

A Swedish scientist, Svante Arrhenius, used Langley's observations of increased infrared absorption where Moon rays pass through the atmosphere at a low angle, encountering more carbon dioxide (CO2), to estimate an atmospheric cooling effect from a future decrease of CO2. He realized that the cooler atmosphere would hold less water vapor (another greenhouse gas) and calculated the additional cooling effect. He also realized the cooling would increase snow and ice cover at high latitudes, making the planet reflect more sunlight and thus further cool down. Overall Arrhenius calculated that cutting CO2 in half would suffice to produce an ice age. He further calculated that a doubling of atmospheric CO2 would give a total warming of 5-6 degrees Celsius.

Another scientist during the same time had been quantifying the natural sources of CO2 emissions for the purpose of understanding the carbon cycle and discovered

that the industrial CO2 emissions (mainly from coal burning) was comparable to that from natural sources back in that time. Arrhenius observed this data and saw that human emissions of CO2 was going to cause warming. However since the rate of CO2 production was relatively low back in 1896, he thought that it would take thousands of years for the warming to actually register on the global scale and even thought that it might be beneficial to humanity.

The early part of the 20th century was spent mainly disputing and recalculating what Arrhenius had done and the whole world subsumed into a larger debate as to whether the atmospheric changes had caused the Ice ages. Various other theories were suggested but none fared better. In 1938, a British engineer called Guy Callender set about reviving Arrhenius' theory. He presented evidence that both temperature and the CO2 level in the atmosphere had been rising over the past half-century, and he argued that newer spectroscopic measurements showed that the gas was effective in absorbing infrared in the atmosphere. Nevertheless, most scientific opinion continued to dispute, sometimes even ignore, the theory.

There was a period of increasing concern during the 50s and 60s about the harmfulness of greenhouse gases and its effects on climate change. By the end of the 1960s, aerosol pollution had become a serious local problem in many cities, and some scientists began to consider whether the cooling effect of particulate pollution could affect global temperatures. Scientists were unsure whether the cooling effect of particulate pollution or warming effect of greenhouse gas emissions would predominate, but regardless, began to suspect that human emissions could be disruptive to climate

Almost everyone's notion strengthened when the first evidence of warming due to the continuous use of fossil fuels began to surface in the 1970s, thanks to considerable improvements in scientific data collection. By 1975 the evidence pointing to warming had accumulated so much that a three dimensional Global Climate Model was made possible by Manabe and Wetherald. This model predicted a 2 degree C increase in temperature worldwide when the CO2 concentration was doubled. Many more models began to spring up and not one could say that the temperature will remain same if CO2 concentration was increased.

By the early 1990s committees were being formed and scientists were beginning to get involved in political decisions regarding curbing Carbon Dioxide levels in the atmosphere. Fast forwarding to the present day, it would be an understatement if we were to say that global warming has become one of the major environmental issues. Further research work on global warming and its effects is being added to the already burgeoning collection every day, and then some more. The question that arises most is how do we reduce the emission of greenhouse gases and more importantly how do we diminish the ones that are already present in the atmosphere.

1.2.2 Emissions and its effects

Ever since Arrhenius calculated that increasing carbon dioxide concentrations are affecting the earth's balance, there was only one outcome and that was the deterioration of the atmosphere. The negative implications of emissions in general are felt all over the world. (Smith *et al*., 2000)

- Loss of sea ice, particularly in the Arctic regions
- Decreasing snow packs in mountains, more profound in the western mountain ranges of North America

- Frequent, longer and more intense heat waves in cities that are currently experiencing them
- Increased risk of inland flash floods in parts of Europe
- Reduction of crop productivity in Southern Europe
- Severe decrease of freshwater availability in Asia and increase in deaths related to diseases from floods and droughts in some regions

The other side to the story is that it is not a secret that the source of energy that has been keeping us going in every sense for the past one and a half centuries is extremely limited, as opposed to what was previously thought. The idea that fossil fuels will one day run out has been with us ever since the day it was used but with continued exploration of new reserves, growing installed production capacity and increasing equipment efficiencies, the possibility of facing that reality was being pushed further and further away.

1.2.3 End of an Era

Mankind has been using fossil fuels for ages now. Ever since the discovery of coal in 13th century, we have been utilizing fossil fuels to improve our quality of life in all aspects imaginable. Primitive uses of coal were for cooking and providing heat. At present, coal is the major energy source that powers almost every home in the United States. It is safe to say then that our society is still dependent on fossil fuels. Not until recently has there been a firm interest in renewable sources of energy. Given that fossil fuels emerged so way back in time it's not unreasonable to expect that newer and cleaner sources of energy would have been the norm by now. There is a reason behind the apparent late coming of renewables. Fossil fuels provide us with immense

convenience. Oil proved to be so versatile and useful that the thought of it being non-renewable did not matter. At least until now.

There is increasing evidence (Hook and Tang, 2012) that the world will reach a so called ‘Oil Production Peak’ beyond which the production rate of oil will always be a terminal decline, no matter how many new reserves we discover (Behrens *et al*., 2011).

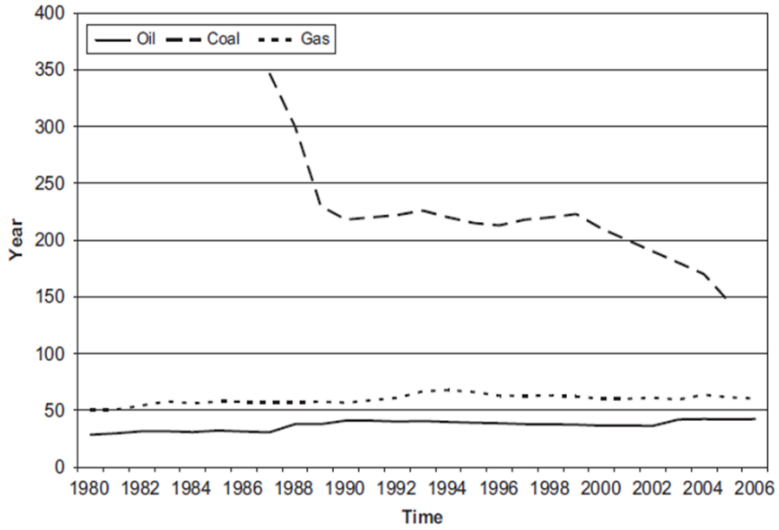

Figure 1-3. Plot showing the relative time left for complete exhaustion of fossil fuels (Photo courtesy of Shafiee and Topal, 2009)

The United Kingdom Energy Research Council predict that this will happen before 2020. It is estimated that even with the most conservative use of our fossil fuels, they will be able to last us only for 43 years in the case of oil, 148 years in the case of coal and 61 years in the case of natural gas. (Shafiee and Topal, 2009)

Contrary to popular belief, renewable sources of energy are not new and we have been fiddling with them as long back as the 17th century. Over time, the one aspect of renewable energy sources that has seen tremendous improvement is the efficiency of energy production from any source. In the modern day, of all the renewable sources being used, one type occupies a fair share of the usage charts, biomass.

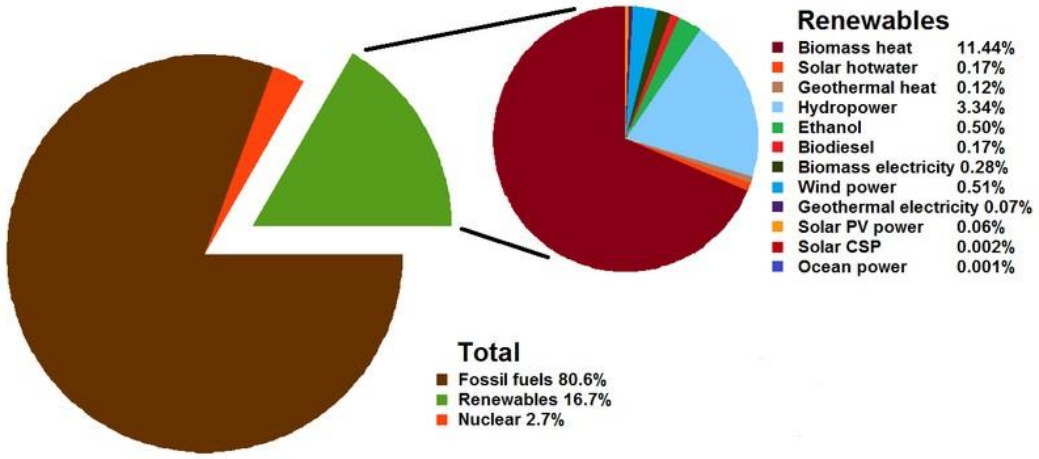

Figure 1-4. Total Energy Consumption with split-up of Renewable Sources as of 2010 (Photo courtesy of Hook and Tang, 2012)

Biomass production involves using garbage or other renewable resources such as corn or other vegetation to generate electricity. When garbage decomposes, the methane produced is captured in pipes and later burned to produce electricity. Vegetation and wood can be burned directly to generate energy, like fossil fuels, or processed to form alcohols. Brazil has one of the largest renewable energy programs in the world, involving production of ethanol fuel from sugar cane, and ethanol now provides 18% of the country's automotive fuel. Ethanol fuel is also widely available in the USA.

The burnout of our current resources is a serious problem even without considering other factors like urbanization, increase in population, modernization, developing economies, consumerism and numerous others. The U.N. projects that world population will increase 41 percent by 2050, with nearly all of this growth in developing countries (Behrens *et al*., 2011). This surge in human numbers threatens to offset any savings in resource use from improved efficiency, as well as any gains in

reducing per-capita consumption. Developed countries like the USA (Behrens *et al*., 2011) have their share of problems too. Consider this:

- The United States, with less than 5 % of the global population, uses about a quarter of the world's fossil fuel resources—burning up nearly 25 % of the coal, 26 % of the oil, and 27 % of the world's natural gas.
- The U.S. has more private cars than licensed drivers, and gas-guzzling sport utility vehicles were among the best-selling vehicles.
- New houses in the U.S. were 38 % bigger in 2002 than in 1975, despite having fewer people per household on average

The need for newer technologies and newer energy sources that can successfully replace fossil fuels is higher than ever before. In more recent times, Energy Security is a term that is coming to the fore whenever there is any discussion on fossil or renewable fuels. Energy security is the constant availability and supply of affordable energy for consumers and industry. Risks to energy security include disruptions to the supply of imported fossil fuels, limited availability of fuel, and energy price spikes. All these factors drive the search for new technologies that can implement some processes which can produce energy from cleaner sources.

1.3 Enter Cyanobacteria

There has been no dearth of research and implementation of plant based biofuels to date. Corn, rapeseed, linseed, sugarcane, palm oil, soybeans, sunflowers, cottonseed, jatropha and countless other crops have been used in producing bioethanol, biodiesel or biomethanol in the United States through different treatment processes, with corn being the crop most used primarily due to its abundance. However the disadvantages of using land based crops for producing renewable energy far outweighs the advantages (Parmar *et al*., 2011):

- Deforestation: Cultivating crops require huge expanses of arable land leading to cutting down of trees, resulting in adding to greenhouse gas emissions, which is extremely undesirable
- Water Usage: The water demands of some crops used for biofuel production puts unsustainable pressure on local water resources
- Food Security: The use of food stocks like corn and soybeans for bioethanol production will result in their price increasing due their increased demand
- Use of Fertilizers: No crop is grown these days without fertilizers. The Nitrogen fertilizers in use today are a known source of Nitrous Oxide, a greenhouse gas that destroys ozone
- Monoculture: As demand for corn-based bioethanol increases, more corn will have to be cultivated. If new land is unavailable, farmers will resort to growing corn alone and will not rotate crops which deprives the soil of nutrients

The other option apart from land based crops in producing biofuels is using plant derived lignocellulose from agricultural and agro-industrial resources such as wood chips, corn stover, sugarcane, bagasse and rice and wheat straw. These are a potentially vast source of renewable energy production that is not linked to food crops and also have the added advantage that most are considered waste in their respective manufacturing processes, therefore not limited in supply. At the moment the primary challenge is to find an economically viable method of converting the lignocellulosic material into simple sugars that can be degraded by microorganisms to produce fuel. A lot of research is being focused on this aspect and it is a long term goal.

Photosynthetic microorganisms like cyanobacteria and microalgae have the potential to be used as feed stocks for biofuel production because:

- The high growth rate of these microorganisms makes it possible to satisfy the massive demand on biofuels using limited land resources without causing potential biomass deficit.
- Microalgal or Cyanobacterial cultivation consumes less water than land crops.

- The tolerance of these microorganisms to high CO2 content in gas streams allows high-efficiency CO2 mitigation.
- Nitrous oxide release could be minimized when photosynthetic cyanobacteria are used for biofuel production.
- Cyanobacterial harvest and farming could be potentially more cost effective than conventional farming.

1.3.1 Polysaccharide Production

As is the case with the strain under study in this work, many cyanobacteria have the ability to produce exocellular polysaccharide, purposes of which range from nutrient storage to, as in case of hypersaline or desert microorganisms, protection from desiccation. A widely accepted theory is that exocellular polysaccharide production is, directly or indirectly, related to environmental constraints imposed on the microorganism. In most cases the polysaccharide serves as a boundary between the cell and its immediate surroundings. Figure 1-5 is a nice example.

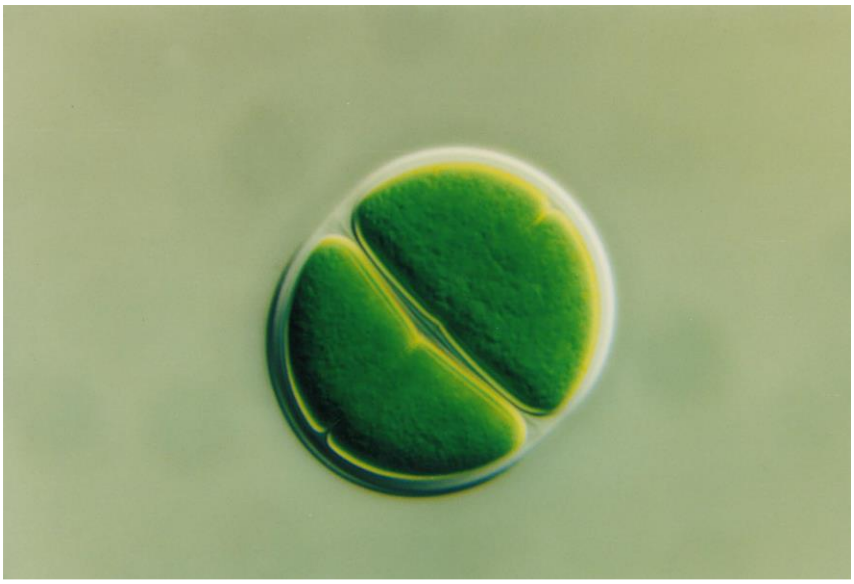

Figure 1-5. A Nomarski contrast photomicrograph of a sheathed *Chroococcus* sp. (1000x) (Photo courtesy of Fattom and Shilo, 1984)

Being more specific one could argue that the layer of polysaccharide provide protection against, apart from desiccation, antibiotics, antibodies, predatory protozoans, surfactants etc. In extending this theory, some scientists suggest that since light is the one and only source of energy for these microorganisms, some cyanobacteria use the secretion of polysaccharides to maximize the availability of light (by flocculating solid particles and clearing the surrounding water column), resulting in higher rates of growth, a direct consequence of increased light utilization and nutrient uptake (Fattom and Shilo, 1984). Other suggested uses of the polysaccharide are nitrogenase protection and a sort of adhesive. The main conclusion we can arrive at from studies of cyanobacterial exocellular polysaccharide is that it can fulfill various roles, depending on the strain and the characteristics of its natural habitat. This work is a beginner's step in trying to understand if the exocellular polysaccharide produced by Synechococcus sp. BG0011 can, in some way, fulfill the role of an energy source.

1.3.2 Scope of Work

Frankly, cyanobacteria provide us with an exciting opportunity to tackle the problem that has confounded society for a long time. Their unique properties and potential serve as a good platform to begin the long process of damage limitation. This work examines the advances that can be made in making the use of biofuels a reality using polysaccharide producing algae. It focuses on formulating a model that takes the first steps in describing the process of growing an algal species and producing polysaccharide in a laboratory scale.

CHAPTER 2
MATERIALS AND METHODS

2.1 Synechococcus BG0011

The cyanobacterial strain that was used in this project is a polymer producing strain of unicellular cyanobacteria that was isolated from a coastal lagoon in Florida Keys, more specifically 25 degrees 00‘ N x 81 degrees 30’ W (Phlips *et al*., 1989). The three most important characteristics of this algal strain that set it apart are: (1) it can fix its own nitrogen (2) it produces a highly viscous polymer that can be used to generate biomethane without separation or bioethanol after separation from culture broth (3) it can grow in a wide range of environmental factors like salinity and temperature (Phlips *et al*., 1989). The fact that it can fix its own nitrogen from the atmosphere makes the use of nitrogen supplements unnecessary. Furthermore, unlike other cyanobacteria that require considerable amounts of freshwater on a comparatively higher scale, Synechococcus BG0011 has been reported to grow with relatively high rates in conditions of varying salinity (Phlips *et al*., 1989). This enables us to use lesser quantities of limited freshwater resources and maybe even some saline media that might have otherwise been unsuitable for cyanobacterial growth. This type of tolerance to thermal changes and ability to grow in such conditions make this strain unique and provides us the perfect platform to explore the possibility of developing a clean source of energy.

2.2 Process Description

The algal strain was cultivated in a closed photobioreactor with aeration and active in-situ sterilization. Aeration was provided by sparging atmospheric air at the rate of 0.5 L/min through each of the reactors. The air was passed through filters prior to

entering the reactors to remove any airborne contaminants. The air being sparged serves two purposes: (1) provides nitrogen and carbon dioxide which are essential for growth (2) creates turbulence inside the reactors resulting in a satisfactory degree of mixing. The air exiting the photobioreactors is sent into a flask to account for some back pressure in the system before being let out into the atmosphere.

2.2.1 Stages of growth

The whole process was split into two distinct growth periods:

- Batch Growth Phase: This begins once the reactors are inoculated and continues till the 18th day, depending on the amount of growth observed during that period, and ends with dilution of reactor contents
- Semi-Continuous Phase: This growth phase begins with the dilution of the reactor contents and is maintained for as long as the run itself

The two phases of growth have different sampling regimes and dilution rates as explained later. The main reason for carrying out the experiment in such a fashion is the loss of reactor contents, primarily water, through evaporation and entrainment. This causes the volume to drop and concentrates the biomass present in the reactors. Furthermore, a loss of reactor contents translates into a loss of vital nutrients for the cyanobacteria which might deter its growth, hence the addition of new growth medium and water during the semi-continuous phase.

2.2.2 Reactor setup

A mechanical 1.5 Amp pump was used to pass air through the system. To prevent any throttling of the pump, and thereby consequent heating up, the bulk of the air from the outlet of the pump was purged and the active line was fed into a reservoir. A reservoir was used to negate the unwanted effects of using a mechanical pump, more specifically the infrequent variations in pressure and errors in measuring flow therein.

The air from the reservoir entered a Humidifier, which does exactly what its name suggests. The Humidifier is a sealed flask having one inlet and two outlets containing autoclaved water. The main function of the Humidifier is to increase moisture content of process air thereby decreasing water loss in the reactors due to evaporation. One of the initial designs had the air exiting the Humidifier passing through a set of flowmeters and filters before being let into the reactors. However, this resulted in some rare cases of water buildup inside the flowmeters. To prevent this, a column of glass tubes with varying cross-sectional area is used to catch any excess water present in the process air before being fed through the flowmeters.

As mentioned before, the air is filtered using 0.45 µm filters to remove any sorts of contaminants. This happens just before the air enters the reactors. The reactors are a set of four Erinmeyer bottles with a total volume of 250 ml and a working volume of 200 ml. Three of the bottles are inoculated with the algal strain while the fourth is used a control to gauge the level of contamination, if any, in the system. All reactors are fitted with porous, fused ceramic spargers in the inlet tube which creates the aeration. The ceramic spargers are cylindrical with a diameter of 0.875 in and an average pore size of 60 µm.

The air, on exiting the reactors, is sent to an exit flask containing a solution of pH 12 made by dissolving Potassium Hydroxide in distilled water. The level of solution inside the flask provides the system with some backpressure preventing small gusts of outside air entering the system, in case the pressure inside drops momentarily due to the infrequent variations in pumping. The high pH maintained in the exit flask also serves to sterilize the system, preventing growth of unwanted bacteria.

Figure 2-1. Picture showing one of the reactors and the control

Additionally, the Humidifier and the exit flask are housed in a black box with a UV light of 350 nm wavelength to further the sterilization efforts and eliminate chances of contamination. All the tubing used in the system is general purpose food-grade Norprene® tube with an inner diameter of 3.2 mm made from polypropylene-based material with USP mineral oil. The components of the system that come in contact with the process air or culture broth were autoclaved at 121 degrees C at 20 psi for a total of 30 minutes excluding cooldown period. Three of the reactors were inoculated at the start of each run with 1 ml of exponentially growing cultures. Temperature inside the growth chamber was maintained at 28-30 degrees C.

The irradiance for algal growth was provided by two white fluorescent light bulbs with a power rating of 20W each. The light unit was attached to a timer that was set to a 13/11 light/dark cycle ensuring the light was on for 13 hours and off for 11. This was done to mimic the light conditions in the natural growth habitat of the algae.

2.3 Sampling

Each photobioreactor is fitted with a sampling port that remains pinched at all times except during sampling. Sampling was accomplished using sterilized needle syringes with the needles snipped off. The syringe was inserted into the sampling port when needed only. Sampling was done in 24 hour intervals beginning with the time of inoculation. During the batch phase of the experiment, sampling volume was kept at 1.5 ml per reactor. During the semi-continuous phase, 5 ml of sample per reactor was withdrawn. The samples after analysis were stored in Eppendorf tubes in a freezer that was maintained at -4 degrees C. Dilution of the reactor contents was also done with another sterilized syringe through the sampling port. Care was taken to ensure: (1) proper mixing was observed before sampling, making sure the unmixed culture broth in the sampling tube is pushed back in and (2) sampling was done before dilution if the experiment was in the semi-continuous phase.

The optical density of the withdrawn samples was measured using a Milton Roy Spectronic 401 spectrophotometer at a wavelength of 540nm. 1 ml of sample was vortexed and placed inside the spectrophotometer to obtain the measurement. It should be noted that vortexing significantly affects the final measurement displayed by the spectrophotometer. It was observed that different times and speeds of vortexing caused the spectrophotometer to display ambiguous readings for the same sample. So each

sample was vortexed for 3 seconds at a speed setting of 4 before measuring its optical density.

2.4 Modeling

The model used to describe the experimental system was coded using Visual Basic for Applications (VBA) in Microsoft Excel. VBA was chosen to write the code because of the simple user interface, ease of learning and its interchangeability with Microsoft Excel where all our data lies. As with all models, a set of assumptions were made right at the beginning. These are:

- Growth kinetics follows the Monod's kinetics characterized by the Monod's parameters – maximum specific growth rate and half-saturation constant
- Changes in volume of reactor content does not affect the end result
- Dilution of reactor contents happens instantaneously
- Transfer rates of all components except CO2, namely Oxygen, Phosphorus and Nitrogen, are either negligible or of no consequence
- There is no significant change in pH of the system
- Salinity of reactor contents is always kept constant throughout the runtime
- Each reactor is completely mixed with the optical density measurements considered as indicative of the entire reactor contents at that period of time
- Light, though essential for growth, is not considered to affect the cell density. In other words, no cell shading occurs.

2.4.1 Characteristic equations

The physical system under study comprised of two important processes with respect to modeling purposes: the growth of microorganisms and the transfer of carbon dioxide from gas phase to liquid phase. The equations describing these processes are described here. All symbols are defined in Appendix C.

The growth of the algae has been assumed to follow Monod's kinetics. It is a mathematical model for prediction of growth rate of a microorganism having a structure similar to Michaelis-Menten equation. The rate of change of biomass following Monod's kinetics is given by the expression

$$\frac{dC1}{dt} = \left(\frac{\mu_{max}.C_2}{K_s + C_2}\right).C_1$$

The mass transfer of carbon dioxide to culture broth depends on the mass transfer coefficient and is governed by the expression

$$\frac{dC_2}{dt} = -\frac{1}{Y_{xs}}\left(\frac{\mu_{max}.C_2}{K_s + C_2}\right).C_1 + \alpha.\left(\frac{P_t.y_{C_2}}{H} - C_2\right)$$

These two equations are solved simultaneously in the model using explicit first order Euler numerical integration technique.

2.4.2 Model Objectives

- The model should successfully be able to predict the occurrence and time of growth characteristics such as exponential growth phase and stationary phase
- It should estimate vital growth parameters like specific growth rate and half-saturation constant by curve fitting with observed experimental data
- The model should also yield itself to a primitive One Factor at a Time (OFAT) sensitivity analysis

CHAPTER 3
RESULTS

The experimental studies on growth was carried out in terms of batches, running from Batch 1 through 5. Batch 1 was the first attempt in the newly built reactor setup and sampling was infrequent as first priority was to evaluate the setup's capabilities and drawbacks. The loss of reactor contents was evident and there was flooding of flowmeters due to oversaturated air passing through them. The columns previously mentioned were added after Batch 1. Batch 1 was also entirely run in Batch phase. The second run proved to be more successful in terms of data recovery and provided us with the fastest growth characteristics. Dilution of reactor contents was introduced in Batch 2 making it the first batch to operate in semi-continuous phase. Batch 3 was not pursued beyond the first 3 days due to startup problems. Batch 4 was operated for a total of 19 days and was stopped due to precipitation issues in the media used in that particular run.

Batch 5 proved to be the most consistent in terms of growth and data collection and was operated for a total of 52 days with distinct batch and semi-continuous modes of operation. The dilution was carried out on the 18^{th} day after inoculation. Due to its numerous data points and consistency, the model uses the data accumulated from this batch for its validation.

The primary parameters considered in the model for curve fitting was the maximum specific growth rate, mass transfer coefficient, dilution ratios, half-saturation constant and the biomass yield coefficient on substrate. The model was also used to

study the effects of inlet substrate concentration and mass transfer coefficient (kla) at first.

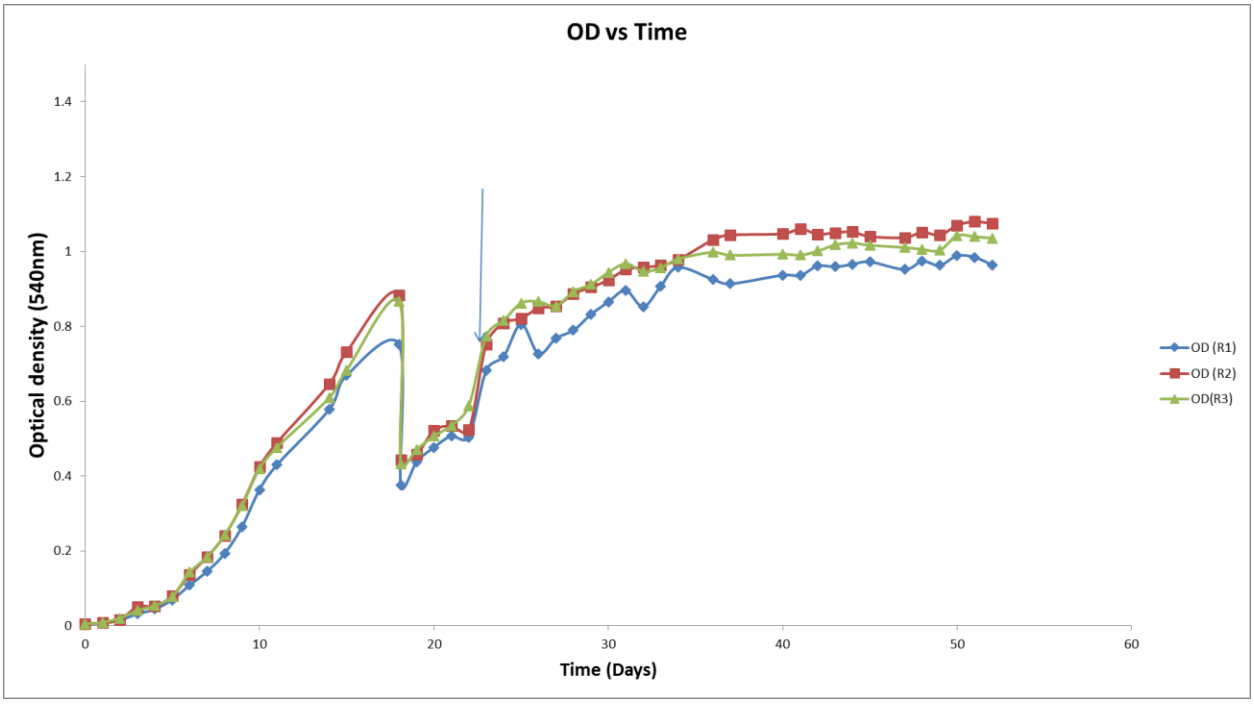

Figure 3-1. Experimental data used in optimization of model

The plot above (Figure 3-1) shows us the values obtained from experimental runs that helped in optimization of the model. This graph belongs to the run Batch 5.

3.1 Model Output

The model's first run was carried out with initial guesstimates based on literature values and observed growth in the laboratory. The culture conditions were taken into account as well. Dilution factors were set at values that were experienced during actual dilution of reactor contents.

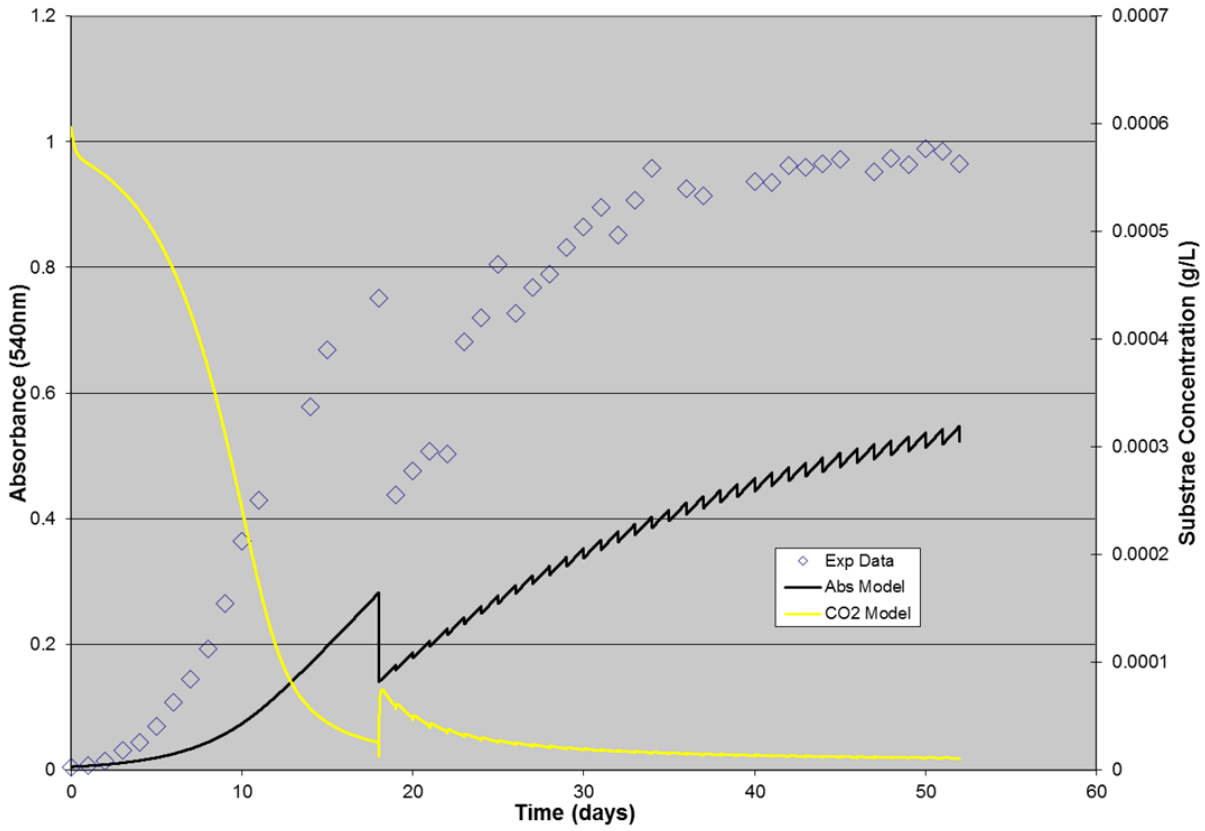

Figure 3-2. First run with estimates for parameter values. The dots represent the experimental curve which is what we are aiming at. The black plot is the model output for biomass growth in terms of absorbance measurements. Yellow line corresponds to the substrate consumption rate.

Clearly the initial values weren't giving acceptable results though this was to be expected. Before the second run, the model output was used to calculate the error in the system and consequently the Sum of Squares Error (SSE). This was then minimized using the four parameters – specific growth rate, half-saturation constant, mass transfer coefficient and the yield of biomass from substrate. These were manipulated by the Microsoft Excel Solver to arrive at the least possible value for SSE. The second run produced results that were much closer to actual observations.

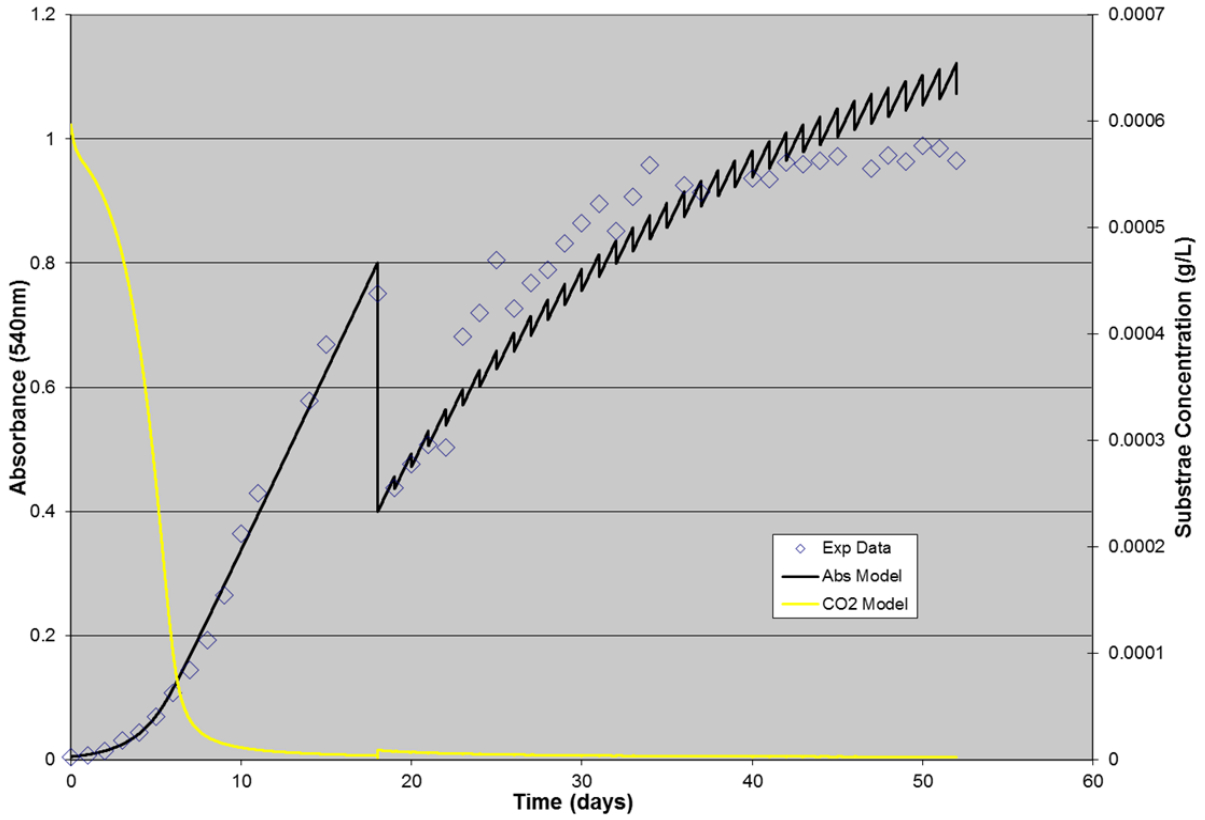

Figure 3-3. Second run of model with minimization of error resulting in a good fit with experimental observations.

As can be seen, the model now predicts a better growth curve that seems to fit reasonably well with our observations. Again the only parameters changed were the four previously discussed. The dilution factors were still kept the same. This output predicts a final absorbance reading of 1.2 (540nm) that translates to a final cell density of 0.45 g/L. The Solver, however, came up with an unusually high value of the yield coefficient to make fit the curve. This is not acceptable as the yield coefficient depends only on the stoichiometry. Hence, using stoichiometric calculations a yield coefficient of 4.67 grams of carbon dioxide per absorbance was used in the third run to make the fit more realistic. Also the second dilution factor, r2, was included in the optimization.

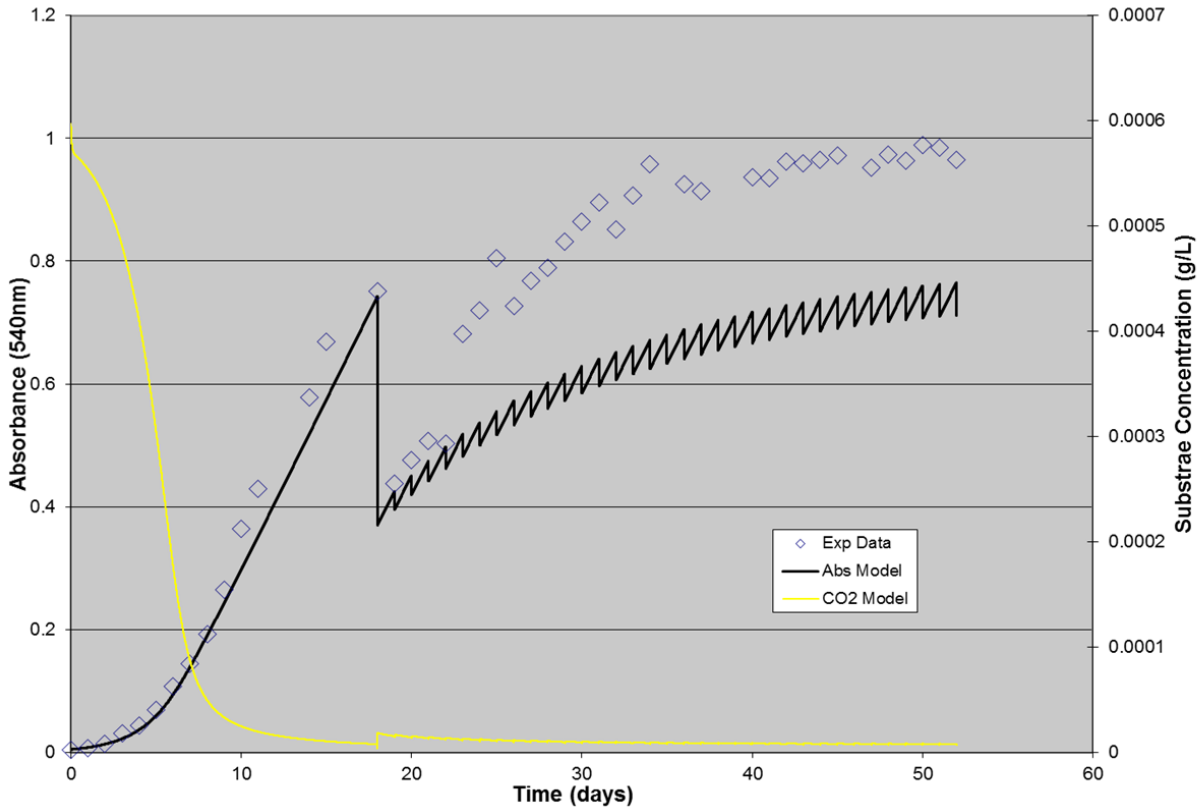

Figure 3-4. Third model run with Ysx = 4.67

From Figure 3-4, it can be seen that the error in the second part of the growth phase, namely the semi-continuous phase is higher than before. The reason for this is that the error was split between exponential and semi-continuous growth phases as including both in the optimization for this run did not return any feasible solution. Hence, only the error in exponential phase of growth was minimized. To obtain a better fit overall, the parameter values produced by curve-fitting was input into the model code and the final plot generated by the model showed a decent fit for both phases of growth. This is shown in Figure 3-5 below.

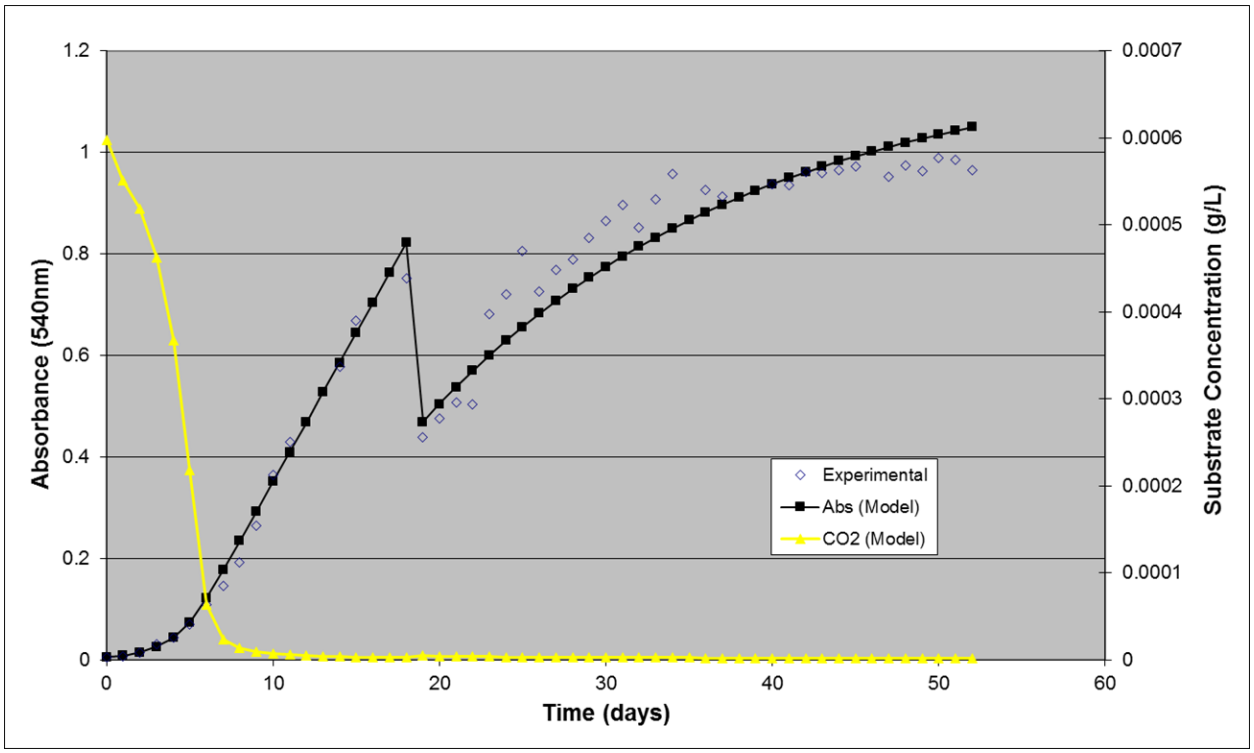

Figure 3-5. Model generated plot showing growth and substrate concentration predictions with parameter values obtained by optimization

3.2 Comparison of Strain Characteristics

To get a better idea of the results obtained from this work, a rudimentary comparison between various algal strains was carried out with emphasis on the specific growth rate of the microorganisms. Even though one could argue that some of the growth studies performed on the cyanobacteria used in the comparison were not geared towards increasing their growth rate, we shall assume, for purposes of simplification, that the noted values of specific growth rate were the maximum values of the said cyanobacteria. We can see from Table 3-1 that the maximum specific growth rate of Synechococcus BG0011 is significantly lower than most, with a strain from the same genus showing a value of 0.85 $days^{-1}$ as compared to 0.56 $days^{-1}$ of BG0011.

Table 3-1. Comparison of strain characteristics

Strains	μmax ($days^{-1}$)	Doubling Time (days)	Source
Aphanizomenon gracile	0.85	0.81	Chi-yong *et al*. (2003)
Anabaena minderi	0.71	0.97	Chi-yong *et al*. (2003)
Anabaenopsis NE1	0.90	0.77	Chi-yong *et al*. (2003)
Microcystis aeruginosa	0.65	1.06	Moisander *et al*. (2002)
Nodularia FL2f	0.40	1.73	Moisander *et al*. (2002)
Synechococcus Elongatus	0.85	0.81	Gonzalo-Barreiro *et al*. (2004)

CHAPTER 4
DISCUSSIONS

4.1 Model Performance

The studies carried out in the laboratory were attempted to be defined by a set of equations in the form of a mathematical model. The model that has been presented here is a basic black box model with some telling assumptions to simplify the process. This is in no way a complete model as this growth study of Synechococcus BG 0011 is a complex process with numerous parameters and factors that all have a significant impression on the final results, be it biomass growth or polysaccharide production.

The model has been found in general to agree with experimental observations. The exponential growth phase was well predicted by the model with accurate dilution factorization into the growth curve. The same couldn't be said of the semi-continuous phase of growth as one of the optimization runs returned a non-feasible solution coupled with a very high value of the yield coefficient. However, the model did generate a reasonable fit for the semi-continuous phase with the optimized parameter values. The reason for this ambiguity could be the negligence of the effect of volume change during dilution, which is more prominent in the semi-continuous phase.

The final set of parameter values obtained from the model are: $\mu max = 0.56$ $days^{-1}$, $Ks = 1.69*10^{-5}$ g/L. mass transfer coefficient $\alpha = 21.56$ $days^{-1}$ and the yield coefficient Ysx = 4.67 grams carbon dioxide per absorbance at 540nm.

4.2 Future Work

The model described in this work is a simple non-linear model that attempts to begin the characterization of the growth of Synechococcus BG 0011. There have been

some global assumptions made right from the start that help in quantifying our process and also in estimation of the parameters. Whenever any assumption is made there is always going to be a drawback associated with it.

The very first assumption made, apart from Monod's kinetics assumption, is that the volume changes in the system doesn't affect out outcome. This was primarily a theoretical assumption as clearly decrease in the reactor content volume concentrates the culture broth. This assumption could be mitigated in future models by using a program structure that accounts for the volume change and its effects.

Additionally, the possibility of growth of the algae with two substrates, dissolved carbon dioxide and dissolved nitrogen, can be explored in subsequent work. The curve shown for carbon dioxide consumption in this work is purely model generated and it could be validated in future using appropriate experimental data. Finally, since we are most interested in the production of polysaccharide from this algae, a model incorporating the quantification of polysaccharide production and effects on it due to changes in environmental conditions can be looked into.

APPENDIX A
MODEL CODE

The Visual Basic code is presented here.

```
Sub SubstrateLimiting()

c1 = Range("N4") 'od

c2 = Range("N6") 'mol/L


t = 0

Dt = 0.00001

tfinal = 52 'days

'miu = Range("N5")


alpha = Range("N7")  '1/days kla

ks = Range("N8")  'od

mumax = Range("N9") '1/days

Ysx = Range("N10") 'g/g

pt = 1 'atm

h = 0.67 'L.atm/mol

yc2 = 0.0004 'mole fraction


tsc = 18

irow = 7 'beginning print row


'Dilution factors
```

```
r1 = Range("N2")
r2 = Range("N1")

c2sol = (pt * yc2) / h

Do
'dilutions
  If Abs(t - tsc) < 0.000001 Then
    c1 = c1 * r1
    c2 = c2 * r1
  End If
  If t > tsc + 0.5 And Abs(t - Int(t + 0.000001)) < 0.000001 Then
     c1 = c1 * r2
     c2 = c2 * r2
  End If
'rates
  dc1dt = mumax * c2 * c1 / (ks + c2)
  dc2dt = -1 / Ysx * mumax * c2 * c1 / (ks + c2) + alpha * (c2sol - c2)
  c1 = c1 + Dt * dc1dt
  c2 = c2 + Dt * dc2dt
  t = t + Dt
  If Abs(t - Int(t + 0.000001)) < 0.000001 Then
    'MsgBox (dc1dt)
```

```
    Cells(irow, 4) = c1
    Cells(irow, 5) = c2
    'Cells(irow, 8) = t
    irow = irow + 1
  End If

Loop While t < tfinal

End Sub
```

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BIOGRAPHICAL SKETCH

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The author, Raghavendran Murali, was born in the southern metropolitan city of Chennai in Tamil Nadu, India. He did his schooling in Padma Seshadri Bala Bhavan Senior Secondary School in his hometown. He completed his Bachelor's degree in National Institute of Technology, Durgapur where he discovered his passion for the unlimited possibilities of Chemical Engineering. He also loves football, or soccer as its otherwise known, and loves playing the sport whenever he finds time.