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ENHANCING THE INDUSTRIAL POTENTIAL OF FILAMENTOUS
CYANOBACTERIA

BY

TYLOR J. JOHNSON

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Biological Sciences

Specialization in Microbiology

South Dakota State University

2016

ENHANCING THE INDUSTRIAL POTENTIAL OF FILAMENTOUS
CYANOBACTERIA

This dissertation is approved as a credible and independent investigation by a candidate for the Doctor of Philosophy degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this dissertation does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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I dedicate this dissertation to my beautiful wife Katie. This would never have been possible without her sacrificing every day so that I could chase my dream of earning my PhD. She is the foundation that keeps our family strong and a wonderful mother to our children: Maddie, Ryan, and Hayden.

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ABBREVIATIONS

2PG: 2-phosphoglycerate

3PGA: 3-phosphoglycerate

AC: Activated carbon

AD: Anaerobic digestion

AISIM: Algae Income Simulation Model

ANOVA: Analysis of variance

ARID: Algae Raceway Integrated Design cultivation system

ARTP: Atmospheric and room temperature plasmas

ATP: Adenosine triphosphate

BOD5: Five-day biochemical oxygen demand

CCM: Carbon concentrating mechanisms

Chl α : Chlorophyll α

CIP: Clean-in-place units

CLSM: Confocal laser scanning microscopy

DAF: Dissolved air flotation

DALY: Disability adjusted life years

DCW: Dry cell weight

DEQ: Damage to ecosystem quality

DHH: Damage to human health

DR: Damage to resources

EC: Electrocoagulation

EMS: Ethyl methanesulfonate

FARM: Farm-level Algae Risk Model

FBPase: Fructose-1,6-bisphosphatase

FU: Functional unit

GADPH: Glyceraldehyde-3-phosphate

GOGAT: Glutamate synthase

GS: Glutamine synthetase

GWP: Global warming potential

HRAP: High rate algal wastewater pond

HTL-CHG: Hydrothermal liquefaction-catalytic hydrothermal gasification

IPTG: Isopropyl β -D-1 thiogalactopyranoside

KOV: Key output variable

LB: Luria broth

LBA: Luria broth agar

LCA: Life cycle analysis

LCIA: Life cycle impact assessment

LED: Light emitting diodes

MALDI-TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MMS: Methyl methanesulfonate

MNNG: N-methyl-N'-nitro-N-nitrosoguanidine

mPt: Millipoint

MRT: Duncan's new multiple range test

MWWT: Municipal wastewater treatment

NAABB: National Alliance for Biofuels and Bioproducts

NADPH: Nicotinamide adenine dinucleotide phosphate

NPV: Net present value

NREL: National Renewable Energy Laboratory

NW: Natural seawater

OD: Optical density

OMEGA: Offshore membrane enclosure for growing algae

PAF: Potentially affected fraction

PBR: Photobioreactor

PBS: Phycobiliproteins

PC: Phycocyanin

PI: Propidium iodide

PSII: Photosystem II

Pt: Point

PVC: Polyvinyl chloride

RubisCO: Ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP: Ribulose-1,5-bisphosphate

SBPase: sedoheptulose-1,7-bisphosphatase

TFTC: Too few to count

U: Fluorescence intensity units

VOC: Volatile organic compound

VS: Volatile solids

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ABSTRACT

ENHANCING THE INDUSTRIAL POTENTIAL OF FILAMENTOUS
CYANOBACTERIA

TYLOR J. JOHNSON

2016

The objectives of this project were to improve the industrial potential of filamentous N₂-fixing cyanobacteria by increasing its biofuel tolerance, and to evaluate the economic feasibility and environmental impacts of a theoretical, cyanobacteria-based biofuel production facility. To develop a method to quantify filamentous cyanobacteria in dilute culture media, a dual-stained fluorescence assay was evaluated. While the viable cell stain (SYTO[®] 9) was accurate, the non-viable cell stain (propidium iodide) also bound to viable cells. Additional non-viable cell stains were evaluated, but none were accurate at quantifying viability. Thus we concluded that the viable cell stain SYTO[®] 9 is a reliable assay and can be used in high-throughput assays. To develop cyanobacteria strains with increased tolerance to biofuels, directed evolution under the pressure of higher biofuel concentrations was used. As these biofuels are highly volatile, it was necessary to conduct experiments in sealed test-tubes. Thus, cyanobacteria growth in a sealed environment was optimized using BG11 as the basal medium supplemented with 0.5 g/L NaHCO₃ as the carbon source. Subsequent directed evolution trials yielded 3 confirmed mutants with increased biofuel tolerance: *Nostoc punctiforme* ATCC 29133 with a 20% improvement in linalool tolerance, *Anabaena variabilis* ATCC 29413 with a

60% improvement in linalool tolerance, and *Anabaena* sp. PCC 7120 with a 220% improvement in farnesene tolerance. To determine the optimal nitrogen source, dinitrogen (N₂ gas) was compared to various fixed nitrogen sources. Ammonium chloride was determined to be the preferred nitrogen source for large scale cyanobacteria cultivation based on growth rate and environmental impacts. Finally, an economic feasibility and a life cycle analysis were conducted on a theoretical limonene production facility that used a genetically engineered filamentous cyanobacteria strain. The facility was not economically feasible at current limonene productivity rates, but would be feasible if productivity can be increased 56.7-fold. The life cycle analysis showed that increasing limonene productivity worsens the environmental profile of the facility. While using filamentous N₂-fixing cyanobacteria as industrial microorganisms is currently in its infancy, there is a great deal of potential for this microbe to become a significant contributor to renewable biofuels and high-value chemicals.

Chapter 1 - Literature Review

1.1 Introduction to biofuels

Currently, approximately 80% of the world's energy demand is supplied by fossil fuels (Chen et al., 2011). It is becoming increasingly important that alternative forms of energy be developed before these non-renewable resources are completely depleted. As of 2009, it was estimated that fossil fuel reserve depletion times for oil, coal, and gas are approximately 35, 107, and 37 years, respectively (Shafiee and Topal, 2009). Other driving forces involved in moving away from fossil fuels are environmental impacts such as production of greenhouse gases which leads to ozone depletion, global warming, and smog formation (Von Blottnitz and Curran, 2007).

Biofuel production from renewable biomass is a promising alternative to petroleum-based transport fuels (Hasunuma et al., 2013). Brazilian production of sugarcane ethanol and U.S. production of corn ethanol are currently the world's leading sources of biofuel (Crago et al., 2010). According to Goldemberg (2007), after more than 30 years of production, Brazilian sugarcane ethanol is an energy commodity that became fully competitive with gasoline on the international markets in 2004. Ethanol production from Brazilian sugarcane increased 47-fold, from 0.58 billion liters 1975 to 28.0 billion liters in 2010 (Chen et al., 2015). In 2013/2014, ethanol production from Brazilian sugarcane was 24 billion liters (Sugarcane.org, 2015). In the U.S., corn ethanol production has increased from 1.8 billion liters in 2006 to 54 billion liters in 2014 (RFA, 2015b). Both of these industries are helping to reduce the use of fossil fuels for transportation. Sugarcane ethanol in Brazil accounts for 16.7% of automotive fuel needs

(SCA, 2013), while corn ethanol in the U.S. has displaced ~10% of gasoline usage (RFA, 2015a).

Current research on biofuels is focused on finding more suitable, productive, and sustainable types of non-food biomass to produce in various locations, and on developing efficient and economical methods to convert biomass into infrastructure-compatible fuels on a large scale (Parmar et al., 2011). The most significant challenge for biofuels is reducing production costs to levels comparable to petroleum-based fuels (Lee et al., 2008). One significant cost factor is transportation of the feedstock from the field to the processing facility. Due to the relatively low density of many solid and liquid forms of biomass compared with fossil fuels, numerous vehicle movements are inevitable (Sims et al., 2010).

Biofuel production processes must both utilize renewable feedstocks and be capable of sequestering atmospheric CO₂ to make a carbon neutral or negative process (Schenk et al., 2008). A controversy related to the production of biofuel feedstocks is competition for arable land with food or feed crops (Hasunuma et al., 2013). These are issues that will need to be addressed for biofuel production to be competitive with fossil fuels.

1.1.1 Importance of biofuels

There is an increasing need for biofuel development as Earth's fossil fuels are being depleted by the growing world population. Moreover, the increase in greenhouse gases being released into the atmosphere from fossil fuel use is problematic. It has been

well established that the massive use of fossil fuels has led to pollution, detrimental health in many organisms, and global climate change (Chen et al., 2011).

Many regions and countries have made strong commitments to increasing the use of biofuels. The EU has mandated that over 10% of transportation fuel originate from biofuels by 2020 (Trostle, 2010). The U.S. has a goal of displacing 30% of the gasoline demand with biofuels by 2030 (Foust et al., 2009). A key to achieving this goal will be unlocking the potential of lignocellulosic ethanol. It is expected that within the next decade, lignocellulosic ethanol will be commercialized as renewable energy for transport (Han et al., 2015). Lignocellulosic feedstocks are lower cost than corn and sugarcane, and may have improved energy balance values (Gnansounou and Dauriat, 2010). Third generation biofuels, which utilize CO₂ as a feedstock, are also being explored as an option for biofuel production. As the inevitable decrease in fossil fuels occurs, increasing the production and availability of biofuels is a global necessity.

1.1.2 Categories of biofuels

Biofuels can be categorized based on the type of feedstock used and/or the type of fuel produced (Table 1.1). Each new generation of biofuel has been developed to overcome limitations or disadvantages of prior types of biofuels. While each biofuel type has its own advantages and disadvantages, together they have begun to lessen the burden of fossil fuel consumption globally. One key element in the development of new categories of biofuels is the underlying biosynthetic pathway. Constructing these pathways is the first step towards making biofuels economically viable (Lee et al., 2008). With continued progress, biofuels will have a greater impact on our global energy consumption.

Table 1.1: Different generations of biofuel: Major source, process, and their examples.

(Awudu and Zhang, 2012; Demirbas, 2009; Dutta et al., 2014).

Generation	Feedstocks	Processing technology	Examples of biofuel
First	Edible oil seeds, food crops, animal fats	Esterification and transesterification of oils and fermentation of sugars, thermochemical process	Biodiesel, bioethanol, biobutanol
Second	Nonedible oil seeds, waste cooking oil, Lignocellulosic feedstock materials: cereal straw, sugarcane bagasse, forest residues	Physical, chemical, biological pretreatment of feedstock and fermentation, thermochemical process	Bioethanol, biobutanol, Fisher-Tropsch diesel, wood diesel, biohydrogen
Third	Algae	Algae cultivation, harvesting, oil extraction, transesterification, or fermentation, or thermochemical process	Biodiesel, bioethanol, biobutanol, drop-in biofuels (i.e. hydrocarbons) biohydrogen, methane
Fourth	Algae and other microbes	Metabolic engineering of algae with increases in carbon entrapment ability, cultivation, harvesting, fermentation or oil extraction, transesterification, or thermochemical process	Drop-in biofuels (i.e. hydrocarbons)

1.1.2.1 1st generation biofuels

First generation biofuels were developed in the 1970s and 80s and consist of either: 1) ethanol produced via fermentation of sugar (primarily from sugar cane) or

hydrolyzed starch (primarily from corn), or 2) biodiesel produced via transesterification of vegetable oil (primarily soybean oil or animal fats). The fuel ethanol process is well known and consists of feedstock pretreatment (milling, crushing, and solubilizing in water), saccharification (converting starch into sugars for the corn ethanol process), fermentation, distillation, and co-product recovery (Cardona and Sánchez, 2007; Kwiatkowski et al., 2006).

The biodiesel production process typically involves degumming the oil and conducting a base-catalyzed transesterification in the presence of an alcohol (methanol or ethanol). A co-product of this process is glycerol (Li et al., 2008). Compared to fossil fuels they replace, greenhouse gas emissions are reduced 12% by the production and combustion of ethanol and 41% by biodiesel (Hill et al., 2006). Biodiesel has been considered as an alternative source of energy for public transport as it is not only renewable but also reduces sulfur oxide production that occurs in current diesel fuels (Jaeger and Eggert, 2002).

First generation biofuels have three major disadvantages: production costs, market access, and competition for arable land with food crops. Because 1st generation biofuel feedstocks are also used for food, the feedstock usually accounts for more than 33% of total production costs, and this situation is unlikely to change as the world population and food demand continues to rise (Dien et al., 2003). Further attention must be paid to the issues of deforestation, irrigation water use, and crop price increases due to expanding 1st generation biofuel acreage (Havlík et al., 2011).

The market access problem with ethanol is referred to as the blend wall issue. Initially, US Federal regulations only allowed up to 10% ethanol (E10) in mixtures with gasoline (EIA, 2010). More recently the US Environmental Protection Agency (EPA) has approved up to E15 in vehicles manufactured after 2001 (Aguilar et al., 2015). This has effectively established an upper limit to the amount of ethanol that can be blended in the US gasoline supply. Engines of certain vehicles have been designed to accommodate an 85% blend of ethanol (E85), but this represents a relatively small proportion of the national vehicle fleet (Aguilar et al., 2015). An additional constraint on ethanol is that pipelines and fuel distribution systems are designed for hydrocarbon fuels, and many will not accept pure ethanol for transport (EIA, 2010).

The other hurdle 1st generation biofuels have encountered is land availability for production of the feedstocks. First generation biofuels cannot replace a significant amount of petroleum without impacting food supplies. Dedicating all U.S. soybean and corn production to the biofuel industry would only supply 12% of the gasoline demand and 6% of the diesel demand (Hill et al., 2006).

1.1.2.2 2nd generation biofuels

Second generation biofuels are typically defined as ethanol or other biofuels produced from lignocellulosic biomass, which includes a diverse range of by-products, wastes, and dedicated feedstocks (Sims et al., 2010). Approaches used in the production of lignocellulosic ethanol are shown in Figure 1.1. Lignocellulose is the most abundant biopolymer in the world (Carere et al., 2008). Agricultural residues such as corn stover in the Midwest (Zheng et al., 2009) and wheat straw in the Great Plains (Miner et al., 2013)

are significant potential feedstocks for biofuel production in the United States. Woody biomass and forestry wastes are dominant feedstocks in the East and Southeastern regions. The chief advantage of these feedstocks over 1st generation biofuel feedstocks is that the former are not used for direct human consumption, although corn stover and wheat straw are used as roughages for livestock feed. These feedstocks are also much lower in price compared to corn, soybeans, and sugar cane.

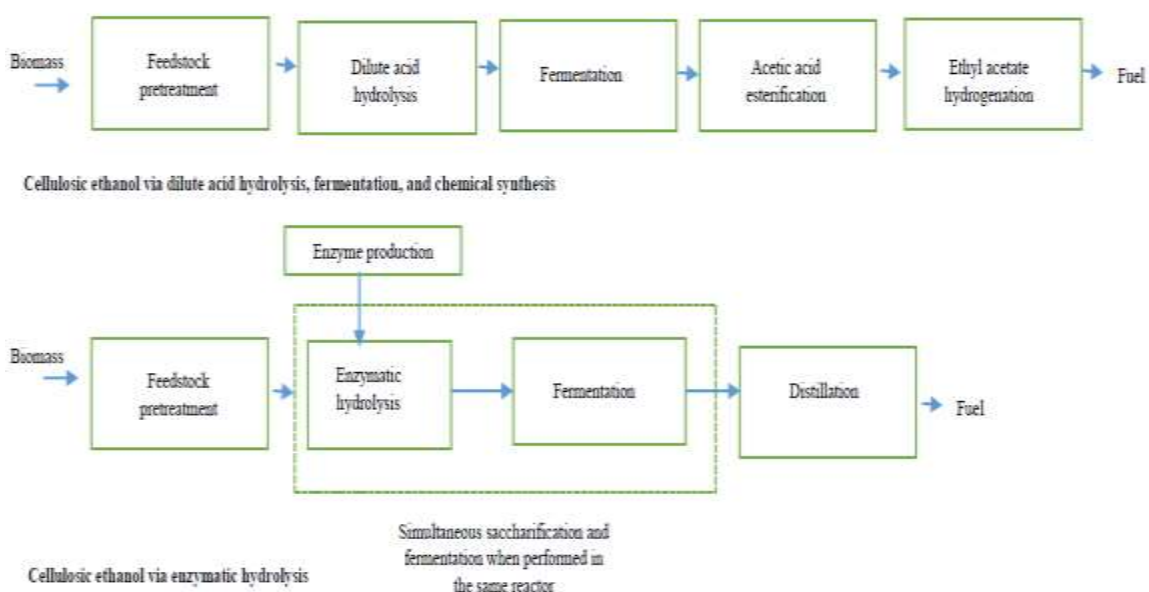


Figure 1.1: Approaches used in the production of lignocellulosic ethanol
(Brown and Brown, 2013)

Two general approaches are used in production of 2nd generation biofuels, and these are described more fully below. Biochemical conversion relies primarily on enzymes and microbes to hydrolyze biomass into simple sugars and then ferment these sugars into ethanol (Foust et al., 2009). Thermochemical conversion uses high

temperatures and pressures to degrade biomass into simple chemicals that are then catalytically synthesized back into more complex fuel molecules (Awalludin et al., 2015). Although biochemical and thermochemical processes have individual strengths and weakness, there is not yet a distinct economic or environmental advantage of one platform over the other (Foust et al., 2009). Marketplace economics should decide which conversion approaches are utilized (Zeman, 2007).

Due to the cost and limitations of feedstocks used for 1st generation biofuel production, 2nd generation biofuel production processes are currently being commercialized. At least ten cellulosic biorefinery projects are scheduled to begin operations by 2014 (Brown and Brown, 2013). This transition was catalyzed in part by the 385 million dollars provided since 2007 by the U.S. Department of Energy toward lignocellulosic bioethanol projects (DOE, 2007). Details of several commercial-scale cellulosic biofuel projects are provided in Table 1.2.

Table 1.2: Details of commercial-scale cellulosic biofuel projects expected to be in operation by 2014.

(Brown and Brown, 2013).

Company	Pathway	Location	Capacity (MGY)	Feedstock	Capital Cost (million)
KiOR	Catalytic pyrolysis & hydrotreating to hydrocarbons	Natchez, MS	41	Yellow pine	\$350
ClearFuels	Gasification & F-T synthesis to hydrocarbons	Collinwood, TN	20	Woody biomass	\$200
SunDrop Fuels	Gasification & MTG synthesis	Alexandria, LA	50	Mixed biomass, natural gas	\$500
ZeaChem	Dilute acid hydrolysis & acetic acid synthesis to ethanol	Boardman, OR	25	Agricultural residue, hybrid poplar	\$391
Abengoa	Enzymatic hydrolysis to ethanol	Hugoton, KS	25	Corn stover	\$350
Beta Renewables	Enzymatic hydrolysis to ethanol	Sampson County, NC	20	Arundo, switchgrass	\$170
DuPont Biofuel Solutions	Enzymatic hydrolysis to ethanol	Nevada, IA	25	Corn stover	\$276
POET	Enzymatic hydrolysis to ethanol	Emmetsburg, IA	20	Corn stover, corn cobs	\$250
Mascoma	CBP to ethanol	Kinross, MI	40	Hardwood pulpwood	\$232

POET is commissioning “Project Liberty” in Emmetsburg, IA. This is a 94.6 million L/year facility that will convert corn stover to ethanol using a biochemical process (pretreatment, enzymatic saccharification, and yeast fermentation) (DOE, 2014). Abengoa is similarly starting a 94.6 million L/year facility (Abengoa, 2015) in western Kansas that will convert wheat straw to ethanol using a biochemical process (Bacovsky, 2010). Mascoma is constructing a 151 million L/year cellulosic ethanol facility in Kinross, Michigan, as part of a joint venture with Valero. This project will use consolidated bioprocessing pathways to convert hardwood pulpwood feedstock into ethanol (Brown and Brown, 2013). DuPont’s \$225 million cellulosic plant opened on October 30, 2015 in Nevada, IA. It is currently the world’s largest cellulosic ethanol plant and expected to produce 30 million gallons of ethanol per year from 375,000 dry tons of corn stover (Ashraf, et al., 2016). Eventually, 2nd generation biofuels could contribute significantly to the future energy supply mix (Carrquiry et al., 2011; Zheng et al., 2009). The billion-ton study funded by the U.S. Department of Energy confirmed that over a billion tons of lignocellulosic biomass is available annually in the U.S., and this un- or under-utilized resource has the potential to generate 442 billion L/year of bioethanol (Bohlmann, 2006).

Along with the commercialized biochemical processes previously described, there are also commercial thermochemical processes. For example, KiOR has a 155 million L/year production facility that creates biooil via catalytic pyrolysis and hydrotreating of yellow pine (Brown and Brown, 2013; Corredores and Iglesias, 2014). ClearFuels has a production facility that utilizes Fisher-Tropsch technologies to produce 76 million L/year hydrocarbons (Bastos and Etanol, 2012; Brown and Brown, 2013).

Second generation biofuels do have disadvantages. The sustainability of these biofuels depends mainly on the choice of feedstock, and this is affected by limited land availability and competition for land use, limited waste feedstocks and competing uses, and the logistical issues and costs of collecting, storing, and transporting low density lignocellulosic feedstocks to the processing facility (Balat, 2011; Deenanath et al., 2012; Mabee et al., 2011; Petersen et al., 2015). Significant progress is also needed to improve conversion efficiency and reduce costs to enable 2nd generation biofuels to be a significant factor in the renewable fuel industry.

1.1.2.2.1 Biochemical conversion of lignocellulosic substrates into ethanol

Lignocellulose can be converted to bioethanol via pretreatment, enzymatic hydrolysis, fermentation, and distillation (Zah, 2010). In biochemical conversions there are many pretreatment and fermentation approaches (Mosier et al., 2005). The biochemical route is less mature than the thermochemical process and probably has a greater cost reduction potential (Sims et al., 2010). Advantages of the biochemical approach in comparison to the thermochemical approach are higher selectivity and conversion efficiencies (Himmel et al., 2007; Houghton et al., 2005).

Biochemical conversion of lignocellulose to bioethanol is difficult due to: 1) the resistant nature of the biomass to breakdown, 2) the variety of sugars that are released when the hemicellulose and cellulose polymers are hydrolyzed, and 3) the need to find or engineer microorganisms to efficiently ferment the mixture of sugars. Pretreatment is the most expensive processing step in the biochemical conversion process (Zheng et al.,

2009). Pretreatment is necessary to disrupt the matrix of lignin that surrounds and protects the carbohydrate polymers (cellulose and hemicellulose) from enzymatic hydrolysis to fermentable sugars (Yang and Wyman, 2008). Costs associated with hydrolytic enzymes are a major barrier associated with the industrial conversion of cellulosic biomass to biofuels (Meng and Ragauskas, 2014). The low efficiency of hydrolytic enzymes due to the natural recalcitrance of lignocellulose to deconstruction is another major bottleneck of the technology (Saritha and Arora, 2012). Universities and companies have worked since the early 1980s to develop effective and efficient pretreatment and hydrolysis methods for lignocellulose (Skoog and Hahn-Hägerdal, 1988; Waldron, 2010).

1.1.2.2 Thermochemical conversion of lignocellulosic substrates into drop-in biofuels

Several approaches have been developed to thermochemically convert lignocellulose into biofuels. Thermochemical conversion relies on heat and pressure to convert biomass into intermediate gas, liquid, and solid fractions. Then, catalytic conversion processes are used to convert some of these intermediate compounds into biofuels. The two major categories of thermochemical processing for biofuels are gasification and pyrolysis. Advantages of the thermochemical process compared to the biochemical process are technology robustness and the ability to accept a wider range of feedstocks (Hallen et al., 1988; Milne et al., 1998).

Gasification is the process of converting carbon-based feedstocks into synthesis gas (syngas) and biochar under high temperatures in the presence of some oxygen

(Higman and Van der Burgt, 2011). Gasification typically yields 5-10% of the feedstock mass as biochar (Brewer et al., 2009). Biochar is the carbon-rich product obtained when biomass undergoes thermal decomposition under limited supply of oxygen and at relatively low temperatures (<700°C) (Lehmann and Joseph, 2012). Biochar has been used for soil remediation (Sizmur et al., 2015), environmental remediation (Xie et al., 2015), and as a precursor of activated carbon (Azargohar and Dalai, 2006). Syngas produced from gasification contains a mixture of methane, hydrogen, carbon monoxide, carbon dioxide, and small amounts of ash and tar in different proportions (Pirc et al., 2012). Syngas is an important intermediate product for many processes. It is mainly used for the production of ammonia, hydrogen, methanol, oxo-chemicals, Fischer-Tropsch fuels, heat, and power (van Rossum et al., 2007).

Syngas can be converted into biofuels by using chemical catalysts known as the Fischer-Tropsch (FT) process or by using microbial catalysts in a process known as syngas fermentation (Munasinghe and Khanal, 2010). The FT process converts syngas to a range of hydrocarbons (Anderson et al., 1984; Dry, 1981) via highly exothermic reactions that take place in either a multi-tubular fixed bed or fluidized bed reactors (Dry, 1981; Dry, 1996). Only iron, cobalt, nickel, and ruthenium based catalysts have adequate activity to be considered for FT commercial processes (Dry, 2002). Examples of microbial catalysts capable of converting syngas into biofuel are: *Clostridium ljungdahlii*, *Clostridium autoethanogenum*, *Acetobacterium woodii*, *Clostridium carboxidivorans*, and *Peptostreptococcus productus* (Munasinghe and Khanal, 2010). These catalysts are capable of fermenting syngas into biofuel more effectively than the chemical catalysts (Heiskanen et al., 2007; Henstra et al., 2007).

Pyrolysis is the process of decomposing organic material into biooil, synthesis gas, and biochar at high temperatures and pressures in the absence of oxygen (Bridgwater, 1995; Goyal et al., 2008). Different temperatures and particle sizes leads to formation of the products in different proportions (Goyal et al., 2008). For example, at 400°C, pyrolysis yields 24% biochar, 42% biooil, and 26% syngas (Onay and Koçkar, 2004). However, by choosing the proper process conditions, up to 70% of the biomass can be converted to biooil via pyrolysis (van Rossum et al., 2007). Pyrolysis biooil is the liquid product produced via pyrolysis, and has potential as a fossil fuel replacement, source of valuable chemicals, fuel in mobile applications, and production of heat, power and syngas (Stoš et al., 2015).

Biooil produced from pyrolysis can be upgraded into transportation fuels (Bridgwater, 1999; Bridgwater et al., 1999). Upgrading into transportation fuels takes place via hydroprocessing or catalytic cracking processes (Carlson et al., 2008; Furimsky, 2000, 2013). Hydroprocessing involves the stabilization and removal of oxygen from untreated biooil through its catalytic reaction with hydrogen over alumina-supported, sulfide CoMo, NiMo, or noble metal catalysts. Catalytic cracking involves the simultaneous scission and hydrogenation of naphthenic and heavy aromatic molecules into lighter aromatic and aliphatic molecules (Shemfe et al., 2015). The syngas generated from pyrolysis is generally combusted to provide all the necessary energy needed to drive biomass pyrolysis and drying operations (Bridgwater, 2012), while the biochar generated is used as described above.

1.1.2.3 3rd generation biofuels

Third generation biofuels were initially defined as fuels derived from fixation of CO₂ by photosynthetic algae and cyanobacteria (Singh et al., 2011b). In this definition, the photosynthetic organism served as both the photocatalyst and producer of biofuel using CO₂ as the feedstock (Lindberg et al., 2010). Much of the early work consisted of algal production of oil, which was then recovered from the harvested algal cell mass (Seo et al., 2015). While algal oil can potentially be used directly as a fuel, in most cases the oil was subsequently processed through traditional oil refinery or biodiesel technologies into biofuels (Chernova et al., 2010; Chernova et al., 2012; Senko et al., 2012). Therefore, many researchers now suggest that the definition of 3rd generation biofuels be altered to photoautotrophic conversion of CO₂ into oil or algal biomass that is subsequently converted into biofuels (Dutta et al., 2014).

Cyanobacteria and algae are capable of growing rapidly while converting solar energy into chemical energy via CO₂ fixation (Chen et al., 2011). Productivity from microalgae can be 50 times higher than switchgrass, which is the fastest growing land plant (Bentley and Melis, 2012; Li et al., 2008). At this time, microalgae appear to be the sole source of biofuel able of meeting the global demand for transportation fuel (Brennan and Owende, 2010; Chisti, 2007; Schenk et al., 2008; Singh et al., 2011a). The photon energy densities and process productivities, as well as the benefit of minimal arable land and freshwater displacement can lead to a promising scenario in which the renewable fuel standards set by the U.S. can be met via photosynthetic microorganisms (Robertson et al., 2011). Microalgae's oxygenic photosynthesis, high per-acre productivity, non-food based feedstock, growth on non-productive and non-arable land, as well as utilization of a wide

variety of water sources are combining to capture the interest of researchers and entrepreneurs (Parmar et al., 2011).

Several companies have been developed to exploit photoautotrophic processes to produce chemicals and biofuels of interest. Aquafuel™ has partnered with Greenwich University to develop a glycerine fuel from algae. They claim that algae can produce up to 80% glycerine by volume and that glycerine could be used to power diesel engines (Aquafuel, 2014). Aquafuel Multi Green Fuels™ manufactures drop-in fuels from algae blended with multiple biomass waste streams (Aquafuel, 2014). In 2009, ExxonMobil launched a biofuel program partnered with Synthetic Genomics Inc.® to manufacture 3rd generation biofuel from algae (BusinessWire, 2009; Sims et al., 2010). These are a few of the numerous ongoing processes being conducted with the goal of making 3rd generation biofuel an alternative to fossil fuel based petroleum.

Disadvantages of processes designed to utilize photoautotrophic microorganisms in an industrial setting include: fouling (Singh et al., 2011a), photoinhibition due to excess light (Ruffing, 2011), cost of mixing (Schenk et al., 2008), the cost of artificial lighting necessary in indoor settings (Chen et al., 2011), and the need to subsequently convert algal oil into fungible fuels (Raheem et al., 2015). These are potential targets for research to increase the feasibility of any 3rd generation biofuel process.

1.1.2.4th generation biofuels

To account for the situation where the photosynthetic organism is genetically modified to convert CO₂ directly to a final biofuel product, the term 4th generation biofuel has been proposed (Chernova et al., 2010; Chernova et al., 2012; Senko et al., 2012). This

allows for the differentiation of wildtype photosynthetic organisms that produce oil as a product, from genetically engineered algae and cyanobacteria that produce drop-in biofuels. The 4th generation biofuel designation is also proposed for those microbes being engineered to utilize electricity, instead of sunlight, to drive conversion of CO₂ directly into fuels (Cogdell, 2013).

Production of biofuels from these organisms appears to be a favorable sustainable opportunity (González-López et al., 2012; Lehr and Posten, 2009). The advantages of utilizing photoautotrophic microorganisms such as algae and cyanobacteria for high-value products are numerous. From these microbes, CO₂ can be biologically converted into organic compounds (González-López et al., 2012). Also, they grow rapidly due to their simple structure. These are examples of reasons why they have been investigated for the production of biodiesel, biooil, biosyngas, and biohydrogen (Li et al., 2008). There are several start companies currently attempting to commercialize algal fuels including: Algenol Biofuels, Bionavitas, Inc., Synthetic Genomics, Inc., and Solix Biofuels (Chisti, 2013).

Despite the potential for next-generation biofuel production, many challenges need to be overcome to achieve commercial viability. These include: proper species selection, low photosynthetic efficiencies, development of single species cultivation, evaporation reduction, CO₂ diffusion losses, and a lack of data for large scale plants due to the few commercial plants in operation (Brennan and Owende, 2010). However, 3rd generation biofuel production has the same challenges to overcome. Alleviating these limitations will be essential in order to achieve an economically feasible industrial

process developing next-generation biofuels and high-value chemicals from microalgae and cyanobacteria.

1.2. Photoautotrophs

Photoautotrophs are organisms that can utilize light as an energy source and CO₂ as a carbon source. These organisms include plants, algae, and cyanobacteria. There is much potential for these organisms to reduce our reliance on fossil fuels, however there are constraints on the abilities of these organisms. When solar radiation encounters a photosynthetic array, only the visible portion (400 to 700 nm) of the solar spectrum is useful for photosynthesis (Ducat et al., 2011; Robertson et al., 2011). Thus only approximately 50% of total solar irradiation can be used for photosynthesis (Lehr and Posten, 2009). Also, the wavelengths of light that an individual photoautotroph can utilize is dependent on the specific light-harvesting pigments they contain, which further reduces the amount of available light for photosynthesis to the organism.

Increasing the efficiency of photosynthesis is one target that has been explored to increase the productivity of photosynthetic organisms. For example, because solar energy contains the full spectrum of light energy, several technologies are being developed to up- or down-convert radiation into wavelengths that can be used by photosynthetic organisms (Chen et al., 2011). Targets for improving the photosynthetic efficiency of photoautotrophs are: genetic modifications to increase the specificity of Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Parry et al., 2003), increasing the activity of RubisCO (Whitney et al., 2011; Zhu et al., 2010), increasing the light-gathering efficiency via reduced antenna size (Mussgnug et al., 2007; Tabor et al., 2011),

and improving carbon concentrating mechanisms (Bar-Even et al., 2012; Bonacci et al., 2012; Delebecque et al., 2011; Dueber et al., 2009; Erb, 2011).

Constraints specific to plants compared to photoautotrophic microorganisms are: the need for arable land, increased water footprint, and slower growth rates (Singh et al., 2011b). With these constraints, photoautotrophic microorganisms can still be considered a preferred source of next-generation biofuels and high-value chemicals compared to plants.

1.2.1 Algae

There has been much progress recently in the science of algal biofuel production. The most common approach for algal biofuels is to grow algae that produce and accumulate high levels of intracellular lipids, harvest the algal cells, extract the lipids, and convert these into biofuels such as biodiesel (Hossain et al., 2008; Patil and Deng, 2015; Sander and Murthy, 2010). Compared to plant-based lipid production, algae have much higher photosynthetic efficiency and productivity (Hossain et al., 2008). Algal biofuel production can also have potential positive impacts on the environment, such as the use of wastewater as the growth medium (Schenk et al., 2008). Wastewater contains nutrients that can be converted into microalgal biomass for use as fertilizer, feed, or biofuel. Decreasing the nutrient load of wastewater is essential prior to its discharge into the surrounding environment (Sutherland et al., 2014). A great deal of research has been directed at finding and/or developing algal species with very high lipid productivities and intracellular concentrations (Griffiths and Harrison, 2009). Some species of algae are capable of achieving 50-60% dry weight as lipids (Sheehan et al., 1998). Figure 1.2 shows an overview of steps necessary biofuel production from algae.

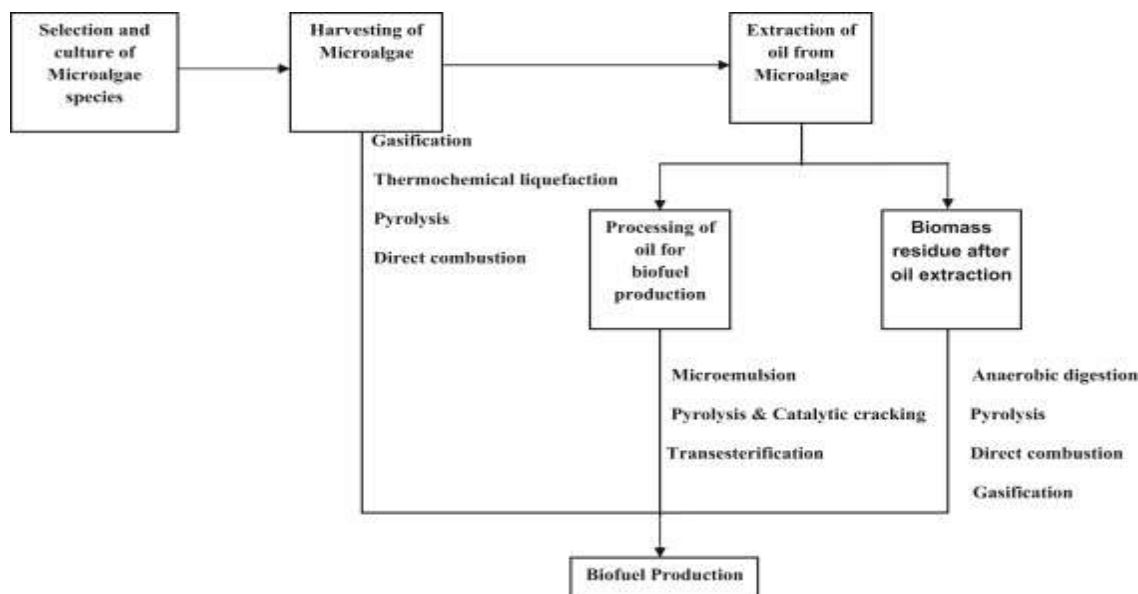


Figure 1.2: Steps involved in producing biofuels from algae.
(Pragya et al., 2013).

Another active area of research with regards to algae is determining strains that are robust for outdoor production. Strains are selected due to their robustness to fluctuating cultivation conditions and their ability to maintain dominance in the growth medium for relatively long periods in outdoor cultures (Rodolfi et al., 2009). Minimizing nutrient requirements for algal strains would also increase the potential for economic feasibility of any production process. Fortunately, nutrient deprivation enhances the lipid content in several species of algae. However, it also decreases the growth rate (Griffiths and Harrison, 2009). The selection of the optimal algal strain for specific production systems will be essential to maximize the economic potential of the system.

One criteria that is often overlooked when selecting industrial algal species is ease of harvesting. Biomass harvest is a significant capital and operating cost in any algal

process, thus it is preferred to select strains with properties that simplify cell recovery (Griffiths and Harrison, 2009). An efficient means of extracting oil from algal cells is also important from an economic perspective. Extraction methods should be fast, effective, easily scalable, and cause minimal damage to the extracted lipids (Rawat et al., 2011). Methods of algal oil extraction include: microwaving (Balasubramanian et al., 2011), autoclaving (Lee et al., 2010), bead-beating (Mercer and Armenta, 2011), and sonicating (Shen et al., 2009). Once the algal biomass is de-oiled it can serve several purposes, such as: adsorbents (Maurya et al., 2014), feed supplements (Han and McCormick, 2014), and feedstock for biohydrogen production (Subhash and Mohan, 2014).

Algal production of biodiesel is technically feasible; however, it is not yet economically feasible (Chisti, 2008a). Algal biofuels have not made a significant impact on the global energy supply because algal productivity is not high enough to offset operating and capital costs (Borowitzka, 1992). Further research to improve lipid productivity and reduce costs associated with oil separation and harvesting is necessary for algal biodiesel to become a meaningful part of the transportation fuel market.

1.2.2 Cyanobacteria

Cyanobacteria are photosynthetic prokaryotes present in diverse habitats, ranging from the tropics to polar regions (Hasunuma et al., 2013; Katoh, 2012; Moreno et al., 1998). Cyanobacteria have existed on Earth for at least 2.7 billion years (Badger and Price, 2003). They have morphologies ranging from unicellular to filamentous, and utilize an identical photosynthesis process as higher plants (Lindblad et al., 2012). Although the process is identical, cyanobacteria are capable of significantly higher

photosynthetic and growth rates than higher plants (Hasunuma et al., 2013) as they are inherently more efficient solar collectors (Dismukes et al., 2008). The maximum rates of photosynthesis have considerable variation with respect to the species of cyanobacteria (Price et al., 1998). Due to their natural metabolic diversity, cyanobacteria are prime candidates for industrial applications (Ruffing, 2011).

1.2.2.1 Morphology and Function

Cyanobacteria are unique in that certain strains are capable of differentiating their vegetative cells into akinetes, heterocysts, and hormogonia (Ireland, 2012). Akinetes are dormant, spore-like cells commonly found in filamentous strains of cyanobacteria. The ability to develop akinetes is a survival trait as these cells can survive harsh conditions and germinate as conditions improve (Sukenic et al., 2012). Heterocysts are capable of fixing atmospheric nitrogen, and these cells are uniquely structured to provide the anaerobic environment necessary for activity of nitrogenase (Buikema and Haselkorn, 1991). This requirement for anaerobic conditions precludes heterocysts from conducting oxygen generating photosynthesis. Hormogonia are shorter filaments involved in cell dispersal and symbiotic associations with plants and fungi (Schuergers and Wilde, 2015). As these cell types serve purposes other than conducting photosynthesis to create metabolites, their presence in biofuel production processes will affect the overall productivity of the system. It would be desirable to minimize the presence of akinetes and hormogonia, however heterocysts may be desired to reduce or eliminate the need for supplementation with fixed nitrogen.

1.2.2.2 Growth Dynamics

Specific growth rates of cyanobacterial strains vary depending on factors such as temperature, pH, and illumination. For example, two members of *Oscillatoria* spp. have been found in Antarctic meltwater ponds which show optimal growth at 8°C, with rates of 0.08-0.12 doublings per day (Nadeau and Castenholz, 2000). Members of the genus *Synechococcus* have been isolated from North American hot springs with an upper temperature limit of 72°C (growth rate not reported) (Papke et al., 2003). Generally, cyanobacteria grow in an alkali environment (pH 9-10). This pH range is typically observed at the end of the exponential phase (Castenholz, 1988). Populations of filamentous cyanobacteria have been isolated from an environment with a pH of 2.9 (Steinberg et al., 1998).

Synechococcus elongatus PCC 7942, *Arthrospira platensis*, and *Synechocystis* sp. PCC 6803 are commonly used industrial strains. Typically, they are cultivated at 30°C with light intensities ranging from 50-150 $\mu\text{mol m}^{-2} \text{s}^{-1}$. *Synechococcus* and *Synechocystis* spp. are typically grown in BG11 medium (pH 7.1) while *A. platensis* is typically grown in Zarrouck medium (pH 9.0) (Lan et al., 2015; Markou et al., 2013; Varman et al., 2013). Their growth rate under optimum conditions is 0.24-0.27 d^{-1} (Bernstein et al., 2014; Leema et al., 2010; Touloupakis et al., 2015).

Many cyanobacterial species are capable of growth in a wide range of conditions, which is one reason they are found in virtually any climate. For example, the optimal temperature for the maximal growth rate of *Anabaena* sp. PCC 7122 is 25.0°C and is 32.5°C for *Aphanizomenon gracile*. However, both species were capable of growth in

temperatures ranging from 20.0-35.0°C. Of several cyanobacterial strains studied, the optimal temperature for the slowest growing strain was 27.5°C (*Cylindrospermopsis raciborskii* CIRF-01). The mean growth rate at this temperature was 0.81 d⁻¹. The growth rate decreased to 0.4 d⁻¹ at 20.0°C. The optimal temperature for the fastest growing strain was 32.5°C (*Microcystis aeruginosa* PCC7941). The mean growth rate at this temperature was 1.16 d⁻¹. Again, both species were capable of growth in temperatures ranging from 20.0-35.0°C (Lurling et al., 2013).

Illumination (wavelength and intensity) also influences specific growth rate and photosynthetic pigment formation (Mohsenpour et al., 2012). Species of cyanobacteria have specific optimal wavelengths based on the composition of light-harvesting pigments they contain. In fact, certain phytoplankton groups are characterized by their pigment composition (Bracher et al., 2009). Compared to plants, both cyanobacteria and algae have higher growth rates (Singh et al., 2011b). Cyanobacteria have lower maximum growth rates than green algae, however at very low light intensities their growth rate is higher (Mur et al., 1999). As mentioned earlier the rate of light intensities commonly used to cultivate cyanobacterial strains with industrial potential is 50-150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Lan et al., 2015; Markou et al., 2013; Varman et al., 2013).

Individual cyanobacterial species have optimal levels for the parameters described. Thus, it is essential for these conditions to be optimized for specific cyanobacterial strains in order for them to be utilized in any industrial process. Some species also have broader ranges of these parameters that they can grow in. These species

are industrially favorable as they can tolerate changing conditions better than species that can only grow in narrow ranges of these parameters.

1.2.2.3 Physiological requirements

The physiology of cyanobacteria is complex for several reasons. While they are photosynthetic organisms, they are also capable of respiration (Schmetterer, 1994).

Diazotrophic, heterocystous cyanobacteria are capable of cellular communication along the filaments as well as transferring fixed nitrogen to adjacent vegetative cells (Omairi-Nasser et al., 2015). There are also various trace metals requirements that play key roles in the photosynthetic electron transport chain in cyanobacteria (Raven et al., 1999). A thorough understanding of the physiological requirements of individual cyanobacterial strains will be necessary in order to utilize them as industrial microbes

1.2.2.3.1 Light

Cyanobacteria utilize light to fuel electron transport and CO₂ fixation, thus it serves to modulate cellular processes (Grossman et al., 2001). Light is harvested via the antennae complex phycobilisomes, which are attached to the surface of the photosynthetic membranes (Grossman et al., 1993). Phycobilisomes are structurally diverse and are optimized to allow maximal absorption of light energy available in the surrounding environment (Blankenship, 2013). The four classes of phycobiliproteins in the phycobilisome are: phycoerythrin (λ_{\max} : 540-570 nm), phycoerythrocyanin (λ_{\max} : 560-600 nm), phycocyanin (λ_{\max} : 610-620), and allophycocyanin (λ_{\max} : 650-655 nm) (Kannaujiya and Sinha, 2016; Marx and Adir, 2014; Rastogi et al., 2015).

Cyanobacteria generally use a combination of membrane-intrinsic and membrane-extrinsic antennas that allows for light harvesting over a wide spectral range (Collins et al., 2012). Cyanobacteria can also alter their photosynthetic pigments based on the wavelength of the light available (Mohsenpour et al., 2012). Not all species of cyanobacteria contain identical light-harvesting pigments. For example, *Spirulina platensis* contains an additional chlorophyll α in Photosystem I, but its phycobilisome lacks phycoerythrin (Akimoto et al., 2013). Ideally, artificial lighting for cyanobacterial cultivation systems would be able to emit light at specific wavelengths that can be utilized by specific strains. However, the only light source capable of emitting light in specific wavelengths which have been used for the illumination of microalgal cultures are light emitting diodes (LEDs) (Wang et al., 2007).

Cyanobacteria are capable of regulating depth within a water column via physiological changes in response to changing environmental conditions. This behavior is termed buoyancy regulation (Reynolds, 1984). This allows cyanobacteria to overcome the vertical separation between light and nutrients that can occur in a body of water (Ganf and Oliver, 1982). This buoyancy also allows cyanobacteria to locate at depths at which their optimal wavelengths occur (Wallace et al., 2000), along with irradiance of the appropriate intensity.

Light is an important stimulus for photosynthetic microbes, where light intensity, quality, and duration are used to control a variety of cellular processes (Agostoni et al., 2013). The optimal intensity of light is variable among different species of cyanobacteria, thus when conducting studies on a specific species, the optimal light intensity should be provided (Richmond, 2000). Light is sensed by photosensory proteins that are activated

by bound organic cofactors or chromophores, transmitting photobiological signals to a downstream output domain (Gomelsky, 2011; Montgomery, 2007; van der Horst and Hellingwerf, 2004). The daily peak in irradiance, which usually inhibits cyanobacterial productivity at the surface (Sournia, 1975), triggers a photoprotective response through an enzymatic-controlled conversion of pigments termed the xanthophyll cycle (Lavaud et al., 2004; Lavaud et al., 2007). Pigments involved in the xanthophyll cycle can be used as markers of the recent light history of photosynthetic microbes (Brunet et al., 2008).

The Photosystem II light harvesting system can undergo structural changes in order to regulate the partitioning of energy between utilization in photosynthesis and dissipation by non-photochemical quenching (Horton et al., 1991; Horton et al., 1996). Non-photochemical quenching of chlorophyll fluorescence is an important short-term reversible photoprotective process in oxygenic photosynthetic organisms (Horton et al., 1996; Müller et al., 2001; Ruban et al., 2012). It works by relieving the excitation pressure in the photosynthetic membrane (Horton and Ruban, 1992; Jahns and Holzwarth, 2012; Ruban et al., 2012). Excess light decreases the pH in the thylakoid lumen, which causes an increase in aggregation of Photosystem II. This results in an efficient pathway for non-radiative dissipation of excitation energy (Horton et al., 1991).

1.2.2.3.2 Photoinhibition

Photoinhibition occurs due to absorption of excess photons which wastes energy via non-photochemical quenching and reduced photosynthesis (Ruffing, 2011). Photoinhibition is caused by photodamage of Photosystem II from irradiation via photosynthetically active and UV radiation (Raven, 2011). Cyanobacteria become

photoinhibited at light intensities only slightly greater than the level required for the maximum specific growth rate to be achieved (Chisti, 2007). Most cyanobacteria exhibit an increase in growth kinetics when light saturation occurs at modest light intensities. Microbial growth kinetics is the relationship between the specific growth rate of a microbial population and the substrate concentration (Kovářová-Kovar and Egli, 1998). Direct light causes a decrease in growth kinetics as well as photoinhibition or photobleaching (Schenk et al., 2008). Photobleaching occurs when a fluorophore (i.e. chlorophyll) is irreversibly, photochemically altered so that it no longer fluoresces (Chen et al., 2003)

A high concentration of dissolved O₂ in combination with intense sunlight can produce photooxidative damage to the cells and have adverse effects on photosynthesis (Chisti, 2007; Fouchard et al., 2008). One source of photooxidative damage is reactive oxygen species which occur after UV-B exposure (He and Häder, 2002; Zeeshan and Prasad, 2009). Photoinhibition will be a major hurdle in scaling-up processes involving cyanobacteria. One approach to prevent photoinhibition in photobioreactors (PBRs) is light dilution (Wilhelm and Jakob, 2011). This works by vertically installing the PBR elements so that light is homogeneously, vertically distributed in the reactors. This is advantageous as it could be constructed in a way that the light dilution is dynamically adjustable according to the ratio of incident irradiance and the actual light demand (Morweiser et al., 2010).

1.2.2.3.3 Temperature

As with light, cyanobacteria have varying optimal temperatures for growth depending on the species. The majority of species have an optimal growth range between 25°C and 40°C (Robarts and Zohary, 1987). However, some cyanobacteria species prefer thermal extremes, such as *Oscillatoria* spp. Members of this genus include psychrophiles that have a growth optima of $\leq 15^{\circ}\text{C}$ (Nadeau and Castenholz, 2000). Psychrotolerant cyanobacteria grow at $\leq 20^{\circ}\text{C}$ and include a member of the genus *Phormidium*, which was isolated at a temperature of 5°C (Tang et al., 1997). Thermotolerant cyanobacteria include *Halomicronema excentricum*, which is capable of growth at 50°C (Abed et al., 2002), and *Chlorogleopsis* sp. SC2, a thermophile that grows very well at 50°C (Ono and Cuello, 2007). As mentioned previously, members of the genus *Synechococcus* have been isolated from North American hot springs with an upper temperature limit of 72°C (Papke et al., 2003).

Microorganisms capable of growth in low temperature environments have adapted several mechanisms to resist the effects of cold which include: loss of membrane fluidity, decrease in enzymatic activity, and a decline in protein stability. This is achieved in several ways including: production of antifreeze proteins, mechanisms to increase membrane fluidity (greater content of saturated lipids), and production of cold-adapted enzymes which have high specific activities at low temperatures (D'Amico et al., 2006). Psychrophilic cyanobacteria must possess regulatory mechanisms to maintain a strict balance between energy absorbed and utilized (Morgan-Kiss et al., 2008). The thermophilic unicellular cyanobacterium, *Thermosynechococcus elongatus* BP-1 has

more genes for heat-shock proteins and fewer genes for typical fatty acid desaturases compared to mesophilic cyanobacteria. They would also tend to have more unsaturated lipids in the membranes and enzymes with optimal activities at high temperatures. These may be genetic features of thermophilic strains of cyanobacteria (Nakamura et al., 2002).

1.2.2.3.4 pH

A pH value between 6 and 9 favors cyanobacterial bloom formations (Wicks and Thiel, 1990). A quantitative measurement that reflects the number of cyanobacterial species in a dataset was highest in soil samples at pH 9.3, while the highest percentage of abundance of heterocyst-forming cyanobacteria was observed at pH 8.1 (Prasanna, 2007). Most cyanobacteria can survive at pH 9-10, which parallels conditions at the end of the exponential growth phase (Castenholz, 1988). This physiological trait could be a valuable tool to hinder contaminant growth in large-scale photobioreactor systems. The ability to survive alkali environments is evolutionarily beneficial as it favors cyanobacterial growth in bloom formations. Bloom formation is generally accompanied with an elevated pH due to increased photosynthesis which depletes CO₂ in the liquid (Summerfield and Sherman, 2008).

An example of an alkaliphilic cyanobacterium is *Arthrospira platensis*. This microbe does not maintain an outward positive pH gradient at its plasma membrane, which is typical for alkaliphiles. Thus, sodium extrusion occurs via an ATP-dependent primary sodium pump, rather than the Na⁺/H⁺ antiport that most cyanobacteria utilize (Berry et al., 2003). The internal pH of alkaliphiles must be lower than the pH of the surrounding environment. Thus, a key feature of alkaliphily is associated with the cell

surface, which differentiates and maintains the intracellular neutral environment separate from the extracellular alkaline environment (Horikoshi, 1999).

Very few publications mention the occurrence of cyanobacteria in acidic environments, however, Steinberg et al., (1998) reported two populations of filamentous cyanobacteria growing in a Bavarian Forest in a lake with a pH of 2.9. The maintenance of an intercellular pH of ~7.0 appears to be necessary for acidophilic microbes to survive acidic environments. The lack of control over internal pH values may be responsible for cyanobacterial growth limitations in acidic environments (Kallas and Castenholz, 1982).

1.2.2.4 Nutrient requirements

One of the most promising aspects of utilizing cyanobacteria for 3rd and 4th generation biofuels is their minimal nutritional requirements. All cyanobacteria are capable of fixing atmospheric CO₂ and some species are capable of fixing atmospheric nitrogen. Thus the chemicals needed for growth are minimal, and primarily include trace elements such as cobalt, molybdenum, and manganese which are utilized as cofactors. Due to their photoautotrophic classification, cyanobacteria have metal requirements that are often absent from other bacteria. Examples of these requirements are: copper in thylakoidal plastocyanins, zinc in carboxysomal carbonic anhydrases, and cobalt in cobalmins. This causes cyanobacteria to have homeostatic systems that differ from other bacteria in which they are able to acquire essential metalloproteins (Cavet et al., 2003).

In addition to carbon, two other nutrients (nitrogen and phosphorus) are especially in high demand by cyanobacteria, possibly accounting for a several-fold higher requirement in comparison to higher plants (Parmar et al., 2011). The cost to supply

nitrogen and phosphorus may be a hurdle for the commercial use of cyanobacteria. For that reason, nitrogen fixing species of cyanobacteria may have more potential as an industrial microbe than non-nitrogen fixing species. The other nutrient demands of cyanobacteria are dependent on whether the organism is a freshwater or marine species.

1.2.2.4.1 Carbon

Cyanobacterial biomass is approximately 50% w/w carbon (Kumar and Das, 2012), thus this represents the largest nutrient requirement. Carbon requirements for cyanobacteria can be met in several ways. As photoautotrophs, cyanobacteria can fix CO₂ via photosynthesis, using CO₂ from the atmosphere, flue gas from coal power plants, and/or fermentor exhaust from ethanol plants (Kumar et al., 2011). CO₂ is commonly supplied to cyanobacterial cultures via sparging (Kajiwara et al., 1997). Two strategies commonly used to supply CO₂ via sparging are continuous bubbling and on-demand injection (González-López et al., 2012). CO₂ can also be provided in the form of NaHCO₃ (Martins et al., 2014; Ungsethaphand et al., 2009; Yilmaz and Sezgin, 2014). This strategy could be useful in situations when cyanobacteria need to be cultivated in a sealed environment, as would be the case when testing tolerances with volatile chemicals that would otherwise be vaporized by CO₂ sparging. This method has previously been utilized with algae (Mayer et al., 2000).

Although cyanobacteria are classified as photoautotrophs, many species can also be categorized as mixotrophic as they have the capability to utilize fixed carbon. Mixotrophy is a growth regime in which CO₂ and fixed carbon are assimilated simultaneously (Subashchandrabose et al., 2013). An example of a mixotrophic

cyanobacterium is *Cyanothece* sp. ATCC 51142. This microbe is of interest to industrial microbiologists as it can produce hydrogen under mixotrophic conditions with glycerol as a substrate and under nitrogen fixing conditions (Min and Sherman, 2010). *Anabaena variabilis* ATCC 29413 can grow in dark conditions in the presence of 5 mM fructose, which indicates that photosynthetic electron transfer is not an obligate growth requirement (Mannan et al., 1991). Other sources of fixed carbon that can be utilized by various strains of cyanobacteria are: glucose (Chojnacka and Noworyta, 2004), acetate (Wan et al., 2015), and glycogen (Gaudana et al., 2013). Some cyanobacteria are both photosynthetic and heterotrophic. Heterotrophic energy generation in cyanobacteria is due to the oxidative pentose phosphate pathway, the glycolytic pathway and tricarboxylic acid cycle (Subashchandrabose et al., 2013).

Photosynthetic carbon assimilation in cyanobacteria results in the accumulation of polysaccharides, mostly in the form of glycogen (Suzuki et al., 2010). Glycogen provides maintenance energy for cell integrity, viability, and function in dark conditions (Gründel et al., 2012). Glycogen accumulation in cyanobacteria is physiologically important for coping with changes in the environment and transient starvation conditions (Gründel et al., 2012; Nakamura et al., 2005). The synthesis of glycogen in cyanobacteria begins with the use of ADP-glucose, which is the glycosyl donor (Ball and Morell, 2003). The synthesis of ADP-glucose from glucose-1-phosphate and ATP, catalyzed by AGPase is the initial committed step of glycogen synthesis and the major rate-controlling step of glycogen synthesis in cyanobacteria (Gründel et al., 2012).

When *Cyanothece* sp. ATCC 51142 is grown in mixotrophic conditions with glycerol, the microbe preferentially consumes glycerol and limits CO₂ uptake. Genes

involved in CO₂ fixation are more highly expressed under photoautotrophic conditions, compared to mixotrophic conditions (Krishnakumar et al., 2015). The ability of many cyanobacterial strains to utilize a wide variety of carbon sources increases their industrial potential. This ability allows for continuous metabolism regardless of light availability, as well as increased robustness for tolerating fluxes in carbon source availability during production.

1.2.2.4.2 Nitrogen

Synechocystis and *Arthrospira* contain 8-12% total nitrogen in their biomass. Protein nitrogen represents $72.1 \pm 1\%$ of the total nitrogen (López et al., 2010). *Anacystis nidulans* ATCC 27144 biomass contains 60-70% protein on a dry basis (Mishra and Kumar, 2007). Approximately 68% of the natural products produced from cyanobacteria contain nitrogen (Singh et al., 1996). Cyanobacteria are capable of growth on various nitrogen sources: nitrite, nitrate, urea, ammonium, several amino acids, atmospheric nitrogen, and nucleosides (Kolodny et al., 2006). While nitrite is inhibitory to many microorganisms, it is widely used as a nitrogen source by many species of cyanobacteria (Frías and Flores, 2015), including *Anabaena* sp. PCC 7120 (Wang and Liu, 2013). *Synechocystis* sp. PCC 6803 is an example of a cyanobacterial species unable to use nitrite. Combined nitrogen sources are taken up through various permeases and reduced into ammonium, which is incorporated into carbon skeletons via the glutamine synthetase–glutamate synthase pathway (Moreno-Vivián and Flores, 2006). Then, nitrogen is distributed from glutamine or glutamate to the other nitrogen-containing organic compounds (Flores and Herrero, 2005).

2.2.4.3 Phosphorus

Arthrospira platensis has an intracellular phosphorous content of 1.85-6.0 mg P g⁻¹ dry biomass depending on the carbohydrate content (Markou et al., 2013). Two *Synechococcus* species have phosphorous contents ranging from 1.8 ± 0.1 – 3.3 ± 0.5 fg/cell (Bertilsson et al., 2003). The majority of cyanobacteria can mineralize organic phosphorus by producing alkaline phosphatases (Fay, 1992; Singh et al., 2006). Phosphorous of cyanobacteria and algae are composed of internal phosphorous pools of polyphosphate (John and Flynn, 2000). Cyanobacteria store large amounts of inorganic phosphate in the form of polyphosphate granules (Shi et al., 2003). There are diverse sets of genes present in different cyanobacterial species that allow for the utilization of various phosphorous sources present in their environments. Thus, the phosphorous assimilation mechanism in cyanobacteria is complex and differs depending on the species according to the availability of different phosphorous sources in the environment (Tiwari et al., 2015).

1.2.2.4.4 Micro-nutrients for freshwater species

Castenholz (1988), listed micro-nutrients commonly required for freshwater cyanobacteria, and these are shown in Table 1.3 compared to BG11, another standard medium used for freshwater cyanobacteria (Allen and Stanier, 1968). While there are some similarities among the freshwater cultivation media, the differences are numerous in both nutrient type and concentration. Thus it is important to survey the literature to determine the standard cultivation medium used for individual cyanobacterial species. It could also be of value to compare the growth of cyanobacterial species in different media

types to determine if there are any differences in growth parameters for specific species of cyanobacteria.

Table 1.3: Freshwater cyanobacteria cultivation media.
(Castenholz, 1988).

Ingredient	Concentration (mg/L)					
	Chu No. 10	Gerloff	BG11 ^a	D Medium ^b	Allen and Arnon	Kratz and Myers
Disodium EDTA	-	-	1	-	4	-
Nitrilotriacetic acid	-	-	-	100	-	-
Citric acid	3	3	6	-	-	165 ^c
NaNO ₃	-	41	1500	700	-	-
KNO ₃	-	-	-	100	2020	1000
Ca(NO ₃) ₂ · 4H ₂ O	40-60	-	-	-	-	25
K ₂ HPO ₄ · 3 H ₂ O	13	-	40	-	456	1000
Na ₂ HPO ₄	-	8	-	110	-	-
MgSO ₄ · 7 H ₂ O	25	15	75	100	246	250
CaSO ₄ · 2 H ₂ O	-	-	-	60	-	-
MgCl ₂ · 6 H ₂ O	-	21	-	-	-	-
CaCl ₂ · 2 H ₂ O	-	36	36	-	74	-
KCl	-	9	-	-	-	-
NaCl	-	-	-	8	232	-
Na ₂ CO ₃ · H ₂ O	20	20	20	-	-	20
Ferric Ammonium Citrate	-	-	6	-	-	-

Table 1.3 Continued: Freshwater cyanobacteria cultivation media.

(Castenholz, 1988).

Ferric Citrate	3	3	-	-	-	-
FeCl ₃	-	-	-	0.3 ^d	-	-
Fe ₂ (SO ₄) ₃ · 6 H ₂ O	-	-	-	-	-	4
Micronutrients	-	-	1 ml ^e	0.5 ml ^f	-	1 ml ^e

^a: pH 7.4 after cooling.^b: Prepared as a 20-fold concentrated stock, stored at 4°C. Micronutrients and FeCl₃ included in stock. pH adjusted to 8.2 with NaOH before autoclaving.^c: Trisodium citrate dehydrate.^d: A stock solution of 0.29 g/L is stored at 4°C.^e: A₅ + Co micronutrient mix (Table 1.4).^f: D micro micronutrient mix (Table 1.4).

Table 1.4: Composition of micronutrient solutions used in freshwater cyanobacteria cultivation media.

(Castenholz, 1988).

Concentration (g/L)		
Ingredient	As + Co	D Micro
H_2SO_4 (conc)	-	0.5 ml
H_3BO_3	2.86	0.5
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	-	2.28
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1.81	-
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.22	0.5
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.08	0.025
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.39	0.025
$\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$	0.049	-
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	-	0.045
$\text{NiSO}_4(\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$	-	0.019
Na_2SeO_4	-	0.004

Presumably, cultivation media such as BG11 have sufficient macronutrients and trace metals for optimal cyanobacteria growth. From an industrial standpoint it would be useful to determine if any of these components could be reduced or eliminated without limiting biomass or byproduct yield. A study conducted by Havel et al., (2006) concluded

that by adjusting components of BG11, the quality of a *Synechococcus* biocatalyst could be increased fivefold while also increasing the biocatalyst yield. Genetic algorithms were utilized to determine how the cyanobacterium responded to increased or decreased levels of specific chemicals. Further research is needed to determine if the components and cost of BG11 could also be reduced for other species of cyanobacteria. BG11₀ is another option for the cultivation of freshwater cyanobacteria. BG11₀ lacks sodium nitrate and is commonly used for culturing nitrogen-fixing cyanobacteria (Castenholz, 1988).

1.2.2.4.5 Micro-nutrients for salt water species

Many species of cyanobacteria grow in seawater, thus there are several varieties of seawater media that have been developed to culture these organisms. It is convenient to work with natural seawater (NW) with salinity generally in the range of 30-35‰ to culture marine cyanobacteria (Castenholz, 1988). However, this creates many variables that are difficult to control, thus several types of media have been developed for specific species of marine cyanobacteria (Berges et al., 2001). Adding 20-30 g/L NaCl to BG11 to emulate seawater will accommodate many marine cyanobacteria (Castenholz, 1988). Compositions of natural and artificial seawater media are provided in Table 1.5.

Table 1.5: Growth media for marine and halophilic cyanobacteria.
(Waterbury and Stanier, 1981).

Ingredient	Concentration (g/L)		
	Modified Chu-11	Yopp	Modified Castenholz D
Deionized H ₂ O (ml)	-	1000	1000
Seawater (ml)	1000	-	-
NaCl	-	116.88	160.0
MgSO ₄ · 7H ₂ O	0.075	10.0	0.1
MgCl ₂ · 6H ₂ O	-	10.68	-
KCl	-	2.0	-
CaCl ₂ · 2H ₂ O	0.036	-	-
CaSO ₄ · 2H ₂ O	-	-	0.06
CaNO ₃ · 4H ₂ O	-	1.0	-
NaNO ₃	1.5	-	0.689
KNO ₃	-	-	0.103
K ₂ HPO ₄ · 3H ₂ O	0.04	0.065	-
Na ₂ HPO ₄	-	-	0.111
Na ₂ SiO ₃ · 9H ₂ O	0.058	-	-
Na ₂ CO ₃	0.02	-	-
Disodium EDTA	0.001	-	-

Table 1.5 Continued: Growth media for marine and halophilic cyanobacteria.
(Waterbury and Stanier, 1981).

Nitrilotriacetic acid	-	-	0.1
Citric acid	0.006	-	-
Ferric ammonium citrate	0.006	-	-
FeCl ₃	-	-	0.0003
Ferric EDTA	-	0.005	-
Trace metals (ml)	1.0 ^a	1.0 ^b	0.5 ^c
Glycylglycine buffer	-	0.5	-
pH after autoclaving	7.5	7.8	7.5

^a: A-5 trace metals.

^b: Sheridan and Castenholz trace metals.

^c: Castenholz D trace metals.

Table 1.6: Trace metal mixes for marine and halophilic cyanobacteria.
(Waterbury and Stanier, 1981).

Ingredient	Concentration (g/L)		
	A-5	Sheridan and Castenholz	Castenholz D
Deionized H ₂ O (ml)	1000	1000	1000
H ₂ SO ₄ (ml)	-	-	0.5
HCl (ml)	-	3.0	-
H ₃ BO ₃	2.86	0.5	0.5
Mn ₂ Cl ₂ · 4H ₂ O	1.81	2.0	-
MnSO ₄ · H ₂ O	-	-	2.28
ZnSO ₄ · 7H ₂ O	0.222	-	0.5
ZnNO ₃ · 6H ₂ O	-	0.5	-
Na ₂ MoO ₄ · 2H ₂ O	0.039	0.025	0.025
CuSO ₄ · 5H ₂ O	0.079	-	0.025
CuCl ₂ · 2H ₂ O	-	0.025	-
Co(NO ₃) ₂ · 6H ₂ O	0.0494	0.025	0.045
VO ₂ SO ₄ · 6H ₂ O	-	0.025	-

1.2.2.4.6 Trace metals

Trace metals play a key role in photosynthetic electron transport that occurs in the thylakoids of cyanobacteria (Merchant and Dreyfuss, 1998). An example of the importance of trace metals to photoautotrophic microorganisms is the biogeochemical cycle of trace metals created in the oceans by these microbes. The uptake of metals by microalgae and cyanobacteria results in extremely low concentrations in the surface seawater, and this limits the rate of photosynthesis. A steady downward flux of the nutrients occurs when the dead biomass or feces of zooplankton settle. This flux is balanced by a slow upward diffusive flux of dissolved elements re-mineralized by heterotrophic bacteria. These fluxes create the cycle of trace metals in the oceans (Morel and Price, 2003).

Quantitatively, iron is the most important trace metal involved in these reactions (Raven et al., 1999). Thus cyanobacteria have adapted a method that allows them to survive with decreased iron availability in their environment via alterations in their cellular iron requirements and by increasing their ability to acquire iron from the environment (Wilhelm, 1995). Certain trace metals are also necessary for nitrogen fixation. Cyanobacterial nitrogenases typically contain molybdenum (Mo) and iron in the active site when there is a sufficient amount of Mo in the environment. Under Mo-deprived conditions, the Mo-nitrogenase is replaced by vanadium-nitrogenase or iron-nitrogenase (Angermayr et al., 2009). Boron is also necessary for nitrogen-fixation in cyanobacteria. Boron-deficiency inhibits cyanobacterial growth and alters heterocyst morphology (Bonilla et al., 1990). Trace metals are an essential part of the physiology of

cyanobacteria and their presence in cultivation media must be considered if any process is to become industrially feasible.

1.2.2.5 Metabolism

Cyanobacterial metabolism is a complex process that varies depending on the physiological and nutritional conditions present. If nutrients are plentiful and physiological conditions acceptable, cyanobacteria have means of storing nutrients, such as glycogen, for future use. However, if nutrients are limited, cyanobacteria have adapted means to generate or substitute for required metabolites. For example, as previously mentioned nitrogenases typically contain molybdenum, however when none is available the molybdenum-nitrogenase is replaced by vanadium-nitrogenase or iron-nitrogenase (Angermayr et al., 2009). If nutrients or physiological conditions are not acceptable, cyanobacteria have the capacity to survive these circumstances for extended periods. One way that cyanobacteria can survive these periods of unfavorable conditions is by forming spore-like cells termed akinetes (Perez et al., 2015).

Cyanobacteria are capable of metabolizing up to half of their total soluble protein (Johnson et al., 2014). *Arthrospira platensis* is capable of degrading the light harvesting apparatus, phycobilisome, to provide nitrogen when exogenous nitrogen is limited (Hasunuma et al., 2013). As mentioned previously, some cyanobacterial strains have the ability to fix atmospheric nitrogen. While they are capable of fixing N_2 , it is not energetically favorable (Stal, 2003). Thus, only in conditions of deprived combined nitrogen will heterocyst formation occur (Zhang et al., 2006). These examples highlight

how cyanobacteria have the ability to survive periods of nutrient limitation, which is one reason they can be found in such diverse habitats.

Cyanobacterial metabolism is important from an industrial standpoint as cyanobacteria could potentially serve as a platform to produce a diversity of natural products, such as terpenoids which contain over 20,000 described compounds (Ghimire et al., 2008). Several types of terpenoids have the potential to be developed as biofuels (Bentley and Melis, 2012). However not all terpenoids are beneficial. For examples, geosmin and 2-methylisoborneol can have adverse effects on conventional water treatment methods as they have a musty smell and are difficult to remove (Agger et al., 2008). Research into terpenoid products is still relatively young, thus the characterization of new sesquiterpene synthases may lead to novel bioactive compounds (Agger et al., 2008).

A great deal of interest is currently being focused on utilizing cyanobacteria to produce metabolites that are valuable in human nutrition such as: vitamins, minerals, and food additives including protein (Lau et al., 2015; Mishra et al., 2008; Singh et al., 2005). There is also the potential for cyanobacteria to be utilized as a source of omega-3 fatty acids, which are long-chain polyunsaturated fatty acids that have therapeutic uses in humans (Gong et al., 2014). Finally, different species of cyanobacteria produce metabolites with biological activities that are beneficial for humans (i.e. anti-tumor, anti-HIV, and anti-inflammatory characteristics). Examples of these include: phycocyanin, cyanoviridin-N, and (+)-8-hydroxymanzamine (Singh et al., 2005).

Since cyanobacterial metabolism is diverse and complex, there is a need to conduct metabolic profiling of strains to assess the metabolites they produce under various conditions and at different stages of their growth cycle. Mass spectrometry (MS) can be used to quickly identify a large number of metabolites to provide a snapshot of the metabolic profile under defined conditions in a process known as metabolomics (Hasunuma et al., 2013). Metabolomics is a widely applied research tool to understand metabolic system behavior in industrially important organisms (Chellapandi and Dhivya, 2010; Schenk et al., 2008). This comprehensive analysis of a wide range of metabolites makes it possible to identify metabolic compounds that play important roles in biological processes (Hasunuma et al., 2013), therefore the functional status of the organism can be related to a phenotype by the metabolomics measurements report (Chellapandi and Dhivya, 2010).

Another technique for quantifying metabolites is Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The major advantage MALD-TOF MS has over conventional MS is speed of analysis (Griffin and Smith, 2000; Lay, 2001). In cyanobacteria, MALDI-TOF MS has been utilized to identify and measure secondary metabolites including: microcystins, micropeptin, anabaenopeptolin, and jamaicamide B (Erhard et al., 1997; Esquenazi et al., 2008). The high-throughput capability of MALDI-TOF MS makes it a preferred method for quantifying cyanobacterial metabolites compared to conventional MS.

1.2.2.5.1 Photosynthesis (light reactions)

In nature, cyanobacteria benefit from being near the air-water surface due to the resupply of CO₂ diffusing from the air (Masojídek et al., 2001). Carbon dioxide fixation is the rate-limiting step in photosynthesis (Wilhelm and Jakob, 2011). Cyanobacteria use the Calvin-Benson cycle for CO₂ fixation (Fig. 1.3). The key enzyme in this cycle is Ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Zarzycki et al., 2012). Photosynthetic rates are generally limited by the amount and activity of RubisCO (Bernacchi et al., 2001), therefore RubisCO is the most abundant protein in photosynthetic organisms and on Earth (Feller et al., 2008; Spreitzer and Salvucci, 2002).

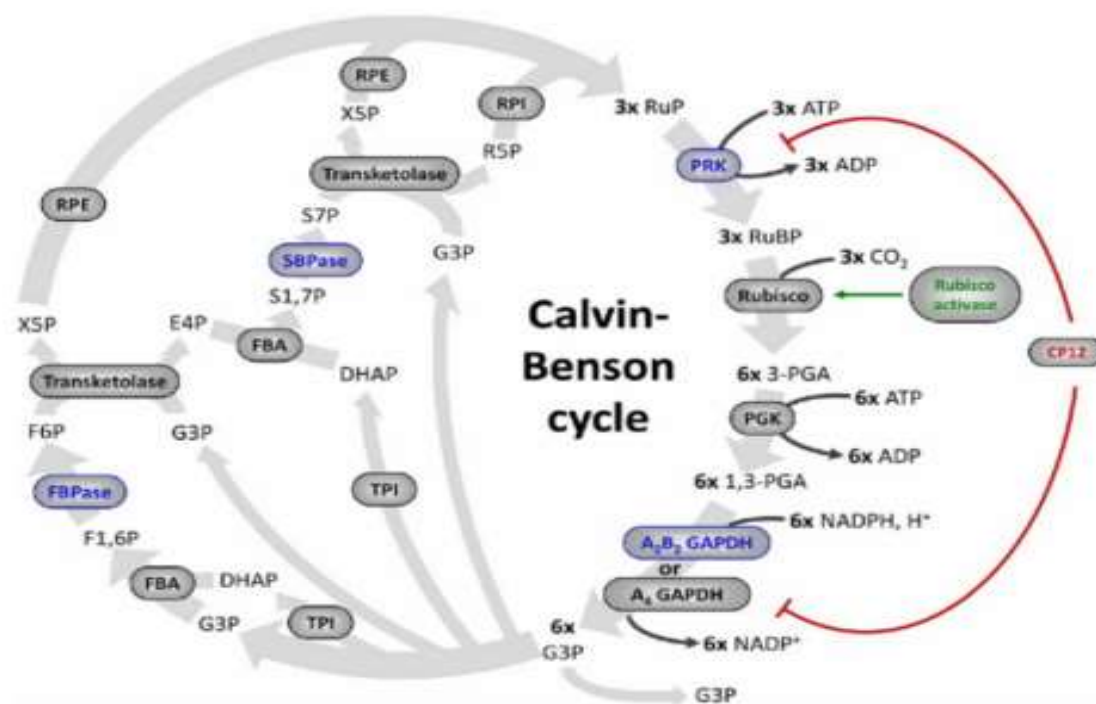


Figure 1.3: The Calvin-Benson cycle.
(Michelet et al., 2013).

As mentioned earlier, carbon content in cyanobacterial biomass is 50% w/w (Kumar and Das, 2012), which corresponds to a capture rate of 1.83 g of CO₂ g⁻¹ dry cell mass produced (Johnson et al., 2014). *Anabaena variabilis* can fix CO₂ at rates up to 96.2-109.0 g CO₂ g⁻¹ dry cell mass h⁻¹ (Markov et al., 1995). In a study by De Morais and Costa (2007), it was determined the maximum daily carbon dioxide biofixation rate for *Spirulina* sp. and *Scenedesmus obliquus* was 53.29% of the daily total carbon fixation per gram of CO₂ added to the medium for 6% v/v CO₂ and 28.08% of the daily total carbon fixation per gram of CO₂ added to the medium for 6% v/v CO₂ respectively. CO₂ influences cyanobacterial metabolism in ways unrelated to being a substrate for photosynthesis (Bowes, 1993). For example, the addition of 1% CO₂ in *Spirulina platensis* cultures did not change the maximal growth rate and decreased the maximum biomass yield. Presumably, this was due to decreased levels of photosynthetic pigments caused by the high titer of CO₂ (Gordillo et al., 1998).

Extensive research has been conducted over the past four decades on photoautotrophic organisms to improve carbon fixation (Rosgaard et al., 2012). One approach is to improve the efficiency of RubisCO (Satagopan et al., 2009; Smith and Tabita, 2003). The purpose of most of these studies has been to elucidate how the enzyme is folded and assembled (Durall and Lindblad, 2015). A major factor in RubisCO engineering is determining how to increase the CO₂ over O₂ specificity. Residues in loop 6 of the large subunit are involved in the specificity of RubisCO, thus numerous amino acid substitutions have been made (Durall and Lindblad, 2015). For example, when alanine 340 was replaced by histidine, the result was a 13% increase of the specificity factor and a 33% decrease in the rate of carboxylation (Madgwick et al., 1998).

Another approach for improving carbon fixation is to use biochemical and enzymatic data to predict the shortest synthetic pathways with highest specific activity and minimum energetic costs for production of glyceraldehyde-3-phosphate (GADPH), a Calvin-Benson cycle metabolite. A proposed synthetic pathway was developed that was predicted to be two to three times faster than the Calvin-Benson Cycle (Bar-Even et al., 2010). Several enzymes have been suggested as potential targets for enhancing CO₂ fixation. They include RubisCO activase, sedoheptulose-1,7-biphosphatase (SBPase), and fructose-1,6-bisphosphatase (FBPase) (Rosgaard, et al, 2012).

Research has also been conducted on improving carbon concentrating mechanisms (CCMs) in plants to enhance CO₂ fixation. CCMs concentrate inorganic carbon levels surrounding RubisCO, which increases the carboxylase activity and minimizes the oxygenase activity (Price, et al., 2002). However, cyanobacteria have highly efficient CCMs which are induced under low CO₂ conditions (Price, 2011), thus it isn't clear if further improvements are possible (Rosgaard, et al, 2012). Any steps that improve the carbon fixation rate of cyanobacteria will be beneficial towards enhancing its capability to produce biofuels.

In cyanobacteria, excess fixed carbon is used to generate glycogen, which is the primary storage polysaccharide (Ball and Morell, 2003). Cyanobacteria accumulate glycogen usually as a result of limiting growth conditions (i.e. nitrogen depletion in the presence of excess carbon) (Ernst and Boger, 1985). Glycogen is catabolized to supply carbon and reductants for ATP production under dark conditions (Osanai et al., 2014). Under optimal conditions, it may account for more than 50% of the dry weight of cyanobacterial cells (Aikawa et al., 2012). This is of specific interest, as enhancing

glycogen production yields a promising strategy for production of 3rd and 4th generation biofuels (Hasunuma et al., 2013). This is due to the ability of glycogen to be converted into biofuels such as butanol, isobutanol, and ethanol (John et al., 2011).

One strategy employed to increase the production of cyanobacterial glycogen is to limit nitrogen. For example, in the absence of nitrate, the glycogen content of *Arthrospira platensis* increased to 63.2% of the dry cell weight compared to 15% in cultures with nitrate at the conclusion of a 72 h experiment. The protein content decreased from 40% to 15.4% in the absence of nitrate, while cultures with nitrate remained steady at 40% protein during a 72 h experiment (Hasunuma et al., 2013). In phosphorous limited conditions, the glycogen content of *Spirulina maxima* reached 34% after the exhaustion of phosphorous in the media (De Philippis et al., 1992). By optimizing light intensity and nitrate supply, glycogen production of *Spirulina platensis* reached 1.03 g/L with a biomass concentration of 1.6 g/L, which at the time was the highest glycogen production ever reported in cyanobacteria (Aikawa et al., 2012). After the initial growth phase in which the maximum glycogen content of *Anabaena variabilis* is reached, the glycogen content began to decrease. This glycogen metabolism presumably takes place due to the light-limitation of photosynthetic activity that occurs ~45 h after inoculation (Ernst and Boger, 1985).

In cases where the target compound produced from engineered cyanobacteria is another metabolite, or the final biofuel compound itself, it would be beneficial to limit glycogen production to shift the carbon flux towards the target compound. For example, the export of sucrose by *Synechococcus elongatus* was increased from 0 to 36.1 mg/L/h by shifting glucose-1 phosphate flux toward sucrose production and away from storage as

glycogen (Ducat et al., 2012). In cases where cyanobacteria are being genetically engineered to produce hydrocarbons and long-chain alcohols (Gu et al., 2012; Halfmann et al., 2014a; Halfmann et al., 2014b; Kiyota et al., 2014), it would be beneficial to inactivate or over-express genes involved in glycogen synthesis or breakdown, respectively. Potentially, this would yield more carbon that could be used in the hydrocarbon or alcohol production pathways (Halfmann et al., 2014b).

The co-product of photosynthesis is O_2 . It is believed that the evolution of cyanobacteria to produce O_2 led to the Earth's atmosphere suitability for aerobic metabolism and complex life (Dismukes et al., 2001). Net photosynthesis can be measured as O_2 evolution in cyanobacteria (Masojídek et al., 2001). Cyanobacteria produce O_2 via the electron transport chain through Photosystem II (PSII). PSII is a large homodimeric protein-cofactor complex located in the thylakoid membrane (Guskov et al., 2009).

While O_2 is produced during photosynthesis, it is also fixed by photoautotrophs in an energetically wasteful cycle known as photorespiration (Price et al., 2013). Photorespiration is the result of the oxygenation of ribulose-1,5-bisphosphate (RuBP) during the Calvin-Benson cycle which leads to the formation of one molecule of 3-phosphoglycerate (3PGA) and one molecule of 2-phosphoglycerate (2PG), which is toxic (Fig. 1.3). If photorespiration did not occur, two molecules of 3PGA and no 2PG would be formed (Zarzycki et al., 2012). Thus, limiting photorespiration is another target for research to enhance the industrial potential of cyanobacteria.

1.2.2.5.2 Dark reactions

During the light phase (photosynthesis) of growth, light energy is used to fix CO₂ and produce carbohydrates that are either used for growth or stored as glycogen or other carbohydrates. During the dark phase, photosynthesis ceases and respiration uses the stored carbohydrates to produce energy while consuming intracellular oxygen. Oxygen uptake in cyanobacterial blooms can be caused by several processes (i.e. dark respiration, photorespiration, and cyclic electron flow around PSII) (Masojíddek et al., 2001). Cyanobacteria are the only organisms capable of performing aerobic respiration and oxygenic photosynthesis in the same compartment (thylakoid membranes) (Schmetterer, 1994). The consumption of intracellular oxygen enables nitrogenases, which are O₂ sensitive, to function properly in the dark phase (Welkie et al., 2014). Thus a balance must be kept by nitrogen fixing cyanobacteria in which oxygen levels in the cell should be low to prevent nitrogenase inactivation, yet the influx of oxygen needs to be at a level to enable respiration for N₂ fixation in dark conditions (Brauer et al., 2013).

In a study by Brauer et al., (2013), the respiration rate of a *Cyanothece* sp. was calculated as the slope of the regression line of oxygen concentration versus time. The respiration rates of the N₂-fixing microbe peaked during the night in N₂-fixing conditions (~100 fmol O₂ cell/h). Respiration rates of the microbe in non-N₂-fixing conditions were low and virtually constant throughout the dark period (~20 fmol O₂ cell/h).

In cyanobacteria, glycogen is consumed in dark conditions during respiration or dark anoxic metabolism (autofermentation) to provide energy as nicotinamide adenine

dinucleotide phosphate (NADPH) and adenosine triphosphate (ATP) for cell maintenance and limited biosynthesis (Stal and Moezelaar, 1997). The average catabolic rate of total reducing sugars equivalents (RSE) of a *Synechococcus* sp. strain PCC 7002 during periods of autofermentation was $35 \pm 16 \text{ mol } 10^{-17} \text{ day}^{-1}$ (Guerra et al., 2013). In glycogen deficient cyanobacterial strains, transient starvation conditions resulted in a significant loss of viability (Preiss, 1984). For example, a glycogen deficient *Synechococcus* sp. PCC 6803 strain had a reduced survival rate during light-dark regimes (Gründel et al., 2012). This showed evidence that glycogen is essential for cyanobacterial survival during photoperiodicity and nutrient starvation.

1.2.2.5.3 Nitrogen Fixation

As mentioned earlier, some species of cyanobacteria are diazotrophic (capable of fixing atmospheric nitrogen). Diazotrophic cyanobacteria are the only known organisms that both fix nitrogen and carry out photosynthesis, thus they must balance the presence of intracellular oxygen with the oxygen sensitive nitrogenase (Berman-Frank et al., 2003). In most diazotrophic filamentous cyanobacteria, nitrogen fixation takes place in specialized cells termed heterocysts. However, in *Trichodesmium* N₂-fixation takes place in anaerobic regions of the filaments and in trichomes (Bergman et al., 2012). All heterocyst-forming cyanobacteria fix nitrogen aerobically. Heterocysts allow for the spatial segregation of photosynthesis and N₂ fixation (Bandyopadhyay et al., 2013), and have higher rates of respiratory oxygen consumption, resulting in an anoxic environment (Bergman et al., 1997).

The majority of non-heterocystous, diazotrophic strains of cyanobacteria require incubation under microoxic or anaerobic conditions for N₂ fixation (Brass et al., 1992; Rippka and Waterbury, 1977). However, some non-heterocystous cyanobacteria are capable of nitrogen fixation under aerobic conditions (Bandyopadhyay et al., 2013). In unicellular, diazotrophic strains such as *Cyanothece* spp. ATCC 51142 and PCC 7822, N₂ fixation and photosynthesis are separated into night and day phases, respectively (Arshad et al., 2014).

Under different light/dark cycles, the average maximum nitrogen fixation rate for *Cyanothece* sp. BG 043511 was 0.87 ± 0.2 pmol N₂ cell/h (Rabouille et al., 2014). The maximum nitrogen fixation rate of an *Aphanizomenon* sp. was ~ 100 mmol N (10⁶ heterocyst)⁻¹ h⁻¹ (Bradburn et al., 2012). The majority of Earth's atmosphere is composed of N₂. Thus due to their diazotrophism, cyanobacteria contain a tremendous potential to produce next-generation biofuels and high-value chemicals with no organic nitrogen input.

1.3 Cultivation Systems

Commercial deployment of 3rd and 4th generation biofuels produced from cyanobacteria will require productive and inexpensive cultivation systems (Sattelle and Sutcliffe, 2008). Wide-ranging research has been conducted regarding cultivation of cyanobacteria using a variety of systems that range from intensive photobioreactor (PBR) systems to extensive outdoor ponds (Parmar et al., 2011). Additional design factors that need to be considered include whether the system is batch, fed-batch, or continuous, open versus closed, or outdoor versus indoor. Each type of system has advantages and

disadvantages. The goal is to minimize capital and operating costs, while maintaining sufficient system productivity and yield so as to achieve competitive production costs (Wijffels et al., 2010).

1.3.1 Pond systems

Outdoor pond systems are the most common means of cultivating algae and cyanobacteria (Parmar et al., 2011). Ponds can be of earthen construction, lined with plastic, or constructed from concrete (Chisti, 2007). The main advantages of open ponds are the lower costs for construction and operation (Borowitzka, 1999; Huntley and Redalje, 2007). The primary disadvantage of pond systems is that performance is generally poor due to difficulty in maintaining optimal operating conditions. Key factors include: thermal control, non-optimal light intensity and uneven distribution, self-shadowing of cells, day-night cycles, mixing, and pH control (Chen et al., 2011). This results in lower photosynthetic efficiency compared to PBR systems and lower utilization of CO₂ from the gas stream (Watanabe and Hall, 1996). Low photosynthetic efficiency is also caused by 85% of the cells being in dark conditions when the optimal photic volume is achieved in ponds (Richmond, 2000).

Another disadvantage of pond systems is the need for large areas of land, however non-arable land, such as much of Southwestern USA, is generally suitable for large scale cyanobacteria and algal cultivation (Singh et al., 2011a). While evaporation rates are higher in the SW USA compared to other regions, it is still a preferred location when scaling-up. A study by Pate et al., (2011) showed that in a proposed 10 billion gallons per year (BGY) algal biofuel facility, the annual evaporative water loss would be less in the SW USA (2800 BGY) compared to the Midwest USA (3300 BGY). This is due to the

increased area needed for ponds in the Midwest to produce 10 BGY biofuel, when compared to the SW region.

The risk of contamination from the surrounding environment is another disadvantage of the open pond system. Thus species are typically selected that can be cultivated under selective conditions (i.e. high salinity or high alkalinity) (Harun et al., 2010). Native species are preferred in open ponds to decrease the effect of contamination, thus utilizing genetically modified organisms is not recommended (Rodolfi et al., 2009). Other disadvantages of open pond systems include evaporative water loss, variation in quality of biomass, and loss of CO₂ in the culture (Harun et al., 2010).

One example of open pond systems that have been used to cultivate cyanobacteria are 0.7 m long, 0.18 m wide, 0.075 m deep ponds in Brazil, that were used to cultivate *Spirulina platensis*. This system was capable of achieving 1.33 g/L biomass using freshwater supplemented with carbon, nitrogen, and metal ions (Costa et al., 2003). Open pond systems have also been used to produce C-phycoerythrin, an ingredient in cyanobacterial-based foods and health foods, as well as a coloring, fluorescent, and antioxidant agent. Productivities ranged from 0.003 g/L/d from pond systems in Spain to 0.024 g/L/d from pond systems in Italy (Eriksen, 2008). Earthrise[®] Nutritionals has an open pond raceway-based biomass production facility in CA, USA, which occupies 440,000 m² (Figs. 4 and 5). This facility is utilized for production of cyanobacterial (*Spirulina*) biomass for food (Chisti, 2007).



Figure 1.4: Earthrise® Farms *Spirulina* production plant. (Spolaore, et al, 2006).

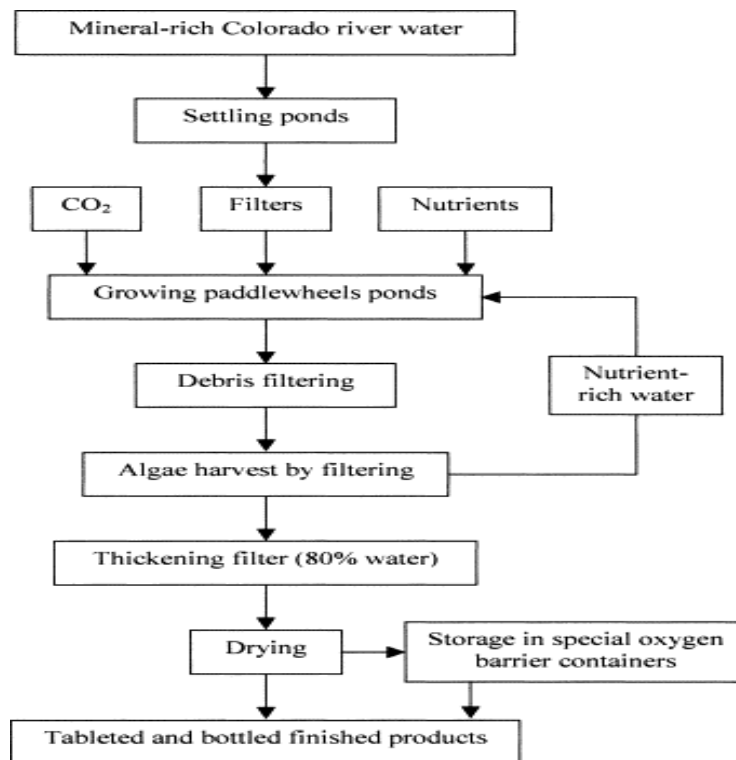


Figure 1.5: Earthrise® Farms *Spirulina* production process. (Spolaore, et al, 2006).

1.3.2 Photobioreactor (PBR) systems

The first PBR systems were developed in the 1940s, and this made it possible to control variables such as light, nutrients, pH, and temperature (Huntley and Redalje, 2007). PBRs are generally closed systems which could be in the form of tubes, plates, or bags made of plastic, glass, or other transparent materials (Lehr and Posten, 2009). The closed nature of PBR systems allows for significant savings in net water use (Schenk et al., 2008). The major problems associated with PBRs are the high operating costs of artificial lighting as well as high power consumption (Chen et al., 2011). One way to decrease lighting costs is to employ a narrow spectral output that overlaps the photosynthetic absorption spectrum. This would eliminate the emission of light at unusable frequencies, thus improving overall energy conversion (Chen et al., 2011). PBRs are also highly dependent on the light supply and reactor geometry to ensure uniform distribution of light (Fouchard et al., 2008). The light source and light intensity are critical factors that affect the performance of cyanobacteria cultivation (Mata et al., 2010). One way that light intensity can be kept below inhibitory levels for cyanobacteria is by installing PBR systems inside greenhouse facilities (Fernández et al., 2012; Gross and Wen, 2014).

The power needed to mix cultures and transport fluid in PBRs can be another significant cost. Mixing is necessary in all PBRs to prevent sedimentation of the cells and evenly distribute CO₂ and O₂ (Schenk et al., 2008). Due to carbon uptake during photosynthesis, pH rises during growth. This can be regulated by monitoring pH and adding buffers as needed (Fouchard et al., 2008). pH control can also be monitored by injection of CO₂ into the PBR (Pawlowski et al., 2014). While the relationship between

hydrodynamics and mass transfer have been thoroughly investigated and correlated in bioreactors for heterotrophic cultures, very few studies on this relationship are available in phototrophic cultures (Ugwu et al., 2008). An increase in aeration rate improves mixing, liquid circulation, and mass transfer between the gas and liquid phases. However, high aeration rates can also cause cellular shear stress (Kaewpintong et al., 2007; Sobczuk et al., 2006; Ugwu et al., 2008). Fluid flow in PBR systems is an essential component on the functionality of the system. Fluid flow dynamics will depend on specific PBR designs; thus they will be discussed individually in the subsections below.

1.3.2.1 Bag systems

Bag PBR systems are an attractive option for cultivating cyanobacteria due to their simplicity, cost-effectiveness, and adaptability (Carvalho et al., 2006; Sathiyamoorthy and Shanmugasundaram, 1994). These systems are commonly constructed of polyethylene plastic bags of various sizes (Cohen and Arad, 1989; Sierra et al., 2008; Trotta, 1981). Sizes ranging from 25 L (Cohen and Arad, 1989) to 1000 L (Borowitzka, 1999) have been utilized for microalgae cultivation.

Sierra et al, (2008) describe a flat panel bag PBR system consisting of a 250 L disposable plastic bag located between two iron frames 0.07 m apart (Fig. 1.6). The frame and plastic bag had a 0.07 m width, 1.5 m height, and 2.5 m length. The bag was constructed of 0.75 μm polyethylene. A gas sparger was placed from side to side on the bottom of the plastic bag for aeration, and a heat exchanger consisting of 4 stainless steel tubes was located above the sparger inside the bag for temperature control. The bag was

replaced when excessive biofilm fouling or contamination occurred. Utilizing the plastic bag led to a substantial cost reduction for this system.

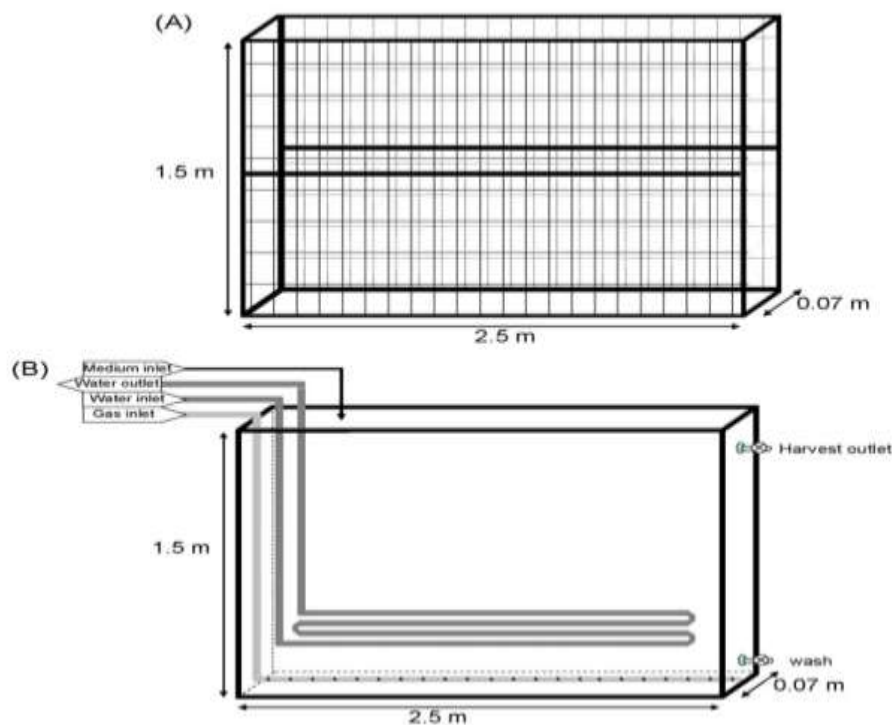


Figure 1.6: Schematic diagram of a flat panel bag PBR system.

(A) Frame; (B) schematic drawing of aeration system, heat exchanger, and medium inlet and harvesting valve for continuous operation (Sierra et al., 2008).

Bag systems can be almost as inexpensive as open ponds, but overcome the challenges of lack of process control, microbial contamination and/or containment, and evaporative water loss (Borowitzka, 1999). The bag PBR system in Figure 1.6 shows how process control is possible in bag systems. Mixing and aeration is controlled by the sparger, and temperature is controlled by the heat exchanger (Sierra et al., 2008). Potentially, a pH probe could be placed in the inlet valve. A study by (Chinnasamy et al., 2010), concluded bag systems performed better than vertical tank reactors and raceway ponds in terms of overall area and volumetric biomass productivity using carpet mill

wastewater as the medium. Disadvantages of bag systems include disposal of used plastic bags and biofilm fouling (Sierra et al., 2008; Wang et al., 2012). Also, scaling up with larger bag volumes does not necessarily lead to increased productivity (Martínez-Jerónimo and Espinosa-Chávez, 1994)

One promising area for bag systems to potentially be deployed is the Offshore Membrane Enclosure for Growing Algae (OMEGA) system. Algae Systems, LLC, has licensed this system from NASA. It consists of flexible bags that could be filled with wastewater and placed in seawater where the treated wastewater would leave the bags via forward osmosis. This system could be used for producing hydrocarbons, including oil (Trent et al., 2013). The OMEGA system consists of individual modules that are closed PBRs filled with wastewater, floating in seawater just below the surface of the water. The modules are 20 cm x 20 cm x 1, 2, or 3 cm, constructed of clear polyurethane with forward osmosis membranes. OMEGA modules were capable of cultivating 6 g/L dry weight *Chlorella vulgaris* using BG11, a standard medium for growing algae and cyanobacteria (Trent et al., 2010).

1.3.2.2 Horizontal tubes

One design for PBR systems uses horizontal, parallel tubes that can be arranged like a fence or they can be arranged in a helical coil to increase the number of tubes that can be used in a given area (Chisti, 2007). Examples of materials used for constructing tubes are glass (Dasgupta et al., 2010; Santiago et al., 2013), acrylic (Roncallo et al., 2013), polyethylene (Dasgupta et al., 2010), and plastic (Slegers et al., 2013). A schematic diagram of a typical horizontal tubular PBR can be found in Figure 1.7. The

flow of fluid, incorporation of gas, and control of temperature and pH occurs in a common tank through which all the fluid flows. The retention time in the tubes cannot be too long, or CO₂ will be depleted and control of optimal pH and temperature will be lost. Thus the flow rate and volume/dimensions of the tubes are critical. Retention time in the tubes and sedimentation of biomass is prevented by maintaining highly turbulent flow, produced by either a mechanical pump or a gentle airlift pump (Chisti, 2007). Mechanical pumps should be avoided as they can damage the biomass (Mazzuca Sobczuk et al., 2006; Sánchez Mirón et al., 2003).

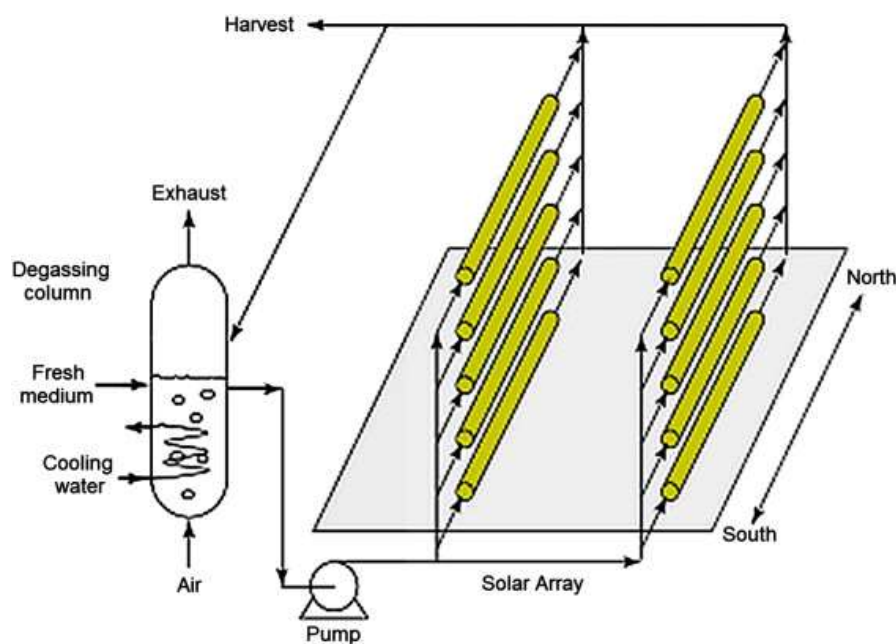


Figure 1.7: Schematic diagram of a horizontal tubular PBR.
(Wang et al., 2012).

The vertical stacking of horizontal tubes is advantageous due to light distribution and control of flow (Pulz, 2001). Another advantage is the largely exposed illumination surfaces (Ugwu et al., 2008). The ground beneath the tubes is often painted white or covered with white sheets of plastic, which increases light reflectance, thus increasing the

total light received by the PBR (Ståhl et al., 1999). A disadvantage of horizontal, tubular PBRs is the difficulty in scaling them up, which requires large amounts of land. Fouling and pH gradients are other disadvantages associated with tubular PBRs (Ugwu et al., 2008).

Horizontal tubular PBRs (5 L working volume) have been used to cultivate *Chlorella vulgaris* for the production of high-value fatty acids. By enriching air with 16% (v/v) CO₂, lipid production reached 24.4 mg L/d. Approximately 80% of these lipids were omega 3 and omega 6 fatty acids (Montoya et al., 2014). Horizontal tubular PBRs have also been used to cultivate *Chlorella vulgaris* as a source of lipids for biodiesel production. Cultivation occurred in 2.0 L PBRs in growth medium enriched with 0.2 g/L NaHCO₃. This system produced 10.3 mg/ L/d lipids (Frumento et al., 2013). To grow *Phaeodactylum tricornutum* in a continuous outdoor culture, 0.2 m³ tubular PBRs were designed. This yielded a biomass productivity of 1.9 g/L/d when the culture was diluted at a rate of 0.04 h⁻¹ (Molina et al., 2001).

1.3.2.3 Vertical tubes

Airlift and bubble column reactors fall into the category of vertical tubular PBRs (Fig. 1.8) (Dasgupta et al., 2010). Materials used for constructing these tubes are identical to horizontal tubes discussed previously. In airlift and bubble column reactors the liquid can be held in a batch mode as long as temperature and pH can be adjusted in individual tubes. Compared to horizontal tubes, the hydrodynamics of vertical columns are quite different. In horizontal tube PBRs, the gas is supplied in a common tank, while in vertical tube PBRs the gas is generally supplied via sparging from the bottom. However, there are

examples of vertical tube systems with spargers on the sides (Dasgupta et al., 2010).

There tend to be many more and larger bubbles in vertical tube PBRs compared to horizontal tube PBRs, and the gas-liquid flow is much less controlled. These differences in bubble size and gas holdup affect gas-liquid mass transfer, light penetration, mixing, and shear stress levels (Miron et al., 1999).

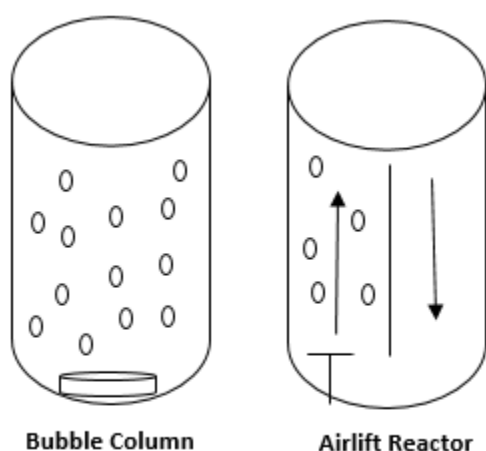


Figure 1.8: Schematic diagrams of vertical tubular PBRs.
(Dasgupta et al., 2010).

Advantages of vertical tubular PBRs compared to other PBRs include compactness of design, low cost, ease of operation, and low shear stress from agitation (Asada and Miyake, 1999; Miron et al., 1999). The main challenge in scaling up vertical tubular PBRs is to reduce construction costs to make them more economically competitive (Borowitzka, 1999). Other drawbacks with tubular PBRs are: fragility of the

material, gas transfer at the top of the reactors, temperature control, small illumination area, and gas holdup (Dasgupta et al., 2010)

Scenario analyses conducted by Slegers et al., (2013) determined that the areal biomass productivity in vertical tubular PBRs is 25-70% higher than in horizontal PBRs. The highest annual area productivity in the analyses was 155.0-ton ha/year *Phaedactylum tricornutum* biomass in outdoor vertical tubular PBRs in Algeria. When grown in 2 L vertical tubular PBRs, *Cyanobium* sp. could be cultivated at a rate of 0.071 g/L/d when the growth medium was enriched with 1.0 g/L NaHCO₃ (Henrard et al., 2011). *Chlorella vulgaris* ESP-31 was cultivated in outdoor 50 L vertical tubular PBRs for lipid production. The tubular PBRs were the first stage of cultivation before the culture was transferred indoors to enhance lipid accumulation. Lipid productivity reached 0.054 g/L/d in this system (Chen et al., 2014).

1.3.2.4 Panel/sheet type

Although raceway ponds are believed to be the most cost efficient systems, vertically oriented, flat-panel PBR systems are expected to play a vital role in future microalgal industries (Li et al., 2015). A schematic diagram of a flat panel PBR can be seen in Figure 1.9. Flat-panel PBRs are transparent, flat vessels where the culture is mixed via aeration, generally at a rate of ≤ 1 L/L/min. Oxygen removal and the addition of CO₂ can be achieved via airlift modules, which are efficient, reliable, and inexpensive (Acién et al., 2001; Molina et al., 2000). These PBRs can be operated in batch mode or in continuous mode with a culture recirculating system. The flat panel PBR described by Li et al., (2015) controlled temperature through two radiating heaters facing the flat panel.

The pH was controlled via CO₂ injection through the sparger. The panel was installed vertically and surrounded with fluorescent light bulbs.

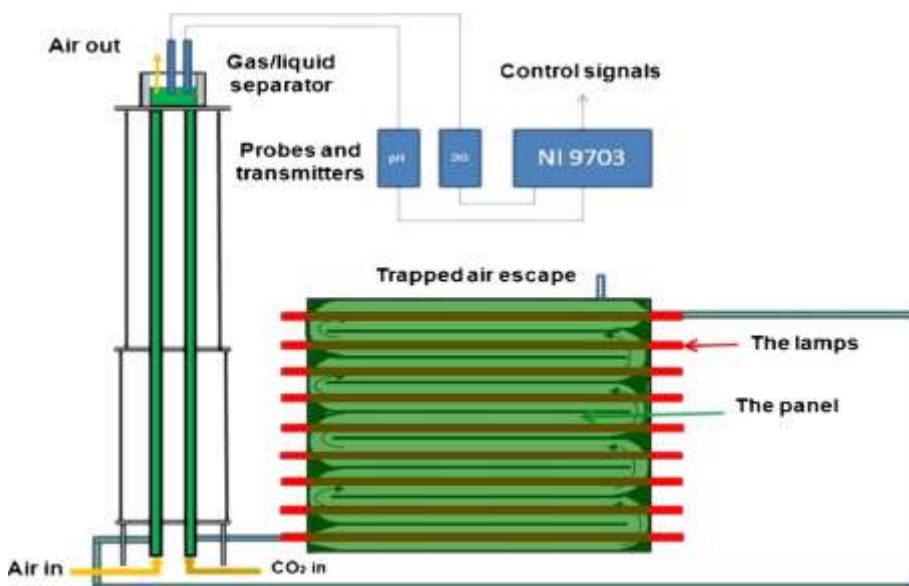


Figure 1.9: Schematic diagram of an airlift flat panel PBR.
(Li et al., 2015).

The optical path length in these systems vary from 1 to 20 cm, thus biomass concentrations from these systems vary greatly (de Vree et al., 2015; Zou and Richmond, 1999). A 100 ha production facility utilizing flat panel PBRs with an optical length of 3 cm had an estimated investment cost of 1.15 M\$/ha (Norsker et al., 2011). Advantages of flat panel PBRs include light utilization efficiency and achievable sterility, while a disadvantage is difficulty in scaling up (Borowitzka, 1999). Flat panel PBRs can be constructed with disposable polyvinyl chloride (PVC) film bags to decrease costs, but these need to be supported by scaffolds which can be expensive (Sierra et al., 2008).

Hydrogen production has been reported in a 3.85 L flat-panel PBR system utilizing a *ΔhupW* strain of *Anabaena* sp. PCC 7120. In this system, a maximal H₂

volumetric production rate of 6.2 ml/L/h was achieved with a light conversion efficiency of 4.0% (Nyberg et al., 2015). A study by Clares et al., (2014), observed that CO₂ capture rate by *Anabaena* sp. ATCC 33047 in a flat panel PBR was 50% higher than that of a tubular PBR on a per area basis, reaching values over 35 g CO₂ fixed m/d. Thus, they concluded that the flat panel PBR system is preferred compared to a tubular reactor for CO₂ capture and biomass generation by *Anabaena* cultures.

1.3.2.5 Other designs

PBR designs are virtually limitless and include: cascade system with baffles, fermentors, and two-stage systems (indoor and outdoor systems) (Borowitzka, 1999). An advantage of the cascade system is a short optical path (< 10 mm), thus light utilization is more efficient leading to high biomass densities (25-35 g/L). A disadvantage of this system is difficulty in controlling temperature (Masojídek et al., 2011). An advantage of fermentor systems is that certain photoautotrophic strain (i.e. *Chlorella*) can be cultivated heterotrophically for biodiesel production. A disadvantage of this system is that a very limited number of species can be cultivated in this manner (Xiong et al., 2008).

Two-stage PBR systems have an industrial appeal by taking advantage of features of both outdoor and indoor systems. The culture is grown in closed systems indoors where there is more control over contamination and growth conditions. Then, the culture is transferred to an outdoor, open system where there is more surface area and the system cost is lower for larger volumes. In the process of scaling up PBRs there is not going to be one clear answer for every situation. Different variables such as regional climate and

available space will dictate the appropriate PBR system. The proper selection of cultivation systems will play a vital role in any cyanobacterial industrial process.

1.4. Product recovery methods

For any industrial process involving cyanobacteria to become feasible, product recovery must be optimized. Efficient harvesting mechanisms are needed to increase the economic competitiveness of microalgal products against traditional sources (Drexler and Yeh, 2014). The various options of product recovery are dependent on whether the desired product is the biomass, within the biomass, or is excreted by the cell into the culture fluid. In this latter case the product may remain in the culture fluid, or be phase separated if it is non-soluble in water. If the product is volatile it may also be displaced into the headspace gas if CO₂ enriched air is bubbled through the reactor. While oleaginous algae are currently the most popular microbes in the microbial biofuel world with respect to producing fuels directly from biomass, cyanobacteria are also being studied for this application. Typically, cells with lipids need to be harvested, dried, and extracted via solvents. The extraction processes are energy intensive, typically accounting for 70-80% of the total cost of the biofuel production process (Grima et al., 2003). However, there is an example of genetically engineered cyanobacteria continuously secreting free fatty acids into the culture medium where they can be collected at a much cheaper cost (Liu et al., 2011).

There are various desired end-products within cyanobacterial biomass, thus product recovery typically involves a cell lysis step. For example, *Synechococcus* sp. PCC 7002 biomass can be hydrolyzed via an enzymatic treatment to release fermentable

carbohydrates that can be utilized by *Saccharomyces cerevisiae* for bioethanol production (Möllers et al., 2014). Other end-products produced from cyanobacteria are excreted from the cell. For example, a genetically engineered *Anabaena* sp. PCC 7120 strain can produce the cyclic monoterpene limonene, which is excreted from the cell and volatilized into the headspace where it can be collected via chromatography (Halfmann et al., 2014b).

1.4.1 Cell recovery

Examples of biomass recovery techniques that can be used with cyanobacterial cultivation systems are: centrifugation, auto-flocculation, bio-flocculation, co-flocculation, and membrane filtration (Mata et al., 2010; Vasel et al., 2014). In any product recovery method involving the recovery of microalgal or cyanobacterial biomass, the composition of the cell wall polysaccharides must be considered as this affects cell flocculation and the recovery of intracellular products (Cheng et al., 2015). One disadvantage of cell recovery is the potential for water and nutrient loss. Zhang et al., (2014) developed a novel filtration PBR in which microalgae are retained on a membrane via filtration, which alleviates nutrient discharge. Keeping nutrients in the culture during a fed-batch process will greatly reduce costs and make for a more efficient system.

Centrifugation is a cell recovery method used to separate particles based on weight. Continuous centrifugation is the fastest and most effective method for total biomass separation (Chen et al., 2011; Rawat et al., 2013). It is a proven technology that has been utilized for harvesting algae and cyanobacteria for many years. However, the associated costs with centrifugation are very high due to the large amounts of water that must be processed (Richardson et al., 2014a). Other disadvantages of centrifugation

include high energetic costs and difficult recovery of the biomass in the case of some microbes (De Godos et al., 2011; Riaño et al., 2012).

Flocculation is the aggregation of suspended solids to form larger aggregates (Riaño et al., 2012). Two different phases can be distinguished in flocculation: the interaction of the flocculant with the surface charge of the suspended solids which creates large flocs; and the merging of the aggregates into even larger flocs that are easier to separate (Knuckey et al., 2006). Advantages of flocculation include: improving biomass sedimentation, centrifugal recovery, and filtration (Grima et al., 2003). Aluminum sulphate is the most commonly used inorganic flocculant. It carries several disadvantages, such as: sludge production, difficult to dehydrate, efficiency is dependent on pH, and it is possibly toxic (Renault et al., 2009).

Membranes can be used for biomass separation from the culture liquid by providing a thin barrier that selectively restricts the passage of solvents and solutes depending on their properties and membrane characteristics (i.e. pore size) (Drexler and Yeh, 2014). Membrane filtration is an appealing method of acquiring the desired end-product as it is minimally disruptive to the biomass and there is no need for chemical additives (Drexler and Yeh, 2014). One example of an application of membrane separation is hydrogen produced by *Chlamydomonas reinhardtii* recovered via a polymeric membrane where the recovery step occurs in a separate reactor than where the microbe is cultivated (Meyer and Weiss, 2014). Disadvantages of membrane systems is that they are more energy intensive and are prone to fouling (Wicaksana et al., 2012).

Of all the biomass recovery methods, centrifugation and chemical precipitation (a type of flocculation) are the only economically feasible options; with centrifugation being slightly better (Chen et al., 2011). Along with separating the biomass from the product, the biomass itself can be used for applications such as biochar or biooil (Maddi et al., 2011). Other applications of microalgal/cyanobacterial biomass are for animal or fish feed (Das et al., 2015; Lum et al., 2013). An estimated 30% of the global algae production is sold for animal feed (Becker, 2007). An effective cell recovery method will be necessary to develop an economically viable production process from microalgae or cyanobacteria.

1.4.2 Direct fuel/chemical recovery

Ideally, any desired end-product generated by cyanobacteria will have the capability to be directly removed from the culture with minimal disruption to the microbes. This would allow for the possibility of a continuous culture rather than fed-batch, which has an obvious economic advantage. Volatile high-value chemicals and next-generation biofuels produced by genetically engineered cyanobacteria have generated a great deal of interest from industrial microbiologists. This is in large part due to their ability to be removed from the culture without disrupting the cells, as the chemicals volatilize from the culture medium into the vessel headspace.

Examples of volatile chemicals that have been produced by genetically engineered cyanobacteria include: linalool (Gu et al., 2012), 1- butanol (Lan and Liao, 2011), limonene (Halfmann et al., 2014b), and farnesene (Halfmann et al., 2014a). Biofuels and high-value chemicals are often toxic to the microbe that produces them (Chubukov et al., 2015; Dunlop, 2011; Jarboe et al., 2011; Kim et al., 2015; Zhang et al.,

2011). Thus, the volatile characteristic of these chemicals is beneficial as they readily separate from the liquid phase into the vessel headspace where they can be recovered directly. One way that the chemicals can be recovered directly is by attaching a column containing resin capable of capturing the volatiles. Then, the chemicals can be eluted from the resin (Halfmann et al., 2014a, 2014b).

In some cases, the desired product is excreted into the culture fluid and can be separated from the culture without cell disruption. Also, a method termed milking can be used to extract the product directly from the cell without having to harvest or kill the cells (Kim et al., 2013). Milking has been used to harvest carotenoids from *Dunaliella bardawil* and *Dunaliella salina* using dodecane. This process achieved 5.3% carotenoid recovery from *D. salina* (Kleinegris et al., 2010). In the case of separation of biodiesel from microalgal biomass, phase separation must be used to harvest the diesel. In the biodiesel production process, after the transesterification step, glycerol is produced as a byproduct. Thus, it is necessary to phase separate the desired biodiesel from glycerol (Atadashi et al., 2011). This can be achieved by homogenous catalysts (alkali and acid) (Helwani et al., 2009), heterogeneous catalysts (solid and enzymes) (Helwani et al., 2009), and membrane reactors (Atadashi et al., 2011; Dube et al., 2007). A cost efficient means of product recovery is essential in order for any production process to become economically viable.

1.5. Cyanobacteria genetics

To enhance the industrial potential of cyanobacteria, a thorough understanding of their genetics is necessary. Many cyanobacteria strains have had their genome sequenced,

which provides the opportunity for comparative genomic analysis of the gene content as well as the diversity of a range of evolutionarily distinct cyanobacteria from different environments (Badger and Price, 2003). As mentioned earlier, many strains of cyanobacteria are also capable of being genetically engineered to produce high-value chemicals and potential biofuels. One potential target for genetic engineering in cyanobacteria is the production of transportation fuels, as the highest-volume application for engineered metabolism is the production of these fuels (Keasling, 2010).

1.5.1 Genetic engineering overview

Genetic/metabolic engineering has enabled the production of numerous biofuels and chemicals using recombinant bacteria (Jarboe et al., 2011). This process has much potential, however the economic viability of these processes are often limited by inhibition caused by the metabolic product (Brennan et al., 2015; Jarboe et al., 2011; Keasling, 2010; Ruffing, 2011). For any cyanobacterial energy production pathway to be considered a success, it must produce a transportable fuel product, achieve high conversion efficiencies, and have low-cost requirements per unit area (Ducat et al., 2011).

Genetically engineering cyanobacteria for increased biofuel production will hinder cell growth, as metabolites will be used for fuel synthesis rather than growth. Thus, the optimal carbon flux distribution to balance cell growth and biofuel production must be achieved (Ruffing, 2011). Metabolic control analysis, which requires experiment based measurements of flux control coefficients, can be used to elucidate this distribution. This involves the prediction of genetic manipulations that leads to optimized cyanobacterial strains, which would maximize the biofuel production rate (Chellapandi and Dhivya, 2010).

Engineering cyanobacteria to produce biofuels directly would bypass the need to synthesize all of the complex chemicals of biomass (Angermayr et al., 2009). One target for engineering is genes that increase photosynthetic efficiency. Any increase in photosynthetic efficiency will enhance downstream biofuel production, as photosynthesis drives the initial stage of all biofuel production processes (Schenk et al., 2008). However, regardless of the enhancement of the photosynthetic efficiency, the upper yield of photosynthesis driven biochemical pathways is limited by the solar radiation available (if no artificial light is utilized), light capture efficiency, and the conversion of photons to chemical energy (Ducat et al., 2011). The criteria for selecting cyanobacteria for biofuel production should include, primarily, the solar-to-biofuel energy conversion efficiency. This efficiency must be as high as possible (Lindberg et al., 2010).

1.5.1.1 Examples of engineering for specific products

Cyanobacteria have been genetically engineered to produce various products such as linalool (Gu et al., 2012), limonene (Halfmann et al., 2014b; Kiyota et al., 2014), farnesene (Halfmann et al., 2014a), sugars (Ducat et al., 2011; Niederholtmeyer et al., 2010), 1-butanol (Lan and Liao, 2011), glucosylglycerol (Tan et al., 2015), and ethanol (Abalde-Cela et al., 2015). Many of the chemicals mentioned (i.e. limonene) are not naturally produced by the cyanobacterial strain, thus no comparison can take place between the wildtype and genetically engineered strains. In the case of glucosylglycerol, the engineered *Synechocystis* sp. PCC 6803 strain had approximately a 3-fold increase in total production during an 8-day trial compared to the wildtype strain (Tan et al., 2015). A *Synechococcus elongates* PCC 7942 strain engineered for enhanced hexose sugar production had a total glucose plus total fructose final concentration of ~250 μM in the

culture supernatant compared to a ~175 μM concentration by the wildtype strain 5 days post-induction with 300 mM NaCl and 100 μM isopropyl β -D-1-thiogalactopyranoside (IPTG) (Niederholtmeyer et al., 2010).

While genetically engineering these strains of cyanobacteria to produce the desired end-product is necessary to move closer to the goal of an economically feasible production process, there are limitations with these microbes. For example, in several of the engineered cyanobacteria previously mentioned, the gene responsible for chemical production is present in a plasmid with an antibiotic cassette. Thus the cultivation medium must be supplemented with antibiotics to maintain the plasmid. To overcome this issue, the necessary chemical synthase genes must be integrated into the cyanobacterial chromosome. Another limitation of the genetically engineered cyanobacteria is their sensitivity to the toxic effects of the chemical that they are engineered to produce (Chubukov et al., 2015; Dunlop, 2011; Jarboe et al., 2011; Kim et al., 2015; Zhang et al., 2011). This limitation could be lessened by developing strains with increased tolerance to the chemical they are engineered to produce.

1.5.2 Mutagenesis

Many mutagenesis techniques can be utilized to create or develop cyanobacterial strains with phenotypes that would enhance their industrial potential. This can be achieved by random or site-directed mutagenesis, and induced by natural mechanisms or via use of chemical or physical mutagens. Mutagenesis is also beneficial for identifying previously unknown genes, and to determine the function of known genes (Koksharova and Wolk, 2002). However, certain atypical characteristics of cyanobacteria may make standard mutagenesis methodologies impractical or unworkable. For example,

cyanobacteria often have ~10 chromosomal copies per cell (Herdman et al., 1979). The filamentous phenotype of some cyanobacteria may make it difficult to isolate individual cells with desired mutations.

1.5.2.1 Directed Evolution

Directed evolution is an *in vitro* process for generating microbes with new and desired properties (Labrou, 2010). Directed evolution works by placing microbes in stress conditions, which induces spontaneous mutations (Singh et al., 2005). Directed evolution techniques that mimic evolution on a laboratory timescale are now well established (Hibbert and Dalby, 2005). While each directed evolution study is different, all involve two steps: making a library of mutants and screening the library for desired phenotypes (Umeno et al., 2005). In many cases, mutants with desired phenotypic changes are then subjected to more cycles of mutation and screening to accumulate beneficial mutations (Kuchner and Arnold, 1997).

1.5.2.2 Chemical mutagenesis

Examples of chemical mutagens that have been used on cyanobacteria are: ethyl methanesulfonate (EMS) (Shari et al., 2014), methyl methanesulfonate (MMS) (Tillich et al., 2012), and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Shari et al., 2014). These mutagens have been used for CO₂ mitigation enhancement (Shari et al., 2014), promoting temperature tolerance (Tillich et al., 2012), and autoflocculation (Kim et al., 2013). Advantages of chemical mutagens are their high efficiency and frequency in achieving saturation mutagenesis (Kim et al., 2006). A disadvantage of chemical mutagenesis is the chemical mutagens create many random mutations in the chromosome

which makes identifying the gene that caused the phenotypic change quite difficult to identify.

1.5.2.3 UV-C mutagenesis

Another method of generating random mutations in cyanobacteria is UV-C irradiation. UV-C light causes mutations by breaking bonds in DNA (Begum et al., 2009). UV-C irradiation potentially produces large numbers of random mutations consistently throughout the entire genome to generate novel strains (Hughes et al., 2012). Cyanobacterial mutants have been generated via UV-C for the purpose of genetic analysis of heterocyst-forming cells (Wolk et al., 1988), chemical resistance (Lanfaloni et al., 1991), and antibiotic resistance (Lambert et al., 1980).

A major disadvantage of UV-C irradiation with cyanobacteria is that it is desirable to have a period of growth in dark conditions immediately after irradiation, which is not ideal for cyanobacteria as many are obligate photoautotrophs (even though some strains are mixotrophic). This is to prohibit photoreactivation, which is the repair of UV-induced lesions in DNA using light energy (Xu et al., 2015). One way to circumvent this issue is growing the culture in yellow light (Wolk et al., 1988). Mannan et al., (1991) used medium supplemented with 5 mM fructose to facilitate growth in the dark. Another disadvantage of UV-C irradiation is that several *Anabaena* strains are resistant to UV light (Golden, 1987). Although there are several disadvantages with attempting to create cyanobacterial mutants with UV-C, it can still be accomplished.

1.5.2.4 Other types of mutagenesis

Traditionally, mutagenesis has been performed using either UV irradiation or DNA-modifying chemicals (Arnold and Georgiou, 2003). However, there are other mutagenesis techniques, such as error-prone PCR, mutator strains, poisoned nucleotides, or concurrently with DNA recombination (Kuchner and Arnold, 1997). The most common method for generating strains with random mutations is error-prone PCR (Arnold and Georgiou, 2003). The key with random mutagenesis is to combine it with a high-throughput screening system. This is a versatile strategy for improving protein functions and yielding desired phenotypes (Arnold and Georgiou, 2003).

1.5.2.5 Screening methods

An optimized mutant screening method is necessary to obtain strains with desired phenotypes. From among the millions or billions of progeny strains, an effective search engine such as genetic complementation and high-throughput screening can identify candidates with phenotypic improvements (Lutz and Patrick, 2004). Filamentous strains of cyanobacteria pose a unique problem when screening mutants, since the “colony forming unit” is a filament. The target of mutagenesis is a chromosome within a single cell, but due to the filamentous morphology, the resulting colony following mutagenesis can contain a mixture of mutant and wildtype cells (Golden, 1987). Another issue that some strains of filamentous cyanobacteria have is that they have 8 to 10 copies of chromosomes per cell (Hu et al., 2007).

There are numerous ways to screen mutant libraries for desired variants. Generating a large library is optional, but the ability to recover the targeted progeny is

mandatory (Lutz and Patrick, 2004). In a study using transposon mutagenesis to isolate *Nostoc punctiforme* ATCC 29133 mutants unable to fix atmospheric nitrogen, the screening method consisted of transferring putative mutants to nitrogen-free solid media. After ~7 d, if the colonies developed yellowing edges, then it was assumed they were unable to fix atmospheric nitrogen. Then, it was determined which gene the transposon interrupted, thus it could be determined which genes were responsible for nitrogen fixation (Cohen et al., 1994).

In a study using atmospheric and room temperature plasmas (ARTP) to generate mutants of *Spirulina platensis* FACHB-904 with increased carbohydrate content and increased growth rate, the screening method consisted of transferring the culture exposed to ARTP to individual wells of a 96-well plate. The plates were then incubated and if wells turned green, indicative of cyanobacterial growth, the culture was transferred to individual wells of 48-well plates for further screening. The cultures' growth was monitored via absorbance (OD₅₆₀) and compared to the wildtype. If the putative mutant's growth rate was increased compared to the wildtype, then the culture was transferred into 50 ml flasks containing 20 ml Zarrouk medium for further cultivation to detect carbohydrate content (Fang et al., 2013). The adage 'garbage in, garbage out' holds especially true in mutant screening, thus it is essential to have an optimized screening methodology in place in order to be able to successfully isolate cyanobacterial mutants with the desired phenotype.

1.6. Analytical methods

To optimize cyanobacteria's capability as an industrial microbe it is essential to be able to monitor growth and then adjust the key parameters affecting growth. One methodology that has been used to monitor growth in cyanobacteria is biomass dry weight (Binaghi et al., 2003; Lürling and Tolman, 2014; Mendez et al., 2015). Binaghi et al., (2003) determined biomass concentration by filtering 10 ml of culture through filters with 0.45 μm pore diameter. The filters were dried until the weight was constant. Archer et al., (1997) developed calibration curves by jointly measuring absorbance (OD_{680}) with biomass concentration during *Anabaena* 7120 growth trials. Linear regression analysis was conducted and in future trials absorbance was monitored, and the biomass concentration was derived from the linear regression equation. Presumably, direct measurement of biomass content would be more accurate as another parameter would introduce a source of error.

One of the challenges with obtaining accurate biomass concentrations is obtaining a uniform sample, especially with strains of filamentous cyanobacteria that aggregate in liquid cultures. The effect of cell aggregation can be decreased by instruments such as homogenizers and sonicators which break up the filaments into smaller filaments and individual cells (Archer et al., 1997; Halfmann et al., 2014b). When using dry cell weight (DCW) to determine biomass concentrations, it is crucial that the samples are absolutely dry. Thus, a limitation of DCW is that the data can't be obtained immediately and a drying oven or freeze dryer is necessary.

Another methodology that can be utilized to monitor cyanobacterial growth is chlorophyll *a* content (Lürling and Tolman, 2014). Many methodologies for chlorophyll determination use solvents such as methanol (Chen et al., 2010) or acetone (Bácsi et al., 2013) for chlorophyll extraction. However, chlorophyll content has also been determined without solvents using the equation: Chlorophyll α (Chl α) ($\mu\text{g ml}^{-1}$) = $14.96 (\text{OD}_{678} - \text{OD}_{750}) - 0.616 (\text{OD}_{720} - \text{OD}_{750})$ (Williams, 1988; Yu et al., 2011).

Absorbance can also be used to monitor cyanobacterial growth. For determining cyanobacterial content via absorbance, Liu et al., (2014) used OD_{680} while Lin et al., (2010) used OD_{720} . Many other studies used wavelengths similar to these. There are situations when using absorbance with cyanobacterial cultures is not recommended. When biomass content is low, absorbance is considered to have borderline sensitivity and precision (Mayer et al., 1997). Another issue to consider is that absorbance and chlorophyll α content can be easily affected by biomass debris formation (Robertson et al., 1998). When designing a study monitoring cyanobacterial growth, it should be determined if absorbance and chlorophyll α content can be measured accurately.

When screening cyanobacterial mutants or monitoring cultivation systems, it is essential to have a method for determining the viability of cells in the culture. It is also desirable to be able to differentiate between viable and non-viable cells. The differentiation of viable and non-viable cells on a cell-by-cell level allows for a causal evaluation of cell viability with respect to changing conditions (Schulze et al., 2011). The LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (Life Technologies[™], Carlsbad, CA, USA) has been successfully used to estimate the viability of bacteria from a broad range

of ecosystems (Filoche et al., 2007; Sato et al., 2004). The BacLight™ Kit uses dual fluorescence dyes to determine cell viability based on the differential ability of the nucleic acid stains SYTO® 9 and propidium iodide (PI) to penetrate viable versus non-viable cell membranes, respectively. This method is based upon the fact that cell membrane permeability is significantly greater in non-viable cells (Agusti et al., 2006; Llabrés and Agustí, 2008). It is important to critically evaluate this kit with each microbial strain tested as several factors need to be considered to ensure its accuracy (Stiefel et al., 2015).

Chapter 2 - Introduction

Fossil fuels are a finite resource. As the world's population continues to grow and fossil fuel reserves continue to be depleted, it is becoming increasingly important to develop renewable, sustainable sources of biofuels and high-value chemicals. The use of fossil fuels has led to global climate change, pollution, and adverse effects on many organisms (Chen et al., 2011), and the detrimental effects of fossil-fuel generated greenhouse gases are well documented (von Blottnitz and Curran, 2007) While policy-makers and the media, particularly in the USA, frequently assert that climate change is uncertain, there is a scientific consensus that human activities are heating the Earth's surface (Oreskes, 2004). One solution to the problems associated with fossil-derived fuels is to use microorganisms to produce biofuels and chemicals. Cyanobacteria, in particular are an attractive option due to their ability to produce chemicals and biofuels directly from CO₂ and solar energy (Machado and Atsumi, 2012).

Cyanobacteria are photosynthetic prokaryotes present in a vast array of ecosystems (Hasunuma et al., 2013; Katoh, 2012; Moreno et al., 1998). They have unicellular or filamentous morphologies, and use the same type of photosynthesis as higher plants (Lindblad et al., 2012). Filamentous strains of cyanobacteria that are capable of fixing atmospheric nitrogen have garnered a great deal of interest as they can be cultivated in medium without combined nitrogen. The ability to fix atmospheric nitrogen may be an attractive trait in industrial applications, as supplying nitrogen is a major cost in large-scale biofuel strategies (Halfmann et al., 2014b; Ortiz-Marquez et al., 2012; Peccia et al., 2013).

The purpose of this research was to enhance the industrial potential of filamentous cyanobacteria by increasing their tolerance to the biofuels and chemicals they are being engineered to produce. To accomplish this, it was first necessary to optimize specific analytical and culture methods. To accurately monitor growth of filamentous microorganisms, classical methods such as viable cell counts, hemocytometer counts, and flow cytometry will not work (Johnson et al., 2015). Therefore, we attempted to quantitate viable/non-viable percentages of filamentous cyanobacteria using a commercially-available dual fluorescence assay. Unfortunately, propidium iodide (PI), the non-viable cell indicator used in the dual fluorescence assay did not work properly with filamentous cyanobacteria. The results of this study are provided in Chapter III. We then evaluated SYTOX[®] Blue as a non-viable cell indicator to use in place of PI. This fluorochrome also did not work as expected with filamentous cyanobacteria due to non-specific binding. We then focused on assessing other viable cell indicators, and correlating their performance to total cell measurements such as optical density, chlorophyll α content, and biomass content. The results of this study are provided in Chapter IV.

Biofuels and high-value chemicals are often toxic to the microbe that produces them (Chubukov et al., 2015; Dunlop, 2011; Jarboe et al., 2011; Kim et al., 2015; Zhang et al., 2011), and this can limit productivity and yield. To develop strains able to withstand these toxic effects, I used directed evolution, a process in which the microorganism is exposed to progressively higher titers of the chemical, random mutations occur, and more tolerant mutants survive and are selected (Singh et al., 2005; Labrou, 2010). Because the chemicals investigated in this project are highly volatile, a

sealed culture environment was necessary in order to maintain a constant titer of the chemical. Therefore, I first determined the optimal conditions for filamentous cyanobacteria growth in a sealed environment. The results of this study are provided in Chapter V. Using the optimized, sealed environment conditions I then used directed evolution to develop strains with increased tolerance to the next-generation biofuels. Three strains of filamentous cyanobacteria were exposed to four different next-generation biofuels, and the results are shown in Chapter VI.

As mentioned previously, nitrogen will be a major cost associated with large-scale cultivation of cyanobacteria. While diazotrophic, filamentous strains of cyanobacteria are capable of growing without a combined nitrogen source, it is unclear if cultivating these strains in the absence of combined nitrogen would be preferred from an industrial standpoint. Nitrogen fixation is an energy demanding process, requiring 8 electrons and ~16 ATP per mole of N_2 fixed (Stal, 2003). Thus, the growth of filamentous cyanobacteria in a medium supplemented with different combined nitrogen sources was compared to growth in the medium with no combined nitrogen source. The results of this study are shown in Chapter VII.

Modeling is an important step in determining whether processes will be economical and environmentally sustainable. Modeling can also be used to identify process components that are bottlenecks or limitations from economic and/or environmentally perspectives. An economic feasibility study was conducted on a theoretical next-generation biofuel production facility that uses a genetically engineered strain of filamentous cyanobacteria to produce the cyclic hydrocarbon limonene. The results of this study are provided in Chapter VIII. A life cycle analysis study was also

conducted on the theoretical production facility to evaluate the environmental burden of the facility, and these results are shown in Chapter IX. The economic feasibility analysis combined with the life cycle analysis can be used to determine if production facilities similar to the theoretical one developed for this project have potential as a future sustainable solution for producing next-generation biofuels.

Chapter 3 - Testing a dual-fluorescence assay to monitor the viability of filamentous cyanobacteria

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Abstract

Filamentous cyanobacteria are currently being engineered to produce long-chain organic compounds, including 3rd generation biofuels. Because of their filamentous morphology, standard methods to quantify viability (e.g., plate counts) are not possible. This study investigated a dual-fluorescence assay based upon the LIVE/DEAD® BacLight™ Bacterial Viability Kit to quantify the percent viability of filamentous cyanobacteria using a microplate reader in a high throughput 96-well plate format. The manufacturer's protocol calls for an optical density normalization step to equalize the numbers of viable and non-viable cells used to generate calibration curves. Unfortunately, the isopropanol treatment used to generate non-viable cells released a blue pigment that altered absorbance readings of the non-viable cell solution, resulting in an inaccurate calibration curve. Thus, we omitted this optical density normalization step, and carefully divided cell cultures into two equal fractions before the isopropanol treatment. While the resulting calibration curves had relatively high correlation coefficients, their use in various experiments resulted in viability estimates ranging from below 0% to far

above 100%. We traced this to the apparent inaccuracy of the propidium iodide (PI) dye that was to stain only non-viable cells. Through further analysis via microplate reader, as well as confocal and wide-field epi-fluorescence microscopy, we observed non-specific binding of PI in viable filamentous cyanobacteria. While PI will not work for filamentous cyanobacteria, it is possible that other fluorochrome dyes could be used to selectively stain non-viable cells. This will be essential in future studies for screening mutants and optimizing photobioreactor system performance for filamentous cyanobacteria.

3.1. Introduction

Microorganisms have been used for thousands of years to produce foods, beverages, and feeds. More recently, microbes are being developed to produce chemicals and fuels from biomass to replace petroleum. Cyanobacteria are capable of producing biofuels by using solar energy and CO₂ as their sole energy and carbon sources (Machado and Atsumi, 2012). Furthermore, many strains of filamentous cyanobacteria are capable of fixing N₂ with specialized cells called heterocysts. This ability makes these strains preferable for industrial application (Yoon and Golden, 1998). There is also increasing interest in engineering cyanobacteria to produce high-value products such as linalool (Gu et al., 2012), limonene (Halfmann et al., 2014b), farnesene (Halfmann et al., 2014a), sugars (Ducat et al., 2011), and 1-butanol (Lan and Liao, 2011). A key consideration for these engineered cyanobacteria is tolerance to the chemicals they are being engineered to produce, as well as their long-term stability in photobioreactor systems (Jin et al., 2014). Therefore, a method is needed to rapidly and accurately quantify the viability of filamentous cyanobacteria.

Because living microbes are responsible for converting substrates to desired end products, monitoring cell viability is an important requirement. For microbes that grow as single cells, methods such as plate counts (Harmsen et al., 1999), hemocytometer counts (Schaeffer et al., 1979), flow cytometry (Berney et al., 2007), and automated analyses of microscopic images such as Image J (Schulze et al., 2011) work effectively.

Unfortunately, these methods do not work well for cyanobacteria that grow as filaments. Although Lee and Rhee (1999a) and Lee and Rhee (1999b) report accurate counts of the filamentous cyanobacteria *Anabaena flos-aquae* using epi-fluorescence microscopy, this approach would be far less accurate in conditions that cause cell aggregation, such as during production of chemicals that could be toxic to the cells.

To assess the tolerance of filamentous cyanobacteria to certain chemicals, a fast and reliable method is needed to quantify cell viability. The LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (Life Technologies[™], Carlsbad, CA, USA) has been successfully used to estimate the viability of bacteria from a broad range of ecosystems (Filoche et al., 2007; Sato et al., 2004). For example, this technology has been used to assess the viability of over-wintering unicellular cyanobacteria (Zhu and Xu, 2013). The BacLight[™] Kit utilizes dual fluorescence dyes to determine cell viability based on the differential ability of the nucleic acid stains SYTO[®] 9 and propidium iodide (PI) to penetrate viable versus non-viable cell membranes, respectively. This method is based upon the fact that cell membrane permeability is substantially higher in non-viable cells (Agustí et al., 2006; Llabrés and Agustí, 2008).

SYTO[®] 9 is a permeant intercalating green fluorescent dye capable of penetrating most plasma membranes and staining all cells containing nucleic acid. PI is a red

fluorescent dye that is membrane impermeable, thus excluded by the intact cell membranes of living cells (Lee and Rhee, 2001; Shi et al., 2007). Therefore, in the BacLight™ Kit, viable cells stain green. PI can pass through the porous membranes of dead cells, where it also intercalates into the nucleic acids, and displaces the SYTO® 9 dye due to intermolecular excitation energy transfer by the resonance mechanism (Samuilov, et al., 2008). Thus, non-viable cells fluoresce in the red spectra at an excitation wavelength of 490 nm and an emission wavelength of 635 nm (Stocks, 2004; Alakomi et al., 2006). This kit is designed to differentiate live cells from dead cells due to the ability of PI to displace SYTO® 9 from nucleic acids of cells with compromised membranes due to a higher affinity for nucleic acids (Stocks, 2004; Biggerstaff et al., 2006; Rath et al., 2014; Nybom et al., 2008; Llabrés and Agustí, 2008). The aim of this study was to determine if this kit was capable of estimating the viability of filamentous cyanobacteria. Such a methodology would be broadly applicable to the study of gene function, biofuel tolerant mutant screening, and culture maintenance of filamentous cyanobacteria.

3.2. Materials and methods

3.2.1 Microbial strains, maintenance, and culture conditions

The filamentous cyanobacteria *Anabaena* sp. PCC 7120, *A. variabilis* ATCC 29413, and *Nostoc punctiforme* ATCC 29133 were obtained from their respective culture collections. *Escherichia coli* strain NEB 10-beta was obtained from New England Biolabs, Ipswich, MA, USA. Cyanobacteria strains were grown in BG11 medium at pH 7.2 (Allen and Stanier, 1968) supplemented with 20 mM HEPES buffer (Thermo Fisher Scientific™, Waltham, MA, USA). For a solid medium, BG11 was supplemented with

1% agar (Benton Dickinson, Franklin Lakes, NJ, USA). For short term maintenance, the cyanobacteria strains were grown on solid BG11 agar and incubated at room temperature under constant illumination of $24 \mu\text{mol m}^{-2} \text{s}^{-1}$, and then stored at room temperature. For long term culture storage, cyanobacteria were frozen at -80°C in 5% v/v methanol. *E. coli* strain NEB 10-beta was grown in 500 ml Luria Broth (LB) in 1 L Erlenmeyer flasks at 37°C . For short term maintenance the *E. coli* strain was grown on Luria Broth Agar (LBA) and stored under refrigeration. For long term maintenance, the strain was frozen at -80°C in 5% v/v glycerol.

Broth cultures of cyanobacteria were grown in 1L Erlenmeyer flasks containing 500 ml of BG11 medium. Flasks were stoppered with a two-hole rubber stopper and a 2 ml disposable polystyrene serological pipet (Thermo Fisher Scientific™, Waltham, MA, USA) was inserted into one of the stopper holes to serve as the gas inlet port. The end of this pipet was submerged in the culture fluid. A section of another 2 ml pipet was inserted through the other stopper hole and was fitted with a sterile filter on the outside of the flask so exhaust gas could flow out. A mixture of 5% CO_2 in compressed air was sparged into the flasks at a constant rate of 0.25 L min^{-1} . Gas flow was regulated by flow meters (Cole-Parmer®, Vernon Hills, IL, USA). The flasks were incubated in a Lab-Line® Incubator-Shaker (Lab-Line® Instruments, Melrose Park, IL, USA) at 30°C and 100 rpm under constant illumination of $19 \mu\text{mol m}^{-2} \text{s}^{-1}$ using fluorescent lights. Light intensities were measured with a Heavy Duty Light Meter with PC Interface (Extech Instruments, Waltham MA, USA). *E. coli* strain NEB 10-beta was grown in 500 ml Luria Broth (LB) in 1 L Erlenmeyer flasks at 37°C and 150 rpm.

3.2.2 Analysis for Autofluorescence

Autofluorescence in the red spectrum occurs in cyanobacteria when excited in the green spectrum due to phycobiliproteins (Baier et al., 2004). PI also fluoresces in the red spectrum, but must be excited in the blue spectrum. Because the BacLight™ Kit uses a blue excitation spectrum, cyanobacteria autofluorescence was not anticipated to be a problem. However previous studies have suggested that red spectrum autofluorescence of cyanobacteria (Grilli Caiola et al., 1996; Dagnino et al., 2006) would interfere with attempts to use PI as a non-viable cell indicator (Sato et al., 2004). Therefore, we assessed whether autofluorescence would interfere with the BacLight™ fluorescence assay by performing a lambda scan on viable cyanobacteria cultures. This test used a blue spectrum excitation wavelength (488 nm), and monitored emissions ranging from 500 to 680 nm in 10 nm segments using an Olympus® Fluoview FV1200 Laser Scanning Confocal Microscope System interfaced with an IX81 Microscope (Olympus® Corporation, Tokyo, Japan). Autofluorescence results were negative, but to confirm similar results in the Synergy 2 Multi-Mode Microplate Reader (BioTek®, Winooski, VT, USA), we repeated the test with the following fluorescence filters as recommended in the kit protocol: excitation wavelength 485 ± 20 nm; green emission 528 ± 20 nm and red emission 620 ± 40 nm. Two hundred μ l of unstained cyanobacteria that had been grown to mid-log phase was pipetted into a microplate well and incubated in the dark at room temperature for 15 minutes. Fluorescence was measured by the microplate reader.

3.2.3 Protocol Development

Each cyanobacterial strain was grown to exponential phase under the conditions previously described, and then processed as shown in Figure 3.1 as per instructions in the BacLight™ kit. Viable and non-viable cells were mixed in ratios of 0:100, 10:90, 50:50, 90:10, and 100:0. The SYTO® 9 and PI dyes were prepared following the manufacturer's instructions. Equal volumes of the viable: non-viable cell solutions were then mixed with an equal volume of the stain solutions. Plates containing the stained cells were wrapped in aluminum foil and incubated in the dark at room temperature for 15 minutes. After incubation, the fluorescence in each well was measured using the microplate reader equipped with the fluorescence filters as previously described. For each trial, an empty well was used for a control to ensure the microplate was working properly.

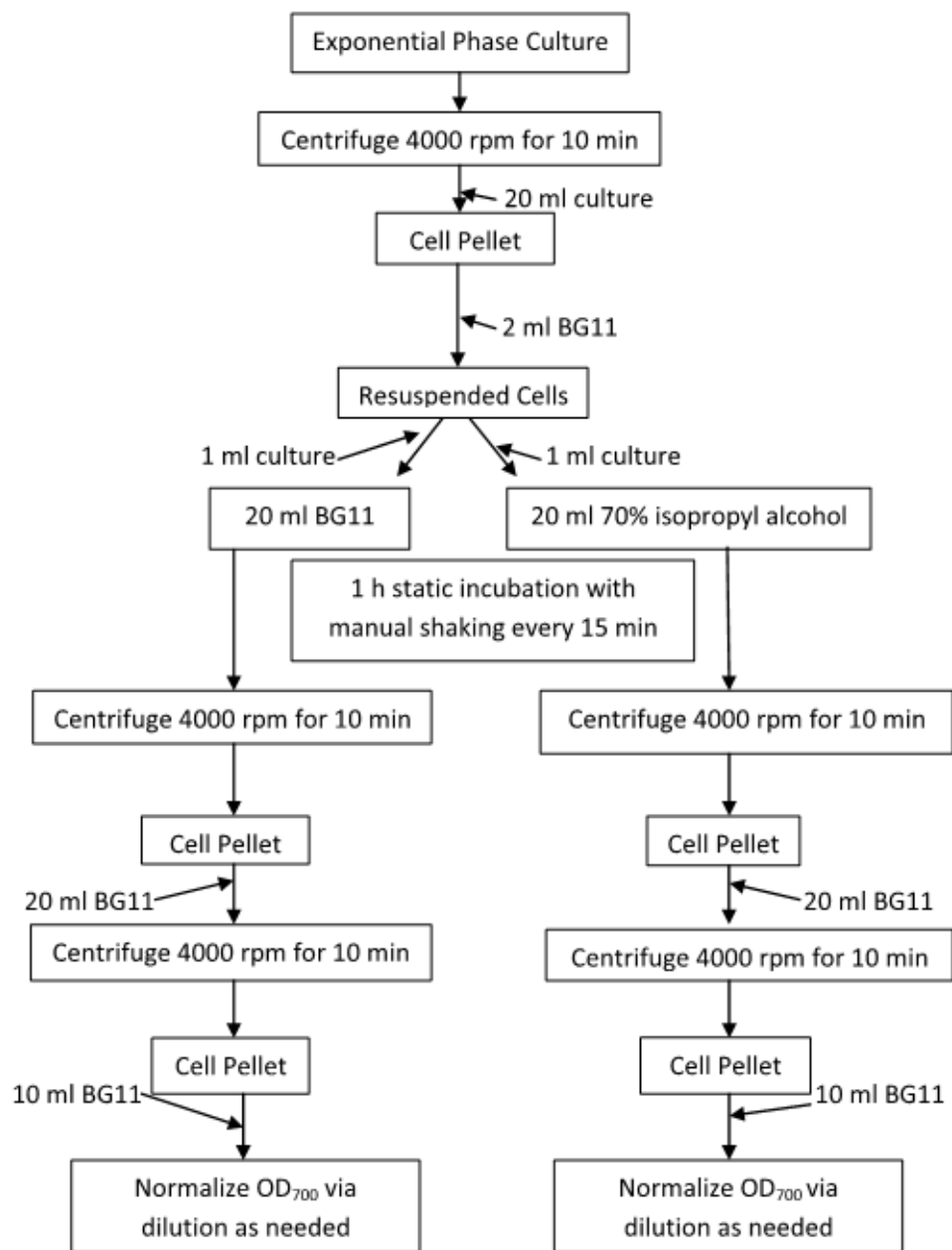


Figure 3.1: Process used to prepare living and dead cells for use in developing calibration curve.

(Isopropyl alcohol used to make cells non-viable. Centrifugations at 4,000 rpm for 10 min at room temperature. The final optical density normalization step was eventually omitted.)

Linear regression was then performed to plot the ratio of green and red fluorescence versus cell viability percentage. The ratios used to generate linear

calibration curves were calculated utilizing the equations in the protocol. (Invitrogen Molecular Probes, 2004) The data was analyzed by dividing the green fluorescence (F) emitted (em) by the red fluorescence emitted, ($\text{Ratio}_{G/R} = F_{\text{cell,em1}}/F_{\text{cell,em2}}$). This ratio was plotted as $\text{Ratio}_{G/R}$ versus percentage of viable cells in the suspension. Linear regression analysis was then performed to calculate the equation and assess the correlation coefficient. Stained and unstained BG11 were tested and acted as a control for the microplate reader to ensure it was working properly.

3.2.4 Microscopy

Confocal Laser Scanning Microscopy (CLSM) was used to evaluate possible binding of PI to cell components other than nucleic acids. For these observations, 488 nm was used as the excitation wavelength and 619 nm as the emission wavelength, and individual scans were made throughout the width of the cells. The staining patterns of the SYTO[®] 9 and PI dyes on viable and non-viable cyanobacteria strains were observed with an Olympus[®] AX70 wide-field epi-fluorescence microscope (Olympus[®] Corporation, Tokyo, Japan). The filter cube (U-MNB) used for these observations contained an excitation filter with a wavelength maximum at 488 nm and a long-pass emission filter that allowed all emitted wavelengths longer than 510 nm to be observed. These filter conditions were chosen to mimic the excitation and emission wavelengths used in the dual-fluorescence spectrophotometric assay of the viability kit, and to allow us to observe the dual presence of green and red photons (seen as a yellow color) within the fluorescence of the filamentous cyanobacteria strains to determine if the kit was working as intended. The presence of non-viable cells was created by 1 h incubation in 70% isopropanol.

3.2.5 Evaluation of Revised Protocol

Initial testing of the protocol shown in Figure 3.1 determined that the final step (normalizing viable vs. non-viable cell density based on optical density) resulted in inaccurate calibration curves. This was due to the presence of a blue pigment that was released from cells killed by the prior 1 h incubation in 70% isopropyl alcohol. Thus we eliminated the optical density normalization step and simply assumed that initially splitting the 2 ml cell re-suspensions into equal 1 ml quantities would maintain equal cell numbers through the rest of the protocol.

To test this modified protocol, we monitored cyanobacteria viability in experiments evaluating tolerance to the long chain alcohol linalool. Trials were conducted in sealed, 27 ml test tubes filled with 26 ml BG11, and supplemented with 0.5 g/L NaHCO₃. Due to the volatility of linalool, it was not possible to bubble CO₂-enriched air through the medium to provide carbon. Tubes were inoculated with an amount of a cyanobacteria culture re-suspended in 1 ml BG11 that yielded a starting OD₇₀₀ of approximately 0.1. Linalool levels of 0, 0.2, 0.4, and 0.6 g/L were added and tubes were incubated for 3 days at room temperature on a tube inverter (Thermo Scientific™, Waltham, MA, USA) at 15 rpm under constant illumination of 24 μmol m⁻² s⁻¹. Samples were taken at 0 and 72 h via a 1 ml syringe and needle pierced through the cap septa on the test tubes to prevent loss of linalool. One hundred μl samples were transferred to a 96 well plate, as well as 100 μl of a 2x stock solution of mixed SYTO[®] 9 and PI. The plate was then covered in aluminum foil, incubated for 15 min, and read as described previously. To measure optical density at the end of incubation, 1 ml samples were

measured at 700 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA).

3.3. Results and discussion

3.3.1 Analysis for Autofluorescence

Previously, researchers have expressed concerns that red spectrum autofluorescence of cyanobacteria (Grilli Caiola et al., 1996; Dagnino et al. 2006) would interfere with attempts to use PI as a non-viable cell indicator (Sato et al., 2004). To ensure that autofluorescence would not interfere with the results of this viability assay, the filamentous cyanobacteria strains used were analyzed for autofluorescence by direct microscopic evaluation. Unstained *Anabaena* sp. PCC 7120, *A. variabilis*, and *N. punctiforme* all exhibited strong autofluorescence in the red spectra (620 nm) when excited in the green spectra (528 nm) (data not shown), confirming prior findings (Grilli Caiola et al., 1996; Dagnino et al., 2006). However, when excited in the blue spectra (485 nm), as would occur with the BacLight™ method, no autofluorescence was observed microscopically (data not shown). However, the studies previously mentioned (Agustí et al., 2006; Sato et al., 2004) indicated that autofluorescence could still present a problem, thus further experimentation was conducted.

To confirm that autofluorescence would not be a problem when using the microplate reader, we tested the cyanobacteria cultures using an excitation wavelength of 485 ± 20 nm (blue), and emission wavelengths of 528 ± 20 nm (green) and 620 ± 40 nm (red). Table 3.1 provides evidence that autofluorescence from cyanobacterial chlorophyll (unstained) would have negligible effects on fluorescence readings from the BacLight™

assay using the microplate reader. Fluorescence in the green spectra was approximately 270-290 U and the red spectra was 300-650 U. While these are higher than the 200 U baseline reading from empty microplate wells, they represent relatively slight increases in intensity due to background noise (typical readings during the BacLight™ assay are several orders of magnitude higher). As noted previously, results from the confocal microscope lambda scan with an excitation of 488 nm also showed very weak autofluorescence in the 570-585 nm spectra range, but none at 620 nm, which is the emission filter wavelength used in the microplate reader for PI (data not shown). Collectively, these data demonstrate that autofluorescence should not significantly affect the PI results from the assay. This is in agreement with the expectations expressed by Agustí et al. (2006).

Table 3.1: Microplate readings of unstained filamentous cyanobacteria using an excitation wavelength of 485 ± 20 nm (blue).

Cyanobacteria Culture	Emission Wavelength		Ratio Green:Red	STD Ratio
	528 \pm 20 nm Green	620 \pm 40 nm Red		
	Intensity Units (U)			
<i>Anabaena</i> 7120	287	587	0.49	
	283	643	0.44	
	277	317	0.87	
Average	282	516	0.547	0.24
<i>A. variabilis</i>	281	302	0.93	
	279	287	0.97	
	278	285	0.96	
Average	279	291	0.955	0.03
<i>N. punctiforme</i>	284	552	0.52	
	288	582	0.50	
	284	611	0.47	
Average	285	582	0.489	0.03

3.3.2 Protocol Development

To establish the BacLight™ calibration curve, we initially followed the manufacturer's protocol that includes a step to equalize numbers of viable and non-viable cells by standardizing absorbance at 700 nm. Unfortunately, the 70% isopropanol treatment used to produce non-viable cells released a blue pigment into the solution, and

these biased absorbance readings compared to the solution containing un-treated (viable) cells. This blue pigment was likely the light harvesting pigment phycocyanin (PC) which exists in cyanobacteria (Eriksen, 2008). Therefore, the optical density equalization step was omitted from the protocol, and physical fractionation was used instead to generate solutions with approximately equal numbers of viable and non-viable cells.

The separate solutions of viable and non-viable cyanobacteria cells were then mixed in various ratios, subjected to the BacLight™ dual-staining protocol, and fluorescence was measured by the microplate reader. Mixtures of viable and non-viable *E. coli* NEB 10-beta were also stained to serve as a reference for performance of the BacLight™ assay, as others have previously reported (Invitrogen Molecular Probes, 2004; Boulos et al., 1999; Berney et al., 2007). Table 3.2 shows the full data set from the *E. coli* strain and all three cyanobacteria strains, as well as the regression equations for the lines plotting the ratio of green divided by red fluorescence versus percent cell viability. Figure 3.2 shows how this data was plotted for *Anabaena* sp. 7120 to generate the regression equation of green fluorescence (viable) divided by red fluorescence (non-viable) versus viable cell percentage. Figure 3.3 shows the plots and regression lines for all the cultures.

Table 3.2: Fluorescence assays of *E. coli* and three filamentous cyanobacteria strains.

(Each trial was completed in triplicate.)

Bacteria Culture	Emission Wavelength				
<i>E. coli</i> NEB 10-beta	528 ± 20 nm Green	620 ± 40 nm Red	Ratio Green:Red	STD Ratio	R²
Viability (%)	Intensity Units (U)				
0	28340	35870	0.790	0.026	
10	34532	28856	1.197	0.025	
50	50386	27546	1.829	0.008	
90	50340	20469	2.459	0.017	
100	54443	19186	2.838	0.003	
Regression equation	$y = 0.0186x + 0.985$				0.985
<i>Anabaena</i> sp. PCC 7120	Green	Red	Ratio	STD	R²
Viability (%)					
0	3520	4972	0.708	0.017	
10	6154	5125	1.199	0.085	
50	10788	5680	1.878	0.469	
90	21886	6377	3.430	0.390	
100	22261	6781	3.284	0.035	
Regression equation	$y = 0.0266x + 0.7703$				0.971
<i>A. variabilis</i> ATCC 29413	Green	Red	Ratio	STD	R²
Viability (%)					
0	2215	3701	0.600	0.039	
10	2773	3529	0.786	0.016	
50	4763	3183	1.496	0.050	
90	6709	2925	2.293	0.092	
100	6313	2790	2.263	0.025	
Regression equation	$y = 0.0175x + 0.613$				0.992
<i>N. punctiforme</i> ATCC 29133	Green	Red	Ratio	STD	R²
Viability (%)					
0	3133	6724	0.466	0.003	
10	3378	6747	0.500	0.029	
50	4431	5604	0.791	0.015	
90	8373	4911	1.705	0.084	
100	9715	4884	1.988	0.064	
Regression equation	$y = 0.0152x + 0.3321$				0.937

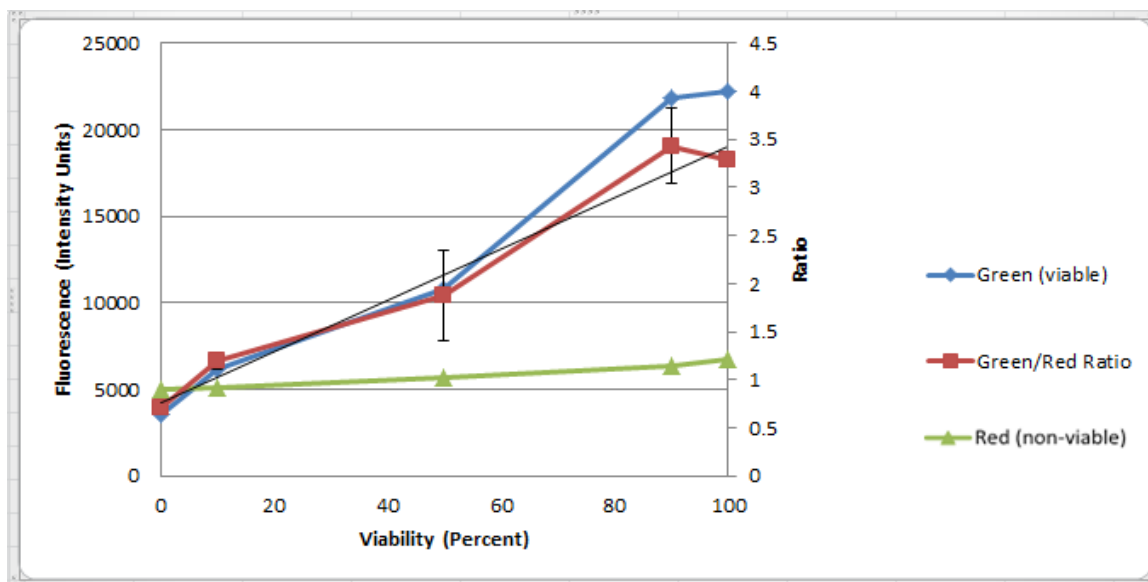


Figure 3.2: Fluorescence of dual-stained *Anabaena* sp. 7120.
(The black line represents the least-squares fit of the green/red ratio versus % viability.)

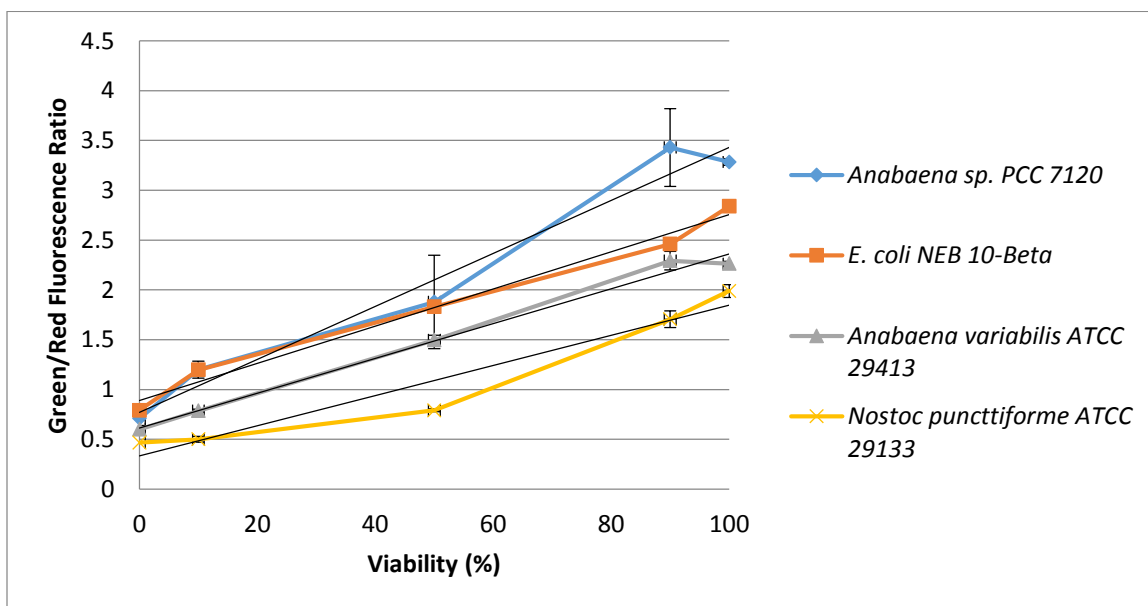


Figure 3.3: Green/red fluorescence versus viability plots and regression lines for *E. coli* and the 3 filamentous cyanobacteria strains.
(Each trial was completed in triplicate.)

E. coli NEB 10-beta performed as expected, with green fluorescence (viable cells) showing a strong positive slope and red fluorescence (non-viable cells) showing an equally strong negative slope as the percentage of viable cells increased (Table 3.2). The regression equation of green divided by red fluorescence versus percent cell viability had a strong positive slope and a high R^2 value.

The cyanobacteria strains showed even greater positive slopes for green fluorescence (viable), especially in the case of *Anabaena* sp. 7120. Unfortunately, the red fluorescence plot of *Anabaena* sp. 7120 had an unexpected slight positive slope, and the slopes for the other two cyanobacteria were only slightly negative (Table 3.2). Based on the *E. coli* results, we had anticipated that the red fluorescence data from the cyanobacteria would also yield negative slopes of approximately the same intensity as the positive slopes of the green fluorescence data. This would have resulted in calibration plots with significantly higher slopes than those shown in Figure 3.3. The poor correlation of red fluorescence with cell viability led us to believe that PI was not staining properly, and appeared to be staining both viable and non-viable cells. Thus we evaluated the stained cells via microscopy.

3.3.3 Microscopy

We stained mixtures of viable and non-viable filamentous cyanobacteria cells with SYTO[®] 9 and PI dyes and then observed them using a wide field epi-fluorescence microscope to mimic conditions of the microplate reader. Assuming these dyes worked as intended in the BacLight[™] assay, we should have only observed either green (viable) or red (non-viable) cells. Figure 3.4 shows micrographs of *Anabaena* sp. PCC 7120, and

similar results were observed for the other strains. While Figure 3.4A does show green and red cells, it also shows numerous cells fluorescing yellow, which indicates that both SYTO[®] 9 and PI are simultaneously staining these cells. This should not occur in viable cells, since PI is not able to cross intact membranes, and thus these cells should stain green. In non-viable cells, PI is supposed to completely displace SYTO[®] 9, resulting in red cells. These principles are the basis of the BacLight[™] viability assay.

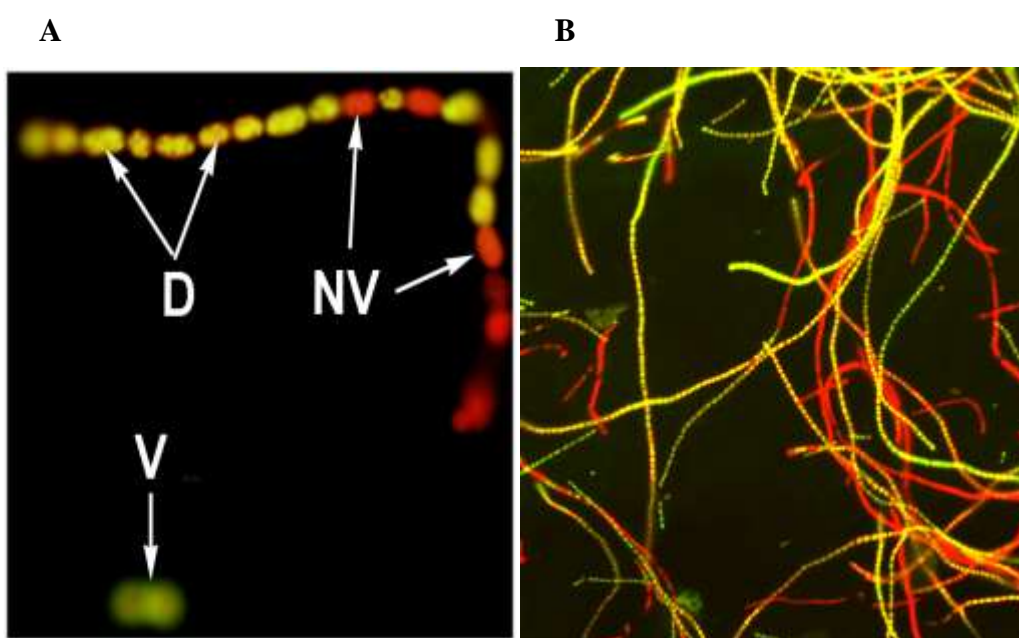


Figure 3.4: Wide-field epi-fluorescence photomicrograph of *Anabaena* sp. PCC 7120 filaments stained with SYTO[®] 9 and PI dyes.

(Green staining viable cells (V), red staining non-viable cells (NV), yellow staining cells that are presumably dying (D)).

This observation of dual binding of SYTO[®] 9 and PI has been reported previously, and could possibly be due to damaged cells that are dying (Stocks, 2004; Alakomi et al., 2006; Nybom et al., 2008). An interesting observation in Figure 3.4A is that the filament contains non-viable (red), damaged (yellow), and even lysed (black)

cells. In another sample (Fig. 3.4B) we noticed several filaments containing viable (green), non-viable (red), and damaged (yellow) cells in the same filament. This does not concur with a study conducted by Agustí et al. (2006) who reported that all cells within a single filament are exclusively either viable or non-viable.

The observation that PI appeared to be binding to viable cells led us to postulate that it was binding to components of the cell other than the nucleic acids, or was somehow able to enter viable cells, perhaps by mechanisms that these filamentous cyanobacteria use to exchange nutrients such as fixed nitrogen (Buikema and Haselkorn, 1991). Therefore, CLSM was used to observe non-viable cells stained with PI to determine if the stain was binding elsewhere. The confocal microscope was initially centered in the middle of the cell, and photos were taken from that point and subsequently “outward” using the same excitation/emission filters that were used in the microplate reader. Since confocal microscopy results in greyscale images, the red color in Figure 3.5 was added to emulate where fluorescence from PI would have appeared. Figure 3.5A is from the middle plane of the bacterial cells and exhibits the most fluorescence, indicating that PI does bind within the cells. Figure 3.5B is a cross-section approximately 3 μm above the prior photo, showing much less fluorescence in the membrane, indicating minimal PI binding to the membrane components of *Anabaena* sp. 7120. These are the expected results for PI staining of non-viable cells.

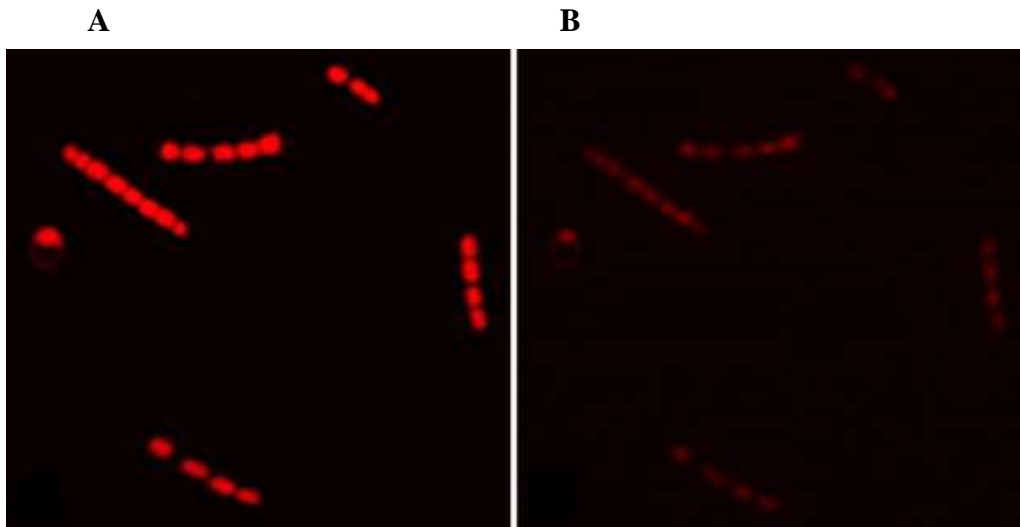


Figure 3.5: Single XY slice photomicrographs of non-viable *Anabaena* sp. PCC 7120 stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kit.

(Figure 3.5A shows a slice through the middle of the cells, while Figure 3.5B shows a slice towards the edge of the cells.)

CLSM was then used to observe viable *Anabaena* sp. PCC 7120 stained with SYTO® 9 (Fig. 3.6A) versus PI (Fig. 3.6B). SYTO® 9 stained the viable cells green as expected, however PI also stained the viable cells red. As noted above, this was not expected, since intact cell membranes are not permeable to PI. Thus, there must be some unique feature in filamentous cyanobacteria cells that allows entry of PI. This may be related to the mechanisms by which cells exchange nutrients such as fixed N that is produced in the heterocysts (Buikema and Haselkorn, 1991).

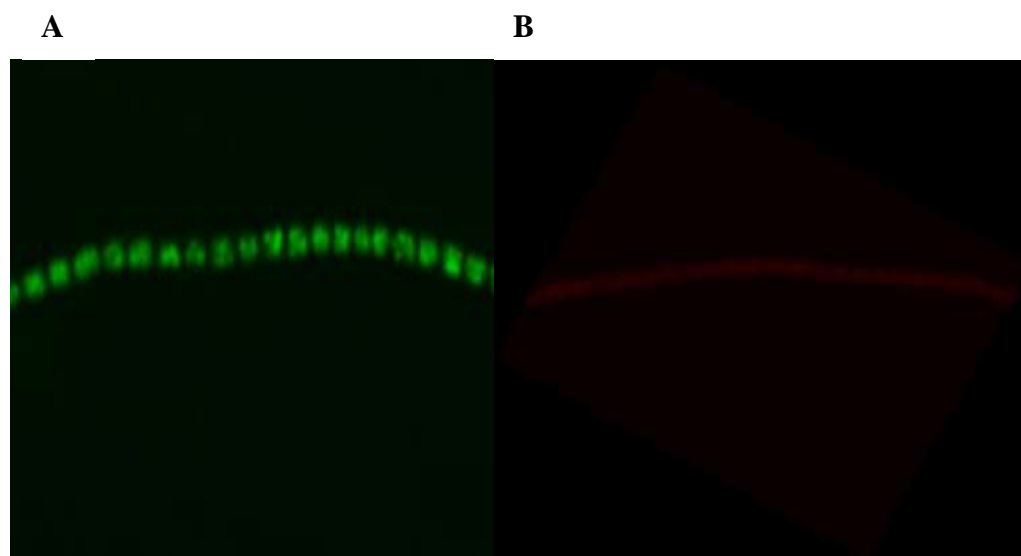


Figure 3.6: Single XY slice photomicrographs of viable *Anabaena* sp. PCC 7120 stained with the LIVE/DEAD® BacLight™ Bacterial Viability Kit.

(Figure 3.6A used excitation/emission filters of 488/528 nm, while Figure 3.6B used excitation/emission filters of 488/620 nm. The photomicrographs are taken from an identical filament.)

3.3.4 Evaluation of Revised Protocol

Since SYTO® 9 appeared to stain viable cells properly and the green/red ratio resulted in a positive slope (in spite of the fact that PI was staining both viable and non-viable cells), we proceeded to test whether the BacLight™ viability kit could still be used on filamentous cyanobacteria. Using *Anabaena* 7120, we first developed several replicate calibration curves that are shown in Figure 3.7. Unfortunately, the correlation coefficients for the lines of best fit are relatively low, showing poor precision. The lines also show a lack of accuracy, in that the slopes and y-axis interception points are quite different. When the calibration curve from Trial 3 was used to assess viability of *Anabaena* 7120 in linalool tolerance trials, initial (0 h) viabilities ranged from -15% to 36%. During subsequent incubation, culture viability exceeded 300% in some tubes (data not shown).

Therefore, the indiscriminate staining of the PI dye resulted in calibration curves that could not accurately predict cell viability.

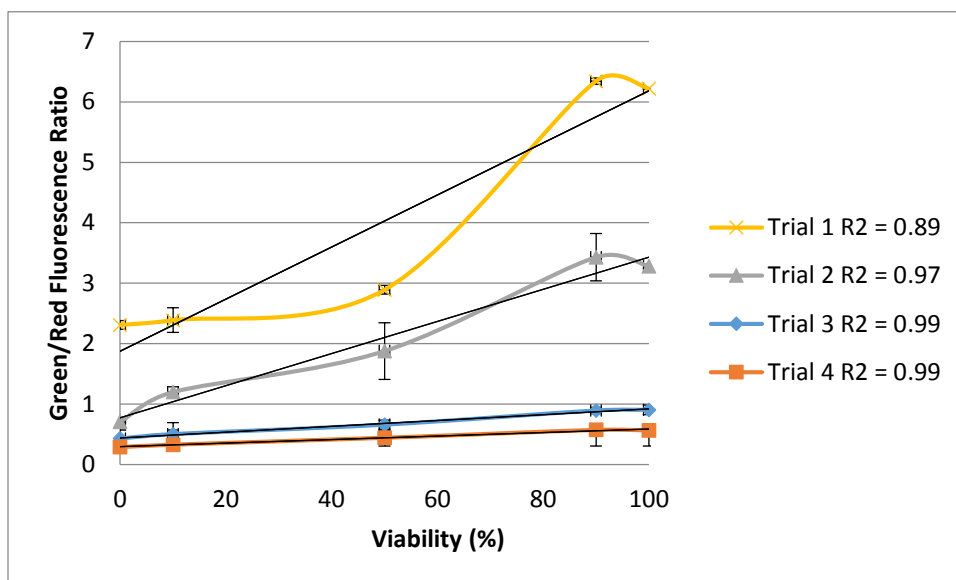


Figure 3.7: Calibration curves and regression lines of *Anabaena* sp. PCC 7120. (Each trial was completed in triplicate.)

3.4. Conclusions

This study showed that red fluorescence from PI is not a reliable indicator of non-viable cells for the filamentous cyanobacteria strains tested. Instead, we observed that PI also stained viable cells, resulting in virtually flat slopes when red fluorescence was plotted against cell viability percentage. In the BacLight™ viability assay, green fluorescence from SYTO® 9 (viable cells) is divided by red fluorescence from PI (non-viable cells) for known mixtures of viable and non-viable cells, and the ratios are plotted against cell viability to generate calibration equations. The inaccuracy of the PI readings resulted equally inaccurate calibration equations that under- and over-estimated viabilities in subsequent tests.

PI is not capable of passing through the membranes of intact cells, and therefore should not be able to stain viable cells. We theorize that the staining we observed may be related to the fact that filamentous cyanobacteria are known to have a system for intercellular communication and sharing of nutrients (Buikema and Haselkorn, 1991). For example, nitrogen fixing heterocysts occur every 10 cells in a filament, and yet the fixed nitrogen is shared with other cells in the filament (Flores and Herrero, 2010). Several other hypotheses have been presented to explain why PI is not a reliable indicator of non-viable cells. One is that DNA may leak from damaged membranes of viable cells, causing background fluorescence (Sato et al., 2004). Another is that PI might bind non-specifically to non-biological materials (Biggerstaff et al., 2006). A third is that membrane permeability may be altered depending on the growth phase of the organism (Shi et al, 2007).

Developing a methodology capable of accurately measuring the viability of filamentous cyanobacteria is essential for research extending from wastewater treatment plants to 3rd generation biofuel laboratories. Other fluorochromes that have been used in cyanobacteria research might prove beneficial in replacing PI as a means of quantifying non-viable cell numbers. These include: 1) SYTOX[®] Orange which has been used in wastewater research (Biggerstaff et al., 2006), 2) SYTOX[®] Green which has been studied with various species of cyanobacteria and algae (Sato et al., 2004), and 3) SYTOX[®] Blue which has been used to study the intracellular delivery of proteins in unicellular cyanobacteria (Liu et al., 2013).

Acknowledgements

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Chapter 4 - Evaluating viable cell indicators for filamentous cyanobacteria and their application

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Abstract

Filamentous cyanobacteria have great industrial potential due to their capability to be genetically engineered to produce next-generation biofuels while using minimal nutrients. One challenge of working with these microbes is that classical methods of quantifying cell viability are not effective due to their filamentous morphology. Therefore, fluorescent assays were evaluated to determine if they could be used as a reliable indicator of cell viability. Initially, a dual-stain assay using SYTO[®] 9 and SYTOX[®] Blue was investigated. Fluorescence from SYTO[®] 9 was accurately correlated with viable cells; however, SYTOX[®] Blue did not work as a non-viable cell indicator in filamentous cyanobacteria due to non-specific binding in both viable and non-viable cells. Autofluorescence from light harvesting pigments was also evaluated as a viable cell indicator. Unfortunately, these pigments resulted in several emission peaks that couldn't be captured by a single emission filter. Moreover, certain light harvesting pigments continued to fluoresce after the cell became non-viable. SYTO[®] 9 was then compared to absorbance and chlorophyll content to quantify viable filamentous cyanobacteria in a chemical inhibition testing protocol. This protocol requires a low initial biomass

concentration to prevent binding of the chemicals to cell biomass, and at low cell densities SYTO[®] 9 was superior to absorbance and chlorophyll content in quantifying viability. It was also determined that SYTO[®] 9 was capable of evaluating different cultivation media on the growth of cyanobacteria in photobioreactors. SYTO[®] 9 is a reliable, accurate indicator of viability of filamentous cyanobacteria and can be used in a high-throughput manner.

4.1 Introduction

Developing processes to produce renewable fuels and chemicals remains important, as fossil fuel reserves are finite and the adverse effects of fossil-fuel generated greenhouse gases are well documented (Chen et al., 2011; Von Blottnitz and Curran, 2007). Using filamentous cyanobacteria for this purpose is an attractive option as they are capable of producing biofuels and chemicals from CO₂ and solar energy (Machado and Atsumi, 2012). For example, filamentous cyanobacteria have already been engineered to produce next-generation biofuels such as limonene (Halfmann et al., 2014b), farnesene (Halfmann et al., 2014a), and linalool (Gu et al., 2012). Many strains of filamentous cyanobacteria are also diazotrophic, using specialized cells called heterocysts to fix atmospheric nitrogen. These attributes have led to cyanobacteria emerging as a promising platform organism for production of fuels and chemicals (Schoepp et al., 2014).

Classical methods of monitoring cell viability, such as viable cell counts and flow cytometry, will not work for filamentous cyanobacteria due to their filamentous morphology (Johnson et al., 2015). Sarchizian and Ardelean (2012), do report using the direct viable count method with epi-fluorescence microscopy on filamentous

cyanobacteria isolated from a mesothermal spring for quantification of viable cells. However, many strains of filamentous cyanobacteria tend to aggregate in liquid media making accurate direct counts via microscopy quite difficult. Cell viability information is critical for research purposes (e.g., screening mutants for increased tolerance to biofuels they are engineered to produce), as well as to monitor cyanobacteria performance in photobioreactor (PBR) systems (Jin et al., 2014).

One potential option for quantifying viability of filamentous cyanobacteria is the LIVE/DEAD[®] BacLight[™] Bacterial Viability Kit (Life Technologies[™], Carlsbad, CA, USA), which has been successfully used to quantify viability of several types of bacteria (Magajna and Schraft, 2015; Zeidán-Chuliá et al., 2015), including cyanobacteria (Zhu and Xu, 2013). However, we determined that this kit does not allow accurate viability estimates of filamentous cyanobacteria, since the non-viable cell indicator propidium iodide (PI) also crossed the intact membranes of viable filamentous cyanobacteria, thereby leading to erroneous results (Johnson et al., 2015). Thus, alternative methods to quantify viability in filamentous cyanobacteria need to be developed.

SYTOX[®] Blue is one of several SYTOX[®] Dead Cell Stains that should not cross intact cell membranes. It has been successfully used to quantify non-viable cells in bio-aggregates (Chen et al., 2007) and in aerobic granules (Adav et al., 2007a). Therefore, in this study it was tested as a method to quantify non-viable filamentous cyanobacteria. Autofluorescence from the light harvesting pigments phycobiliproteins (PBS) and chlorophyll α offer additional options to quantify viability in filamentous cyanobacteria and were also tested in this study. Fluorescence from the viable cell indicator SYTO[®] 9 was compared to absorbance and chlorophyll α content to determine which is a superior

method for monitoring viability in conditions where biomass content is minimal. Finally, SYTO[®] 9 was evaluated for monitoring the effect of different cultivation media on cyanobacterial growth in 40 L PBRs. A reliable, accurate viability assay for filamentous cyanobacteria would have broad applications such as monitoring cultures and studying gene function.

4.2 Materials and methods

4.2.1 Microbial strains, maintenance, and culture conditions

The filamentous, diazotrophic cyanobacterial strain *Anabaena* sp. PCC 7120, a model specie for cyanobacteria (Bryant, 2006; Rippka et al., 1979), was obtained from its respective culture collection. A putative mutant of *Anabaena* sp. 7120, referred to hereafter as A7120.(0.32t).farn was isolated during prior trials in which *Anabaena* sp. 7120 was acclimated to 0.32 g/L farnesene. For long term storage, strains were frozen at -80°C in 5% v/v methanol. For short term maintenance the cyanobacteria were grown on BG11 agar (1.5% agar) (Allen and Stanier, 1968) at pH 7.1 and incubated at room temperature under constant illumination of 24 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and then stored at room temperature. Light intensities were measured with a Heavy Duty Light Meter with PC Interface (Extech Instruments, Waltham MA, USA).

In the experiments described below, cyanobacterial cultures were grown either in 27 ml screw capped test tubes, 250 ml Erlenmeyer flasks, or in 40 L PBRs. The 27 ml test tubes had an open top cap and used PTFE/silicone septa to allow inoculation and sampling via syringe and needles, and yet prevent the loss of volatile chemicals that were being tested for cell toxicity. Tubes were filled with ~27 ml BG11 with 20 mM HEPES

buffer and 0.5 g/L NaHCO₃ for a carbon source. The tubes were incubated at room temperature under constant illumination of approximately 24 $\mu\text{mol m}^{-2} \text{s}^{-1}$ while rotating at 8 rpm in a Thermo Fisher Scientific™ Labquake™ Tube Rotator (Thermo Fisher Scientific™, Waltham, MA, USA). The 250 ml Erlenmeyer flask trials contained 100 ml of BG11 broth at pH 7.1 supplemented with 20 mM HEPES buffer. Flasks were stoppered with a foam stopper and the opening covered with aluminum foil. The flasks were incubated in a Lab-Line® Incubator-Shaker (Lab-Line® Instruments, Melrose Park, IL, USA) at 30° C and 100 rpm under constant illumination of 19 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using fluorescent lights.

PBR trials were conducted in 40 L transparent fiberglass flat bottom tanks (Solar Components Corp., Manchester, NH, USA) that were sparged from the bottom with a mixture of 95-5% air-CO₂ at a rate of 0.25 L/L/min. The culture medium consisted of 30 L of BG11 without HEPES buffer and was inoculated with 1.5 L (5%) of an *Anabaena* sp. 7120 culture that had been grown to mid-log phase. The reactors were incubated until 2 days after stationary phase was reached at room temperature (20-22° C) under constant illumination of approximately 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using fluorescent lights.

4.2.1 Evaluating SYTOX® Blue and SYTO® 9 as a dual-stained assay to quantify viability

To determine if SYTOX® Blue fluoresces non-viable cyanobacterial cells at the expected excitation/emission wavelengths, 1 ml of a mid-log phase culture of *Anabaena* sp. 7120 was chemically killed by exposing it to 10 ml of 70% isopropanol in a 15 ml conical test tube that was incubated at room temperature for 1 h with manual mixing every 15 min. The cells were then recovered by centrifuging at 10,000 rpm for 10 min at

room temperature. The cell pellet was then washed in 1 ml BG11 and centrifuged at 10,000 rpm for 1 min at room temperature. This was repeated two additional times and the final cell pellet was re-suspended in 1 ml BG11. SYTOX[®] Blue was added to a final concentration of 5 μ M. A 100 μ l aliquot of the culture was transferred to a microscope slide for examination by a Cytation[™] 3 Cell Imaging Multi-Mode reader (BioTek[®] Instruments, Inc., Winooski, VT, USA). Cyanobacterial cells were observed for fluorescence in the blue spectra by the reader at the excitation/emission maxima for SYTOX[®] Blue when the dye is bound to DNA (444/480 nm).

To evaluate a dual-stain assay using SYTO[®] 9 (for viable cells) and SYTOX[®] Blue (for non-viable cells), *Anabaena* sp. 7120 was grown to mid-log phase in a 250 ml Erlenmeyer flask under the conditions previously described. Ten ml of the culture was transferred to a 15 ml conical and processed as shown in Figure 4.1 to obtain solutions containing equal numbers of viable and non-viable cells. These were mixed in ratios of 0:100, 10:90, 50:50, 90:10, and 100:0. One hundred μ l of viable: non-viable cell solutions were pipetted into wells of a 96-well plate. SYTO[®] 9 and SYTOX[®] Blue were added to each well at a concentration of 5 μ M for each stain. The plates were then wrapped in aluminum foil and incubated in the dark at room temperature for 15 min. After incubation, fluorescence was measured by a Synergy 2 Multi-Mode Microplate Reader (BioTek[®], Winooski, VT, USA). To measure green fluorescence from SYTO[®] 9 stained cells, a fluorescence filter with excitation wavelength 485 ± 20 nm; emission wavelength 528 ± 20 nm was used. To measure blue fluorescence from SYTOX[®] Blue stained cells, a fluorescence filter with excitation wavelength 440 ± 30 nm; emission

wavelength 485 ± 10 nm was used. For each trial, an empty well was used as a negative control to ensure the microplate reader was working properly.

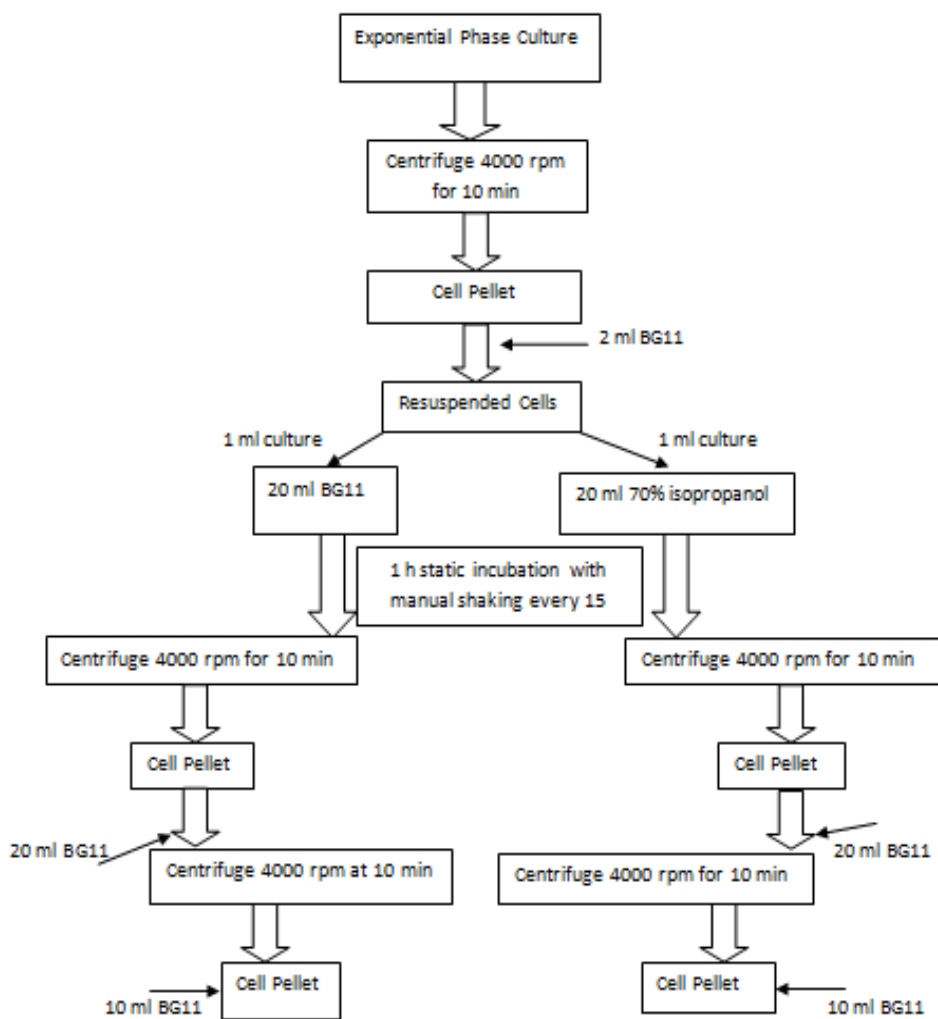


Figure 4.1: Process used to prepare viable and non-viable cells for use in developing calibration curve.

The resulting samples were mixed in ratios of 0:100, 10:90, 50:50, 90:10, and 100:0. (Isopropyl alcohol used to make cells non-viable).

Linear regression was performed to plot the ratio of green and blue fluorescence vs. cell viability percentage. The data was analyzed by dividing the green fluorescence (F) emitted (em) by the blue fluorescence emitted, ($\text{Ratio}_{G/B} = F_{\text{cell,em1}}/F_{\text{cell,em2}}$). The ratio was plotted as $\text{Ratio}_{G/B}$ versus cell viability percentage. Then, linear regression analysis was performed to calculate and assess the correlation coefficient. To further investigate SYTOX[®] Blue as a non-viable cell indicator, fluorescence data was analyzed from the microplate reader. Each well contained 100 μl of either *Anabaena* sp. 7120 or BG11, as well as both SYTO[®] 9 and SYTOX[®] Blue or one stain individually at a final concentration of 5 μM . An empty well was used as a control. To measure fluorescence from the stains the filters that were previously described were used.

4.2.2 Comparison of SYTO[®] 9 and autofluorescence as viable cell indicators

To compare SYTO[®] 9 and autofluorescence as viability assays in filamentous cyanobacteria, a 40 L PBR containing 30 L BG11 was inoculated with 1.5 L (5%) of a mid-log phase culture of *Anabaena* sp. 7120. This inoculum had been grown in a 4 L Erlenmeyer flask containing 2 L BG11 in a Lab-Line[®] Incubator-Shaker under the conditions previously described. After inoculation, the PBR was incubated under the conditions previously described, and samples were taken daily for absorbance (OD_{700}) and viability until stationary phase was reached. For absorbance, 1 ml samples were measured using a Thermo Fisher Scientific[™] Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific[™], Waltham, MA, USA).

To measure viability, 100 μl samples were transferred to a 96-well plate. SYTO[®] 9 was added to each well at a concentration of 5 μM and fluorescence was measured by

the microplate reader using the excitation/emission wavelength spectra described above. For viability measurements via autofluorescence, 100 μ l samples were transferred to a 96-well plate. As there are various wavelengths used in the literature, several excitation/emission filters were tested on the microplate reader to determine which yielded the greatest fluorescence intensity of a mid-log phase culture of *Anabaena* sp. 7120. This was determined to be an excitation wavelength of 540 ± 25 nm and an emission wavelength of 620 ± 40 nm.

4.2.3 Assessing cell viability in a chemical inhibition test

To investigate if fluorescence from SYTO[®] 9 allows for the screening of putative filamentous cyanobacterial mutants, SYTO[®] 9 stain was evaluated for use in measuring the viability of filamentous cyanobacteria that were exposed to farnesene in chemical inhibition tests. Because farnesene is volatile (Asai et al., 2016; Ruther and Hilker, 1998), screening and acclimation trials had to be conducted in sealed test tubes to maintain a constant titer of the chemical. As no atmospheric CO₂ was able to enter the test tube, 0.5 g/L NaHCO₃ was added to the BG11 medium after autoclaving to serve as the carbon source (Hailing-Sørensen et al., 1996; Mayer et al., 2000).

Twenty-seven ml test tubes containing ~27 ml of NaHCO₃-supplemented BG11, along with 0.032 g/L farnesene, were inoculated with 270 μ l (1%) of either a mid-log phase *Anabaena* sp. 7120 culture or a putative mutant A7120(0.32t).farn. Cultures were incubated under conditions previously described. Daily samples were taken via a needle and syringe in order to maintain constant titers of farnesene. For viability determinations, 100 μ l samples were transferred to a microplate. SYTO[®] 9 was added to each well at a

concentration of 5 μM , and fluorescence was determined as described above. Absorbance and chlorophyll α were measured by diluting 100 μl samples with 900 μl BG11 in a cuvette. Absorbance was measured at an optical density (OD) of 700 nm. Chlorophyll α was determined using the equation: $\text{Chl } \alpha \text{ content } (\mu\text{g/ml}) = 14.96 (\text{OD}_{678} - \text{OD}_{750}) - 0.616 (\text{OD}_{720} - \text{OD}_{750})$, and the protocol described by Guoce et al., (2011).

4.2.4 Assessing cell viability in photobioreactors

To investigate if fluorescence from SYTO[®] 9 allows for the comparison of different treatments on the viability of filamentous cyanobacteria in PBRs, *Anabaena* sp. 7120 was cultivated in various growth media. The cultivation media evaluated were as follows: BG11 (standard BG11 includes sodium nitrate), BG11 supplemented with urea rather than sodium nitrate (BG11_U), BG11 without a nitrogen source (BG11₀), and tap water. The cultivation media containing nitrogen sources (BG11 and BG11_U) contained 0.248 g/L nitrogen. The PBRs previously described were inoculated with 1.5 L (5%) of a mid-log phase culture of *Anabaena* sp. 7120. The PBRs were incubated in the conditions previously described. For daily viability determinations, 100 μl samples were transferred to a microplate. SYTO[®] 9 was added to each well at a concentration of 5 μM , and fluorescence was determined as described above.

4.3 Results and discussion

4.3.1 Evaluating SYTOX[®] Blue and SYTO[®] 9 as a dual-stain assay for quantifying viability

SYTOX[®] Blue is a nucleic acid binding fluorochrome commonly used to stain non-viable bacterial cells, as it should not be able to penetrate an intact cell membrane (Adav and Lee, 2008; Krause et al., 2007; Truernit and Haseloff, 2008). SYTO[®] 9 is a fluorescent dye capable of penetrating most cellular membranes and causing all cells containing nucleic acids to fluoresce green (Lee and Rhee, 2001; Shi et al., 2007). SYTOX[®] Blue was used as an alternative to the non-viable cell indicator PI, that was previously shown by Johnson et al., (2015) to not work as a non-viable cell indicator in filamentous cyanobacteria because it also stained viable cells. This was presumed to be due to intercellular channels that allow nutrient passage between cells in the filaments (Buikema and Haselkorn, 1991).

Previously we had confirmed that SYTO[®] 9 caused viable *Anabaena* sp. 7120 cells to fluoresce in the green spectra (Johnson et al., 2015). Using the Cytation[™] 3 microplate reader, we confirmed that SYTOX[®] Blue caused non-viable *Anabaena* sp. 7120 cells to fluoresce in the blue spectra (data not shown). These observations were consistent with other reports and information from the company (Adav et al., 2007b; Chen et al., 2007; Filoche et al., 2007; Sato et al., 2004; Zhu and Xu, 2013).

To determine if a dual-stain assay consisting of SYTO[®] 9 and SYTOX[®] Blue is capable of accurately quantifying the viability of filamentous cyanobacteria, calibration curves were generated to correlate the ratio of green (SYTO[®] 9, viable cells) to blue

(SYTOX[®] Blue, non-viable cells) fluorescence against known mixtures of viable and non-viable cells. Figure 4.2 shows the plots and regression lines for three trials. All trials had high R² values (0.96-0.98). Trials 1 (0.0129) and 3 (0.0101) had similar slopes, compared to Trial 2 (0.006). Trials 1 (0.7186) and 2 (0.8457) had virtually identical y-intercepts compared to Trial 3 (1.6693). The variability in the results between the three trials indicated that there was a problem with the dual-stain assay.

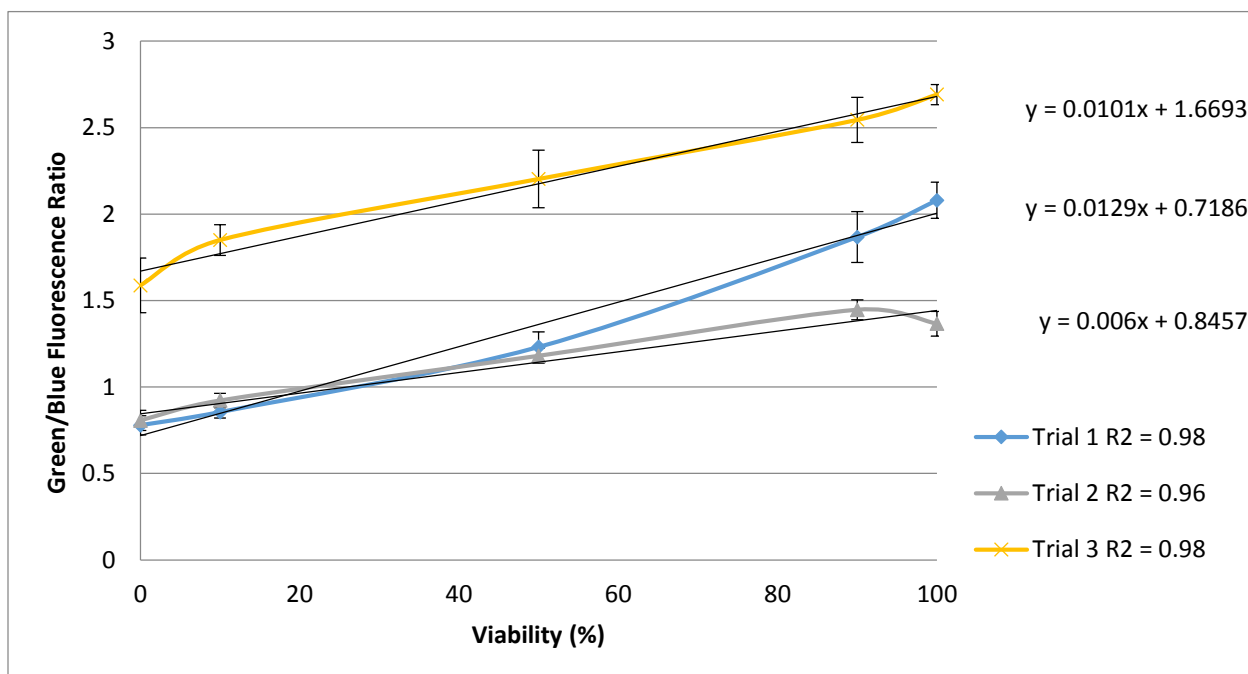


Figure 4.2: Viability vs. green/blue fluorescence ratios for *Anabaena* sp. PCC 7120. In each trial triplicate SYTO[®] 9 green and SYTOX blue fluorescence measurements were taken for each mixture of viable and non-viable cells. This data was used to calculate the green/blue fluorescence ratio. Error bars represent the standard deviation. The black lines represent the least-squares fit.

To investigate why the correlation equations were not identical for the three trials shown in Figure 2, we assessed the underlying data from Trial 1. Figure 4.3 shows the separate plots for green (SYTO[®] 9) and blue (SYTOX[®] Blue) fluorescence vs. % viability from Trial 1, as well as the calculated ratio. Fluorescence from the SYTO[®] 9 stain

provided the expected positive slope of green fluorescence intensity over percent viability. However, fluorescence from the SYTOX[®] Blue stain resulted in a relatively flat slope instead of the anticipated strong negative slope. This indicates that SYTOX[®] Blue is staining both non-viable and viable cells, which is similar to what occurred with PI with filamentous cyanobacteria (Johnson et al., 2015). Thus, it appears that the SYTO[®] 9 and SYTOX[®] Blue dual-stained assay is not precise in this application. Other researchers have noted the occurrence of misinterpretations in live/dead staining results for various reasons (Bridier et al., 2015; Johnson and Criss, 2013; Lu et al., 2014; Stiefel et al., 2015).

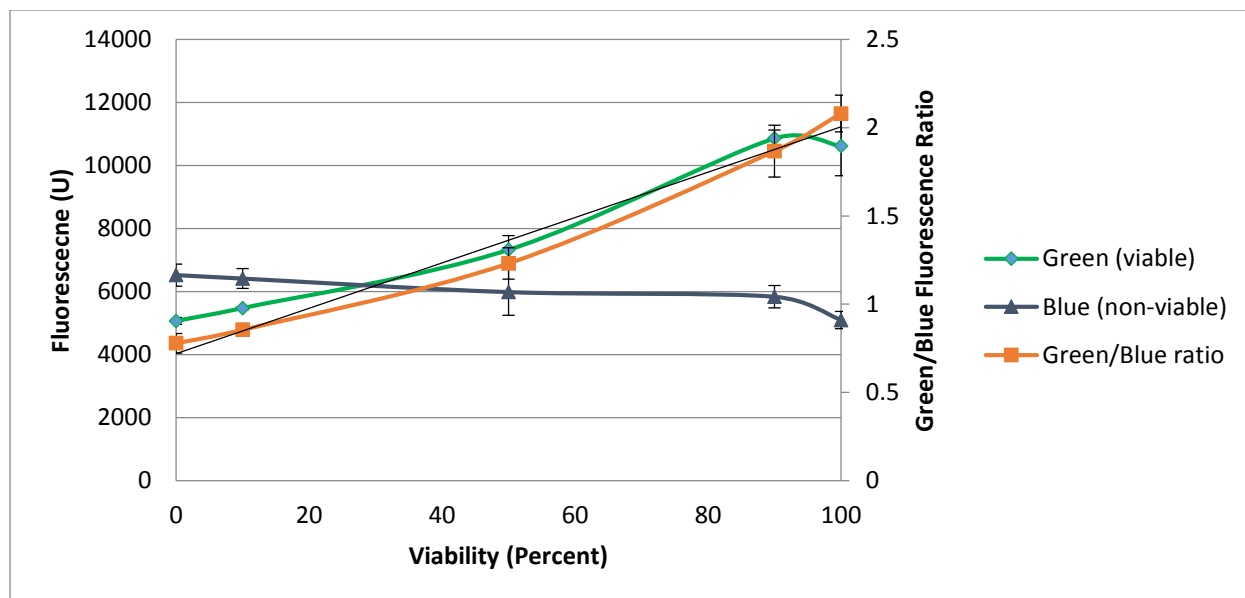


Figure 4.3: Viability vs green (viable) and blue (non-viable) fluorescence for *Anabaena* sp. 7120.

(Each data point represents the average of triplicate readings, and error bars represent the standard deviation. This data was used to calculate the green/blue fluorescence ratio, which is also shown, along with the least-squares fit line (black line)).

To ensure that the BG11 medium was not the cause of inaccuracy in the dual-stain assay, raw data from the microplate reader was also analyzed for samples containing the medium only, as well as the medium with 100% viable cells. This data is shown in Table 4.1. For green fluorescence the average fluorescence intensity readings (arbitrary unit: U) were 433 for the empty well, which could be considered background fluorescence. For BG11 + SYTO[®] 9 the intensity was 1,835 U. This value is about 4x higher than the empty well, and represents the baseline green fluorescence of the medium and the SYTO[®] 9 dye. BG11 + SYTOX[®] Blue intensity was 325 U. This shows that the SYTOX[®] Blue dye did not provide any green fluorescence, since the intensity was about the same as the background fluorescence reading. BG11 + SYTO[®] 9 + SYTOX[®] Blue resulted in a fluorescence reading of 1,767 U. This green fluorescence intensity was approximately the same as observed when just SYTO[®] 9 was added. The data from the controls described above is what would be expected.

Table 4.1: Microplate readings of *Anabaena* sp. PCC 7120 evaluating fluorescence intensity [arbitrary units (U)] of SYTO[®] 9 and SYTOX Blue.
Averages and standard deviations are representative of a triplicate.

	Empty Well	BG11 + SYTO 9	BG11 + SYTOX Blue	BG11 + 2 dyes	<i>Anabaena</i> 7120 + SYTO 9	<i>Anabaena</i> 7120 + SYTOX Blue	<i>Anabaena</i> 7120 + 2 dyes
SYTO[®] 9 (Ex 485 ± 10 Em 528 ± 20 nm)	433.00 ± 9.84	1835.00 ± 84.18	325.33 ± 9.71	1767.00 ± 105.51	26585.33 ± 1373.56	303.00 ± 6.92	28218.67 ± 2752.60
SYTOX (Ex 440 ± 30 Em 485 ± 10 nm)	2745.67 ± 8.62	2095.00 ± 21.07	1830.33 ± 44.16	4160.00 ± 898.86	4609.00 ± 354.23	3051.33 ± 109.74	6111.00 ± 544.47

Viable *Anabaena* 7120 cells in BG11 + SYTO[®] 9 yielded an intensity of 26,585 U. This high green fluorescence reading was consistent with the SYTO[®] 9 accurately measuring viable cells. *Anabaena* 7120 in BG11 + SYTOX[®] Blue resulted in a reading of only 303 U, which was approximately the same as the background fluorescence reading. Thus, the SYTOX[®] Blue dye did not provide any green fluorescence. *Anabaena* 7120 in BG11+ SYTO[®] 9 + SYTOX[®] Blue gave a reading of 28,218 U. This green fluorescence intensity was approximately the same as observed when just SYTO[®] 9 was added. The above data from the treatments with viable cells is what would be expected. SYTO[®] 9 and the resulting green fluorescence correlate accurately with cell viability, and SYTOX[®] Blue does not interfere with the green fluorescence.

For blue fluorescence the average fluorescence intensity for the empty well was 2,745 U, which could be considered background fluorescence. BG11 + SYTO[®] 9 yielded an intensity of 2,095 U. This shows that the SYTO[®] 9 dye did not provide any blue fluorescence, since the intensity was approximately 25% lower than the background fluorescence reading. It also showed that the background fluorescence intensity decreased when the culture medium was added to the well. BG11 + SYTOX[®] Blue resulted in an intensity of 1,830 U. This value is about 33% less than the background fluorescence reading, but almost identical to the BG11 + SYTO[®] 9 reading. This means that SYTOX[®] Blue dye by itself did not add any additional blue fluorescence. This is expected as the stain is supposed to only yield blue fluorescence when bound to DNA or RNA (Estevez et al., 2011).

As BG11 + SYTO[®] 9 and BG11 + SYTOX[®] Blue yielded virtually identical blue fluorescence, it can be concluded that neither stain contributes to blue fluorescence, and

that BG11 medium causes a decrease in background fluorescence compared to the empty well. BG11 + SYTO[®] 9 + SYTOX[®] Blue fluorescence intensity was 4,160 U. This blue fluorescence intensity was higher than what was observed due to the background or presence of either SYTO[®] 9 or SYTOX[®] Blue alone. This finding is logical as the summation of blue fluorescence from BG11 + SYTO[®] 9 and BG11 + SYTOX[®] Blue (3,925 U) was almost identical to fluorescence from BG11+ SYTO[®] 9 + SYTOX[®] Blue (4,160 U), which is only a 5.6% difference.

Viable *Anabaena* in BG11 + SYTO[®] 9 yielded a fluorescence of 4,609 U. This value is double of what was observed in the same test without viable cells present. The green fluorescence from viable cells in BG11 + SYTO[®] 9 was 26,585 U. The high intensity of this fluorescence coupled with the close proximity of green and blue on the light scale was the reason for this increase in background fluorescence. *Anabaena* in BG11 + SYTOX[®] Blue intensity was 3,051 U. This value is 1,200 U higher than in the same test without viable cells present. This suggests that SYTOX[®] Blue was staining viable cells, otherwise the fluorescence intensity wouldn't have increased.

Anabaena in BG11+ SYTO[®] 9 + SYTOX[®] Blue resulted in a fluorescence intensity of 6,111 U. This blue fluorescence intensity was approximately 50% higher than in the same test without viable cells present. Again, this suggests SYTOX[®] Blue was staining viable cells. Although the presence of SYTO[®] 9 may have also contributed to this increase, as it did in the identical case without cells. The above data from the treatments with viable cells is not what would be expected. Thus, it can be concluded that SYTOX[®] Blue also stains viable filamentous cyanobacterial cells. This is a similar result

to what we found with PI, another non-viable cell indicator, with filamentous cyanobacteria (Johnson et al., 2015).

Both PI and SYTOX[®] Blue have now been shown to be ineffective as non-viable cell indicators with filamentous cyanobacteria. Thus it can be assumed that none of the other SYTOX[®] dead cell stains will work for this purpose as they are all supposed to be impermeant to viable cell membranes. This conclusion concurs with a study by Sato et al., (2004) in which strong correlations between expected and measured values in mixtures of live and dead *Anabaena* sp. 7120 cells could not be made when SYTOX[®] Green was the non-viable cell indicator.

While, the SYTOX[®] Dead Cell Stains have been shown to work with certain species of cyanobacteria (Sato et al., 2004; Zhu and Xu, 2013), none were heterocyst-forming filamentous cyanobacteria. Tashyreva et al. (2013) report using a SYTOX[®] dead cell stain to stain *Phormidium* populations. However, this stain was not used in conjunction with SYTO[®] 9, thus the results cannot be compared. As mentioned earlier, other researchers have noted misinterpretations in live/dead staining results. While using SYTO[®] 9 and SYTOX[®] Blue as a dual-stained viability assay does not work as expected with filamentous cyanobacteria, this experiment showed evidence that fluorescence from SYTO[®] 9 appeared to be an accurate viable cell indicator of filamentous cyanobacteria.

4.2.4 Comparison of SYTO[®] 9 and autofluorescence as viable cell indicators

Cyanobacteria are known to autofluoresce in the red spectrum (Caiola et al., 1996; Dagnino et al., 2006) when excited in the green spectrum due to chlorophyll α (Aiken, 1981; Lichtenthaler et al., 1986) and phycobiliproteins (PBS) (Baier et al., 2004). We

theorized that autofluorescence from these light harvesting pigments could be used to assess cell viability, with the additional benefit that no external stains would be needed. The complicating factor of this approach is that photosynthetic pigments can have multiple emission wavelengths. For example, various emission spectra have been used for chlorophyll α determination, including 681 nm, (Guzmán et al., 2015) 672 nm, (Lozano et al., 2013) and 660 nm (Ogawa and Sonoike, 2015). Also, PBS have fluorescent peaks at 635, 645, 654, 659, and 673 nm (Gryliuk et al., 2014).

Figure 4.4 shows that SYTO[®] 9 is a better indicator of viability of filamentous cyanobacteria than autofluorescence from light harvesting pigments. While there are a couple data points in the SYTO[®] 9 plot that cause the plot to not have the standard smooth sigmoidal shape expected with microbial growth curves, this plot has a similar shape to another plot where SYTO[®] 9 was used to monitor cell viability of a microorganism (Yagüe et al., 2010). Both plots have data points that alter the expected smooth shape of the plot, yet still allow for adequate interpretation of the growth dynamic that is occurring.

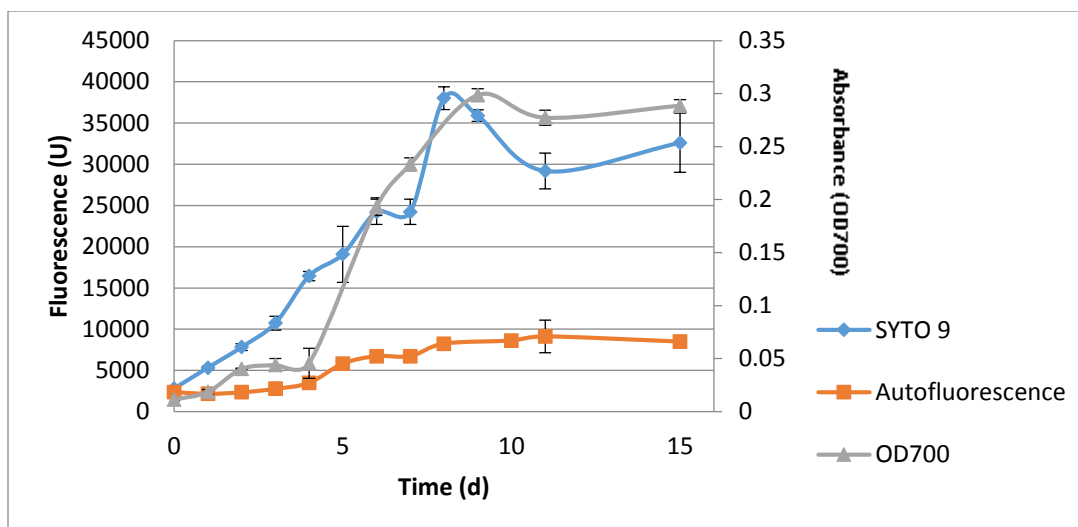


Figure 4.4: Comparison of two viability assays against absorbance for quantification of *Anabaena* sp. 7120 in a 40 L PBR.

The data represents the averages from triplicate replications. Error bars represent the standard deviation.

A possible reason for the weak autofluorescence in Figure 4.4 was that the emission peaks from each of the pigments is different, thus the assay loses sensitivity. Another reason could be that there are no differences in fluorescence from the pigments in viable *vs.* non-viable cells. Regardless of the reason, autofluorescence from light harvesting pigments is not a reliable indicator of viability. This concurs with a study by Sato et al., (2004) who concluded that the intensity of fluorescence from the light harvesting pigments was not related to the relative percentages of viable *vs.* non-viable cells. The fact that the plot generated from fluorescence from SYTO[®] 9 is similar to the plot generated by absorbance readings provides further evidence that SYTO[®] 9 can be used as a reliable indicator of viability in filamentous cyanobacteria.

4.2.4 Assessing cell viability in a chemical inhibition test

High cell densities can affect the bio-availability and toxicity of chemicals that are being assessed for cell toxicity. This is caused by the adsorption of the chemical onto the living or dead cell biomass. The result can be a reduction in the effective dosage of the chemical, leading to an over-estimation of the tolerance of the organism to the chemical. Thus it is desirable to use minimal biomass levels in these tests, so that the dosage and toxic effect of chemicals can be assumed to be a constant (Nyholm and Peterson, 1997; Peterson and Nyholm, 1993). If the chemicals being tested are volatile, then it is also important that the testing be performed in sealed vessels with minimal headspace. This may limit the volume of sample that can be withdrawn, as that would affect headspace. Unfortunately, many cell quantification methods are not very accurate at low cell densities and with low sample volumes. This may be more problematic if one is trying to assess viable cell numbers, instead of total cell biomass.

To determine which viability assay was the most accurate and reproducible under low cell density conditions, we evaluated absorbance, chlorophyll α , and fluorescence from SYTO[®] 9 in chemical inhibition tests involving farnesene. Farnesene is a long-chain hydrocarbon that filamentous cyanobacteria have been genetically engineered to produce (Halfmann et al., 2014a). The antimicrobial property of farnesene, generally as a component of plant oil, is well established (Agnihotri et al., 2011; Aligiannis et al., 2004; Caccioni et al., 1998; Gudžić et al., 2002; Lopes-Lutz et al., 2008). Farnesene has many applications including biofuels, lubricants, cosmetics, and fragrances (Buijs et al., 2013; Halfmann et al., 2014a). We hypothesized that the fluorescent technique would provide

necessary sensitivity compared to absorbance and chlorophyll α when biomass content and sample volumes are low.

Figure 4.5 shows the comparison of three cell quantification methods (2 for quantifying viable cells and 1 for quantifying total cells) for screening cyanobacteria in BG11 medium containing 0.5 g/L NaHCO_3 as a carbon source and 0.032 g/L of farnesene. For each method, growth of a wildtype *Anabaena* sp. 7120 was compared to a strain previously acclimated to tolerate 0.32 g/L farnesene. A lower titer of farnesene (0.032 g/L) was used compared to what the strain was able to tolerate (0.32 g/L), as this allowed for monitoring growth rather than just survivability. These trials used a low starting cell inoculum, and were conducted in sealed test tubes, where sample volume was limited to 100 μl daily samples.

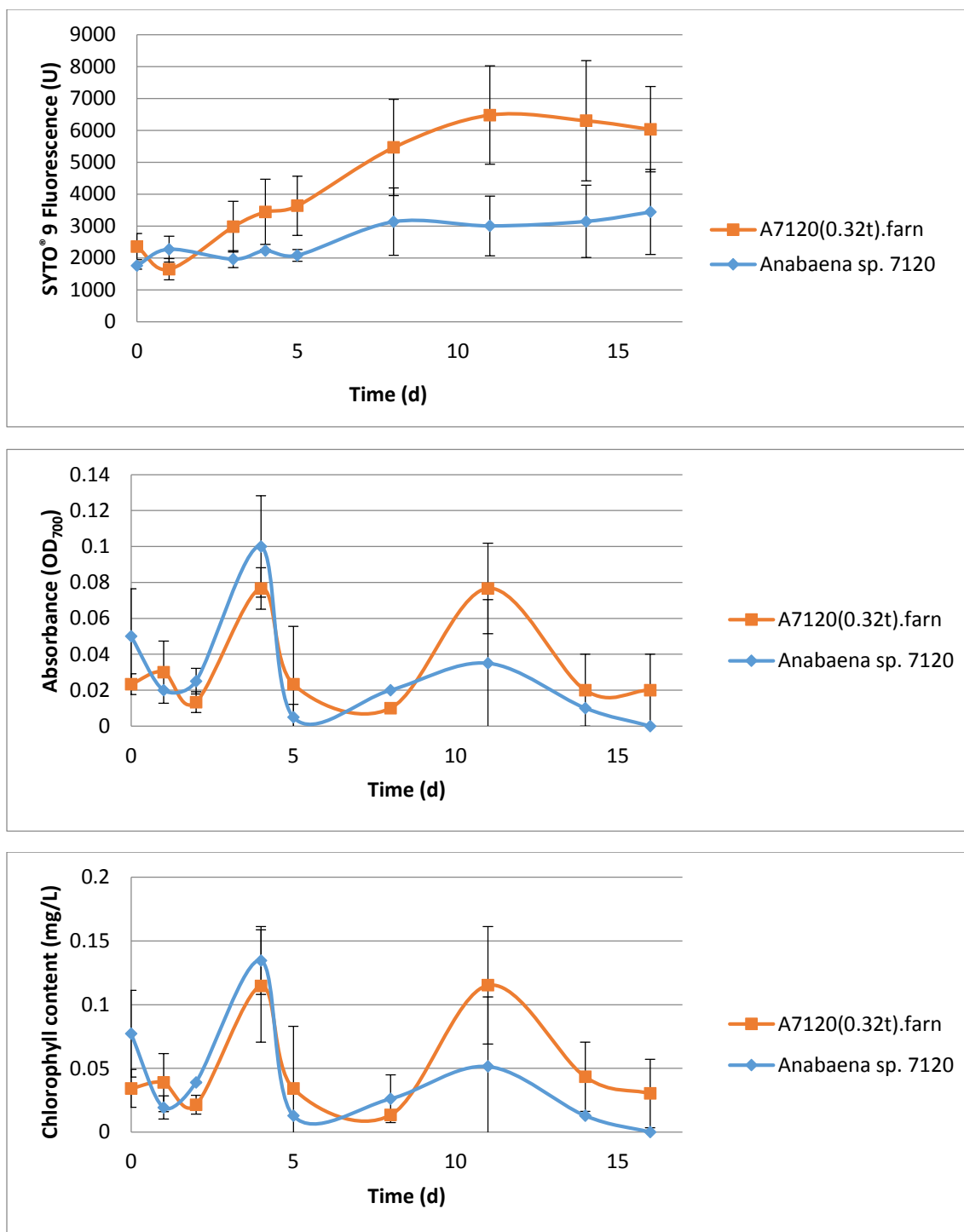


Figure 4.5: Comparison of three cell quantification methods for monitoring cyanobacteria growth at low cell densities and sample volumes. Each trial was completed in triplicate. Error bars represent the standard deviation.

Fluorescence from SYTO[®] 9 was the only method that provided typical growth curves under these conditions of low cell density and small sample size. Even though the error bars are large in the SYTO[®] 9 plot, the assay was still able to distinguish a difference between the acclimated strain and the wildtype. As the daily sample size was limited, it was necessary to dilute the sample to obtain sufficient volumes for measurements in the spectrophotometer. This, in turn, likely diluted the biomass and chlorophyll α levels to or below the sensitivity level of the spectrophotometer. This, at least is partially responsible for the high degree of variability shown in the absorbance and chlorophyll α plots. The large error bars in Fig. 4.5 presumably occurred due to the small biomass levels. Any amount of random or experimental error with values that low would yield large error bars. Further evidence that low biomass content caused the large error bars can be observed by comparing Fig. 4.5 to Fig. 4.4 which had greater biomass content. The error bars are much smaller in Fig. 4.4 even though the method for measuring viability was identical.

As mentioned earlier, high cell densities will yield inaccurate results in chemical inhibition tests, thus must be kept to a minimum. This finding concurs with a study by Mayer et al., (1997) that states that absorbance is considered to have borderline sensitivity and precision at the low biomass levels required for toxicity tests. All three growth parameter measurements were taken from the same culture at the same time, thus fluorescence from SYTO[®] 9 is a superior method when cell density and/or sample size are limited.

4.2.4 Assessing cell viability in photobioreactors

It was previously shown (Fig. 4.4) that absorbance (total cell biomass) and the fluorescence assay (viable cell biomass) are capable of quantifying cyanobacterial growth when biomass and sample volumes are not limited. We postulated the fluorescence assay would be a preferred alternative to absorbance, because the former only measures viable cells. There is also the potential to use smaller sample volumes and detect smaller differences in cell numbers. We therefore evaluated the use of the fluorescence assay to monitor viable cyanobacterial cells in 40 L PBR trials that compared various cultivation media.

Figure 4.6 shows the effect of different cultivation media on the growth of *Anabaena* sp. 7120 as measured by fluorescence from SYTO[®] 9. The definitions for the three growth parameters measured from the fluorescence assay are as follows: maximum viability is the maximum fluorescence value during a trial, % increase in viability is the final fluorescence divided by the initial fluorescence, and viability rate of change is the maximum viability divided by the incubation time. In all cases, BG11 resulted in the best performance. BG11 contains sodium nitrate. Tap water resulted in the least growth, followed by BG11 that was nitrate deficient (BG11₀). Replacing sodium nitrate with urea resulted in reduced growth compared to BG11, but did perform better than BG11₀. A reduction in growth of *Anabaena* sp. 7120 was also observed by Wang and Liu (2013), when sodium nitrate was replaced with urea in BG11. The three parameters allow for a more robust analysis of the dynamics of filamentous cyanobacterial growth. This experiment showed that SYTO[®] 9 was capable of detecting differences in cyanobacterial growth due to differences in growth media. In this application, fluorescence from SYTO[®]

9 is preferred compared to absorbance. Thus, this assay is an attractive option for industrial microbiologists interested in evaluating large-scale processes involving filamentous cyanobacteria.

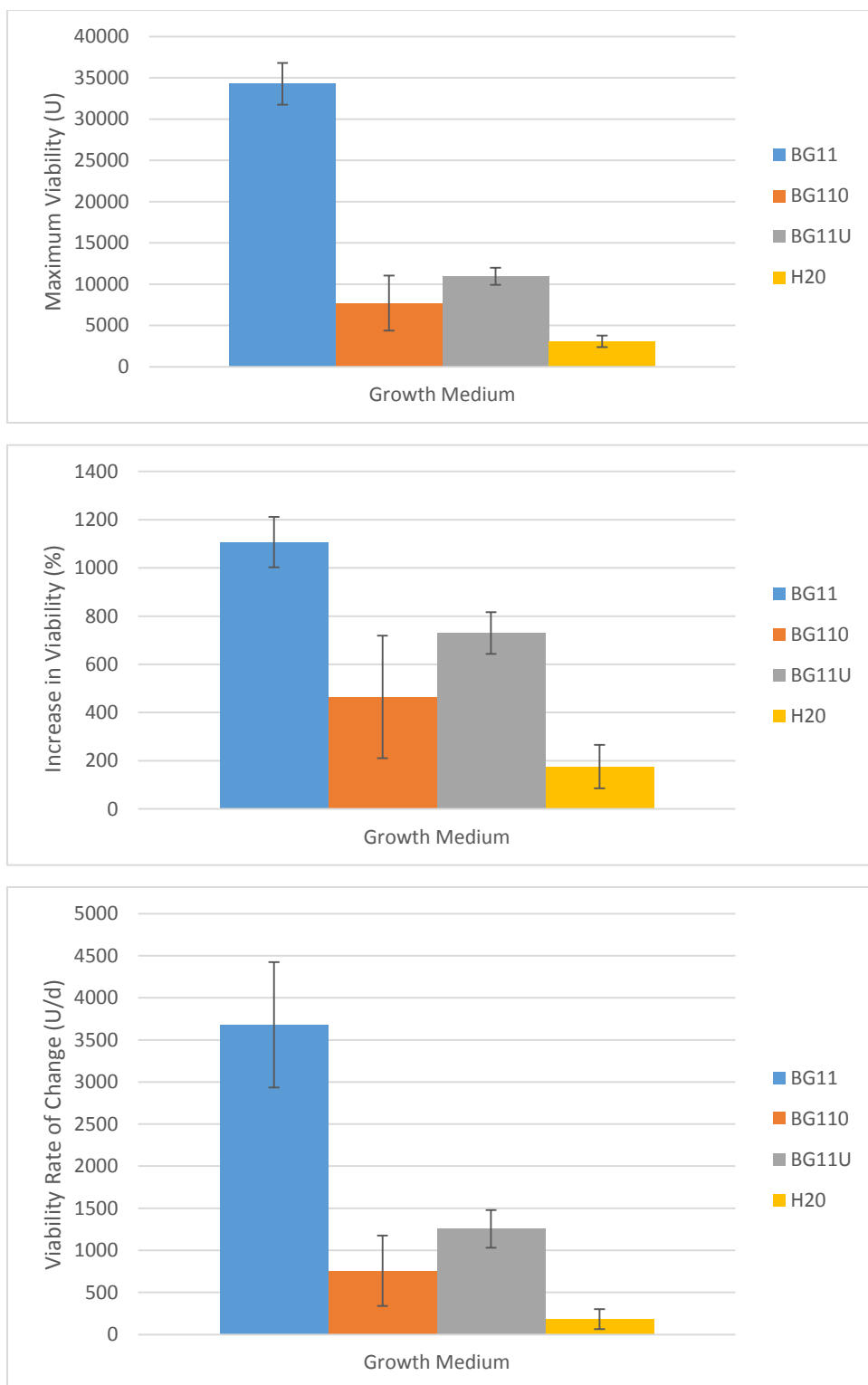


Figure 4.6: Comparison of different cultivation media on 3 growth parameters of *Anabaena* sp. 7120 using fluorescence from SYTO[®] 9 as a viable cell indicator. Each trial contained at least 4 replicates. Error bars represent the standard deviation.

4.4 Conclusions

This study showed that SYTO[®] 9 is a reliable and accurate indicator of filamentous cyanobacteria viability. However, SYTOX[®] Blue did not work as a non-viable cell indicator in filamentous cyanobacteria, and led to erroneous results similar to those we previously reported for the non-viable cell indicator PI (Johnson et al., 2015). Thus, it can be assumed that the other SYTOX[®] Dead Cell Stains will also not work as non-viable cell indicators in filamentous cyanobacteria because they have the same mode of action. Presumably the intercellular modes of transporting nutrients allow PI and SYTOX[®] Blue to penetrate viable cell membranes. Microscopic evidence of this can be seen in a study by Johnson et al. (2015). Autofluorescence from light harvesting pigments was also not a practical method to monitor viability, presumably due to the intensity of fluorescence from the pigments not relating to the relative percentages of viable vs. non-viable cyanobacterial cells.

Fluorescence from SYTO[®] 9 is preferred for monitoring viability of filamentous cyanobacteria under conditions of low biomass concentrations compared to absorbance and chlorophyll α . A sealed system with low biomass content is needed for various applications, such as screening mutants for increased tolerance to next-generation biofuels. Small sample volumes should also be taken so that the headspace of the system does not affect the chemical titer. With the small volumes and the low biomass content, it is not possible to obtain accurate absorbance and chlorophyll α content results. However, it is possible to use SYTO[®] 9 as a viability indicator under these conditions.

In 40 L PBR trials where sample size was not limited, fluorescence from SYTO[®] 9 was capable of detecting differences in cyanobacterial growth caused by differences in growth media. We believe this method is preferred compared to absorbance due to the fluorescence assay's capability of measuring only viable cells, compared to absorbance which measures total cells and cell debris. This study and our prior work (Johnson et al. 2015) show that SYTO[®] 9 is a reliable, accurate indicator of viable cells in filamentous cyanobacteria. Measuring fluorescence from SYTO[®] 9 in a microplate reader allows for high-throughput data collection, which adds to its potential. Applications of this assay include monitoring cultures in PBR systems and screening mutants for increased tolerance to next-generation biofuels.

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Chapter 5 - Optimizing cyanobacteria growth conditions in a sealed environment to enable chemical inhibition tests with volatile chemicals

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Abstract

Cyanobacteria are currently being engineered to photosynthetically produce next-generation biofuels and high-value chemicals. Many of these chemicals are highly toxic to cyanobacteria, thus strains with increased tolerance need to be developed. The volatility of these chemicals may necessitate that experiments be conducted in a sealed environment to maintain chemical concentrations. Therefore, carbon sources such as NaHCO_3 must be used for supporting cyanobacterial growth instead of CO_2 sparging. The primary goal of this study was to determine the optimal initial concentration of NaHCO_3 for use in growth trials, as well as if daily supplementation of NaHCO_3 would allow for increased growth. The secondary goal was to determine the most accurate method to assess growth of *Anabaena* sp. PCC 7120 in a sealed environment with low biomass titers and small sample volumes. An initial concentration of 0.5 g/L NaHCO_3 was found to be optimal for cyanobacteria growth, and fed-batch additions of NaHCO_3 marginally improved growth. A separate study determined that a sealed test tube environment is necessary to maintain stable titers of volatile chemicals in solution. This

study also showed that a SYTO[®] 9 fluorescence-based assay for cell viability was superior for monitoring filamentous cyanobacterial growth compared to absorbance, chlorophyll α (chl a) content, and biomass content due to its accuracy, small sampling size (100 μ l), and high throughput capabilities. Therefore, in future chemical inhibition trials, it is recommended that 0.5 g/L NaHCO₃ is used as the carbon source, and that culture viability is monitored via the SYTO[®] 9 fluorescence-based assay that requires minimum sample size.

5.1 Introduction

Fossil fuels are a finite resource, and it is well established that the massive use of fossil fuels has led to pollution and detrimental health effects in many organisms (Chen et al., 2011). Beyond the long-recognized negative environmental impacts of smog formation and ozone depletion, global warming is a more recently recognized effect of fossil fuel use (von Blottnitz and Curran, 2007). Due to these environmental concerns, it is urgent to develop efficient, clean, and secure systems for the production of biofuels from sustainable sources (Becerra et al., 2015; Gu et al., 2012).

One potential source of renewable biofuels is the photoautotrophic, diazotrophic cyanobacterium *Anabaena* sp. PCC 7120 (herein referred to as *Anabaena* sp. 7120). This microbe is capable of being genetically engineered to produce next-generation biofuels and high-value chemicals such as linalool (Gu et al., 2012), 1- butanol (Lan and Liao 2011), limonene (Halfmann et al., 2014b), and farnesene (Halfmann et al., 2014a). These chemicals are insoluble or have low solubility in water, and this hydrophobic nature leads to high bio-concentration in aquatic organisms such as cyanobacteria, making these

chemicals quite toxic at low concentrations (Mayer et al., 2000). Thus it is important to increase the microbe's tolerance to these chemicals to improve productivity and the industrial potential of these photoautotrophs.

The chemicals previously mentioned are highly volatile, thus chemical inhibition tests must be conducted in a sealed environment with minimal headspace. This will enable maintenance of desired titers of the chemical of interest during the incubation period. Unfortunately, this means that sparging with CO₂ enriched air, or even simple exposure to atmospheric CO₂ cannot be used to supply carbon for cell growth. An alternative carbon source for cyanobacteria is NaHCO₃. Many cyanobacterial species are capable of taking up HCO₃⁻ from the environment via transport across the plasma membrane into the cytosol. There, CO₂ is derived from HCO₃⁻ by carbonic anhydrase maintaining a steady flux to ribulose-1,5-bisphosphate carboxylase/oxygenase for photosynthesis (White et al., 2013).

Studies have been conducted on algal chemical inhibition tests with volatile chemicals, but to the best of our knowledge no studies have used cyanobacteria. Mayer et al., (2000) used 0.3 g/L NaHCO₃, while Herman et al., (1990) used 4 g/L NaHCO₃ for algal chemical inhibition tests. Mayer et al., (2000) also supplemented the medium with 2% CO₂ which was adapted from a study by Hailing-Sørensen et al., (1996). CO₂ was used both for carbon enrichment and to act as a pH buffering agent. However, supplementing the medium with a physiological buffer, such as HEPES could also suit this purpose.

While Herman et al., (1990) did evaluate different NaHCO_3 concentrations, the vessels used had a significant volume of headspace. Also, both Herman et al., (1990) and Mayer et al., (2000) performed these trials with algal rather than cyanobacterial strains. Thus it is necessary to determine the optimal concentration of NaHCO_3 for growth of a cyanobacteria strain (*Anabaena* sp. 7120), and if supplementing with NaHCO_3 in a fed-batch manner would further increase growth in a sealed environment.

An additional challenge with chemical inhibition tests with hydrophobic chemicals is that the chemicals can have sorption interactions with the cyanobacterial biomass itself and/or the walls of the culture vessel, thereby altering the effective concentration exposed to the cells (Mayer et al., 2000). To minimize this problem, it was recommended that trials are conducted with low biomass levels (Mayer et al., 1997; Nyholm and Peterson, 1997; Peterson and Nyholm, 1993). However, at low biomass levels, classical methods of monitoring culture biomass are less accurate. For example, optical density is considered to have borderline sensitivity and precision at the biomass levels of standard algal toxicity tests (Mayer et al., 1997). Another issue to consider is that optical density and chlorophyll α (chl a) content can be easily affected by biomass debris formation (Robertson et al., 1998).

A fluorescence viability assay has previously been shown by Johnson et al., (2016a) to be a superior method of monitoring viability of *Anabaena* sp. 7120 at low biomass titers when compared to optical density and chl a content. Determining if there is a strong correlation between the viability assay and absorbance, chl a content, and biomass content would provide further evidence that the viability assay is an accurate means of monitoring cell viability.

For next-generation biofuels and high-value chemical production from cyanobacteria to become industrially feasible, it is essential to develop strains with increased tolerance to the chemicals that they will be engineered to produce. Because many of these compounds are highly volatile, a sealed environment will be necessary to maintain the chemical titer in solution. Biomass levels must also be minimized to ensure constant chemical-to-biomass concentrations. Therefore, the objectives of this study were to: 1) determine the most accurate and reproducible methods to monitor cyanobacterial growth and viability in a sealed environment, 2) determine the optimal initial concentration of NaHCO_3 and if fed-batch addition of NaHCO_3 would enhance growth, and 3) compare cyanobacterial growth in the sealed test tube environment optimized in the previous objective to growth in test tubes that are not sealed.

5.2 Materials and methods

5.2.1 Microbial strains, maintenance, and culture conditions

Anabaena sp. PCC 7120, a model species for filamentous cyanobacteria (Bryant, 2006; Rippka et al., 1979), was obtained from the Pasteur Culture Collection of Cyanobacteria (Paris, France). For long term storage, strains were frozen at -80°C in 5% v/v methanol. For short term maintenance the cyanobacteria were grown on BG11 agar (Allen and Stanier 1968) (1.5% agar) at pH 7.1, incubated at room temperature under constant illumination of $24 \mu\text{mol m}^{-2} \text{s}^{-1}$, and then stored at room temperature. Light intensity was measured with a Heavy Duty Light Meter with PC Interface (Extech Instruments, Waltham MA, USA).

Inoculum for the experiments described below was grown in 250 ml Erlenmeyer flasks containing 100 ml of BG11 medium at pH 7.1 supplemented with 20 mM HEPES buffer. Flasks were stoppered with a foam stopper and then covered with aluminum foil. The flasks were incubated in a Lab-Line[®] Incubator-Shaker (Lab-Line[®] Instruments, Melrose Park, IL, USA) at 30° C and 100 rpm under constant illumination of 19 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using fluorescent lights.

In the sealed test tube experiments cyanobacterial cultures were grown in 27 ml screw-capped test tubes. The 27 ml test tubes had an open top cap sealed with a PTFE/silicone septum to allow inoculation and sampling via a syringe and needle, and yet prevent the loss of volatile chemicals that were being tested. Tubes were filled with ~27 ml BG11 with 20 mM HEPES buffer and various concentrations of NaHCO_3 for a carbon source. The tubes were incubated at ~22° C under constant illumination of approximately 24 $\mu\text{mol m}^{-2} \text{s}^{-1}$ while rotating at 8 rpm in a Thermo Fisher Scientific[™] Labquake[™] Tube Rotator (Thermo Fisher Scientific[™], Waltham, MA, USA).

5.2.2 Correlation of growth parameters in sealed test tubes

To assess the accuracy and reproducibility of various methods of monitoring biomass levels, 27 ml sealed test tubes containing ~27 ml BG11 with 20 mM HEPES and 0.5 g/L NaHCO_3 were inoculated with 270 μl (1%) of a mid-log phase culture of *Anabaena* sp. 7120. The initial starting concentration of 0.5 g/L NaHCO_3 was chosen as it was previously shown to support cyanobacterial growth in sealed test tubes (Johnson et al., 2016a). Sufficient test tubes were inoculated so that 3 tubes could be sampled and then discarded each day. This was necessary, because the sample volume removed would

have altered the headspace volume and introduced variability. Test tubes were incubated under the conditions previously described in subsection 2.1, and the trials continued for 1 d after the maximum viability was reached as determined by the fluorescence assay. Daily samples of 14 ml were taken for determination of absorbance (OD_{700}), viability via a fluorescence assay, chl a content, and biomass content. These methods are described in detail in the analytical methods subsection 5.2.6.

5.2.3 Optimizing the initial $NaHCO_3$ concentration in sealed test tubes

To determine the optimal initial concentration of $NaHCO_3$, 27 ml sealed test tubes containing ~27 ml BG11 with 20 mM HEPES and 0, 0.25, 0.5, 0.75, or 1.0 g/L $NaHCO_3$ were inoculated with 270 μ l (1%) of a mid-log phase culture of *Anabaena* sp. 7120. The tubes were incubated in conditions previously described. Daily, 100 μ l samples were collected via a 1 ml syringe and a 21-gauge needle. Fluorescence was measured as described below in subsection 2.6. Maximum fluorescence reached during a trial (U), increase in fluorescence from the initial time point to the maximum fluorescence reached (%), and fluorescence rate of change during the trial (U/d) were calculated from these data. The trials continued for 1 d after the maximum viability was reached, which was typically 5-7 days.

5.2.4 Fed-batch trials optimizing $NaHCO_3$ concentrations in a sealed test tube system

To determine if supplementing $NaHCO_3$ in a fed-batch manner would further increase biomass levels in a sealed environment, 27 ml sealed test tubes containing ~27 ml BG11 with 20 mM HEPES and 0.5 g/L $NaHCO_3$ were inoculated with 270 μ l (1%) of a mid-log phase culture of *Anabaena* sp. 7120. The tubes were incubated, sampled, and

measured for fluorescence as described in subsections 5.2.1 and 5.2.6. On a daily basis, 0, 8.0×10^{-4} , 2.4×10^{-3} , or 4.8×10^{-3} g NaHCO₃ was added to the tubes via a 1 ml syringe and a 21-gauge needle. This was accomplished by adding 0, 100, 300, or 600 μ l of an 8 g/L NaHCO₃ stock solution, respectively. The volume of fluid added to the tube was removed after manual mixing to maintain a constant headspace volume. Maximum fluorescence reached during a trial (U), increase in fluorescence from the initial time point (%), and fluorescence rate of change during the trial (U/d) were calculated from the data. The trials continued for 1 d after the maximum viability was reached, which was typically 3-6 days.

5.2.5 Evaluating the efficacy of the sealed environment using a chemical inhibition test

To determine if the sealed environment optimized for filamentous cyanobacterial growth was necessary to maintain stable titers of volatile chemicals in the culture medium during a chemical inhibition test, cyanobacterial growth in sealed test tubes was compared to test tubes that were opened daily for sampling. We postulated that filamentous cyanobacteria in sealed test tubes spiked with a volatile chemical would have significantly inhibited growth compared to test tubes that were opened daily. The volatile chemical tested in this experiment was limonene, which is a highly volatile (Inouye et al., 2001), cyclic hydrocarbon with potential as a biodiesel and a biofuel (Halfmann et al., 2014b). Examples of microorganisms that have been genetically engineered to produce limonene include *E. coli* (Alonso-Gutierrez et al., 2013), the unicellular cyanobacterium, *Synechocystis* sp. PCC 6803 (Kiyota et al., 2014), and the filamentous cyanobacterium, *Anabaena* sp. 7120 (Halfmann et al., 2014b).

Twenty-seven ml sealed test tubes containing ~27 ml BG11 with 20 mM HEPES, 0.5 g/L NaHCO₃, and 0.04 g/L limonene were inoculated with 270 µl (1%) of a mid-log phase culture of *Anabaena* sp. 7120. This limonene titer was chosen based on a concentration that didn't kill the entire culture in the first 24 h before the tubes were opened, yet still inhibited growth compared to the control tubes (data not shown). A set of control test tubes without limonene were also included. Half of the limonene⁺ and limonene⁻ tubes were maintained in a sealed condition throughout incubation. The other tubes were opened daily for 5 seconds during sampling. Otherwise tubes were incubated, sampled, and analyzed as described previously. Maximum fluorescence reached during a trial (U), increase in fluorescence from the initial time point (%), and fluorescence rate of change during the trial (U/d) were calculated from the data. The trials continued for 1 d after the maximum viability was reached, which was typically 5-9 days.

5.2.6 Analytical methods

For absorbance, optical density of 1 ml samples was measured at 700 nm (OD₇₀₀) using a Thermo Fisher Scientific™ Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific™, Waltham, MA, USA). The chl a content was calculated using the following formula: $\text{Chl a } (\mu\text{g/ml}) = 14.96 (\text{OD}_{678} - \text{OD}_{750}) - 0.616 (\text{OD}_{720} - \text{OD}_{750})$ (Williams, 1988; Yu et al., 2011). The 1 ml samples used to measure absorbance were also used to measure chl a content, as both methods were determined via spectrophotometry (OD₇₀₀). Biomass content was determined by filtering 10 ml of culture through a 0.45 µm diameter pore filter (Binaghi et al., 2003). The filters were then dried at 80°C until the weight was constant.

Viability was determined in cyanobacterial cultures via fluorescence from SYTO[®] 9 (Life Technologies[™], Carlsbad, CA, USA) (Johnson et al., 2016a). SYTO[®] 9 is a fluorescent dye capable of penetrating most cellular membranes and causing cells containing nucleic acids to fluoresce green (Lee and Rhee 2001; Shi et al. 2007). The fluorescence intensity units (U) directly correlate with the amount of viable cells in the culture. While, we were unable to determine the number of cells that correlate to fluorescence intensity due to the filamentous morphology, the assay allowed us to make direct comparisons. For example, if one culture yields 2000 U and another yields 4000 U, we can conclude the second culture has 100% more viable cells. For viability measurements via the fluorescence assay, 100 µl samples were transferred to a 96-well plate. SYTO[®] 9 was added to each well at a concentration of 5 µM and fluorescence was measured by a Synergy 2 Multi-Mode Microplate Reader (BioTek[®], Winooski, VT, USA). To measure green fluorescence intensity units (U) from SYTO[®] 9 stained cells, fluorescence filters with excitation wavelength 485 ± 20 nm; emission wavelength 528 ± 20 nm were used.

5.2.7 Statistical analyses

The Shapiro-Wilk univariate normality test was used to test the normality of each dataset (Ghasemi and Zahediasl, 2012). Non-normality can inflate type I error rates (Auer et al. 2016), however if only a few datasets analyzed are not normally distributed then no further statistical analyses will be performed as ANOVA is reasonably robust with respect to the normality assumption (Schmider et al. 2010). Normality was performed with a confidence level of 0.95 using Microsoft[®] Excel along with the Simetar[®] add-in. Simetar[®] is a risk analysis software that been used regularly in business models and

prospective businesses that can perform various statistical tests (Richardson and Johnson, 2015). G*Power statistical power analysis program was used to conduct *post hoc* power analysis to ensure the sample size of each treatment was adequate to achieve statistical power of ≥ 0.95 (Faul et al., 2007).

To determine if significant differences existed among the treatments of filamentous cyanobacterial growth, analysis of variance was performed (ANOVA) using the R statistical power analysis program and the packages ‘reshape2’ and ‘agricolae’ (Hadley 2012; de Mendiburu 2013; R Core Team 2013). In the event of heterogeneity of variances among data sets occurred, a Welch’s ANOVA was performed (Weiss-Schneeweiss et al., 2006; Liu, 2015; Jan and Shieh, 2014). Tukey tests were used for *post hoc* tests of significant differences between means on data sets with heterogeneous variances (Driscoll, 1996). When there was no heterogeneity of variances among data sets, a one-way ANOVA was performed. Duncan’s new multiple range tests (MRT) were used for *post hoc* tests of significant differences between means on data sets with no heterogeneity of variances (Freund et al., 2010).

5.3 Results and discussion

5.3.1 Correlation of growth parameters in sealed test tubes

As the first step of this study, we monitored growth of *Anabaena* sp. 7120 in sealed test tubes using 0.5 g/L NaHCO₃ as the carbon source to determine which growth measurements provided accurate and reproducible results at low biomass levels and small sample volumes. This was necessary, since low cell biomass levels are recommended for chemical toxicity testing (Mayer et al., 1997; Nyholm and Peterson, 1997; Peterson and

Nyholm, 1993). At high biomass levels the chemical can bind to the biomass altering the titer exposed to cells (Mayer et al., 2000). The need for small sample volumes is to minimize creation of additional headspace in sealed tubes, which could otherwise result in the chemicals of interest volatilizing into the headspace and reducing the titer in the liquid phase.

Linear regression analysis was performed on the relationships between absorbance, chl a content, viability, and biomass content. Linear regression analysis was performed with Microsoft Excel using the method of least squares, which resulted in an estimated line that minimized the variance of the residuals (Freund et al. 2010). This line predicts the relationship between two variables. To perform linear regression comparing two variables, the first variable listed was plotted on the y-axis and the second variable was plotted on the x-axis (Table 5.1). Table 5.1 shows that absorbance, chl a content, and viability all had strong correlations to each other (average $R^2 > 0.89$). Biomass content did not correlate strongly with the other parameters (average $R^2 < 0.34$). Presumably, this was due to the low biomass content in the test tubes coupled with low sample volumes (10 ml) which led to high variability in cell dry weight values. The viability assay only required sample sizes of 100 μ l, and because it was strongly correlated with absorbance and chl a content would make it the ideal method to monitor cell growth and viability in chemical inhibition tests conducted in small sealed test tubes. The high R^2 values calculated for the comparisons of viability, chl a content, and absorbance indicate there is a strong correlation between these variables as R^2 is a measure of the strength of the linear relationship between two quantitative variables (Freund et al. 2010). Since chl a content and absorbance are well established methods for

quantifying microbial growth, the fact that the viability assay correlates strongly with these methods indicates that the assay is an accurate method of monitoring growth of cyanobacteria. This method is also advantageous as it can be used in a high-throughput mode using a microplate reader. Thus, the viability assay was used to monitor cyanobacterial growth in the experiments conducted in this study.

Table 5.1: Correlations of growth parameters of an *Anabaena* sp. PCC 7120 cultures in 27 ml sealed test tubes containing ~27 ml BG11 supplemented with 0.5 g/L NaHCO₃.

The length of trials used to determine linear regression plots was 7 days.

Parameters Compared	R ²
Absorbance (OD ₇₀₀) vs. Chlorophyll (µg/ml)	0.96
Fluorescence (U) vs. Chlorophyll (µg/ml)	0.87
Fluorescence (U) vs. Absorbance (OD ₇₀₀)	0.85
Fluorescence (U) vs. Biomass (g/L)	0.42
Absorbance (OD ₇₀₀) vs. Biomass (g/L)	0.31
Chlorophyll (µg/ml) vs. Biomass (g/L)	0.29

5.3.2 Optimizing the initial NaHCO₃ concentration in sealed test tubes

To determine the optimal initial concentration of NaHCO₃ for cyanobacterial growth in a sealed environment, three growth parameters were compared for various

initial concentrations of NaHCO_3 . Cyanobacterial growth was compared in sealed test tubes with initial NaHCO_3 concentrations of 0, 0.25, 0.5, 0.75, or 1.0 g/L. Growth was measured via the fluorescence assay previously described, and in addition to maximum fluorescence (i.e., growth), calculated parameters used for comparison purposes included the percentage increase in fluorescence from the initial reading, and the rate of change in fluorescence (U/d) during a trial.

Due to the heterogeneity of variances in the data sets, Welch's ANOVAs were performed to determine if statistical differences occurred among the means at a confidence level of 0.95. Tukey tests were used for *post hoc* tests of significant differences between means. Figure 5.1 shows the results for the batch trials comparing various NaHCO_3 treatments for three growth parameters. The initial concentrations of NaHCO_3 were chosen due to their proximity to concentrations that were used in the algal chemical inhibition studies previously described. As expected, the 0 g/L NaHCO_3 control resulted in the least growth in all 3 growth parameters measured. There were no significant differences in maximum fluorescence or the rate of change in fluorescence for 0.5, 0.75, and 1.0 g/L NaHCO_3 . These three treatments yielded significantly higher fluorescence values in all three parameters measured than 0 and 0.25 g/L NaHCO_3 . These results suggest that 0.5 g/L yields statistically similar growth as 0.75 and 1.0 g/L NaHCO_3 . It is more cost effective to use less NaHCO_3 , thus 0.5 g/L NaHCO_3 was selected as the optimal initial concentration for future trials.

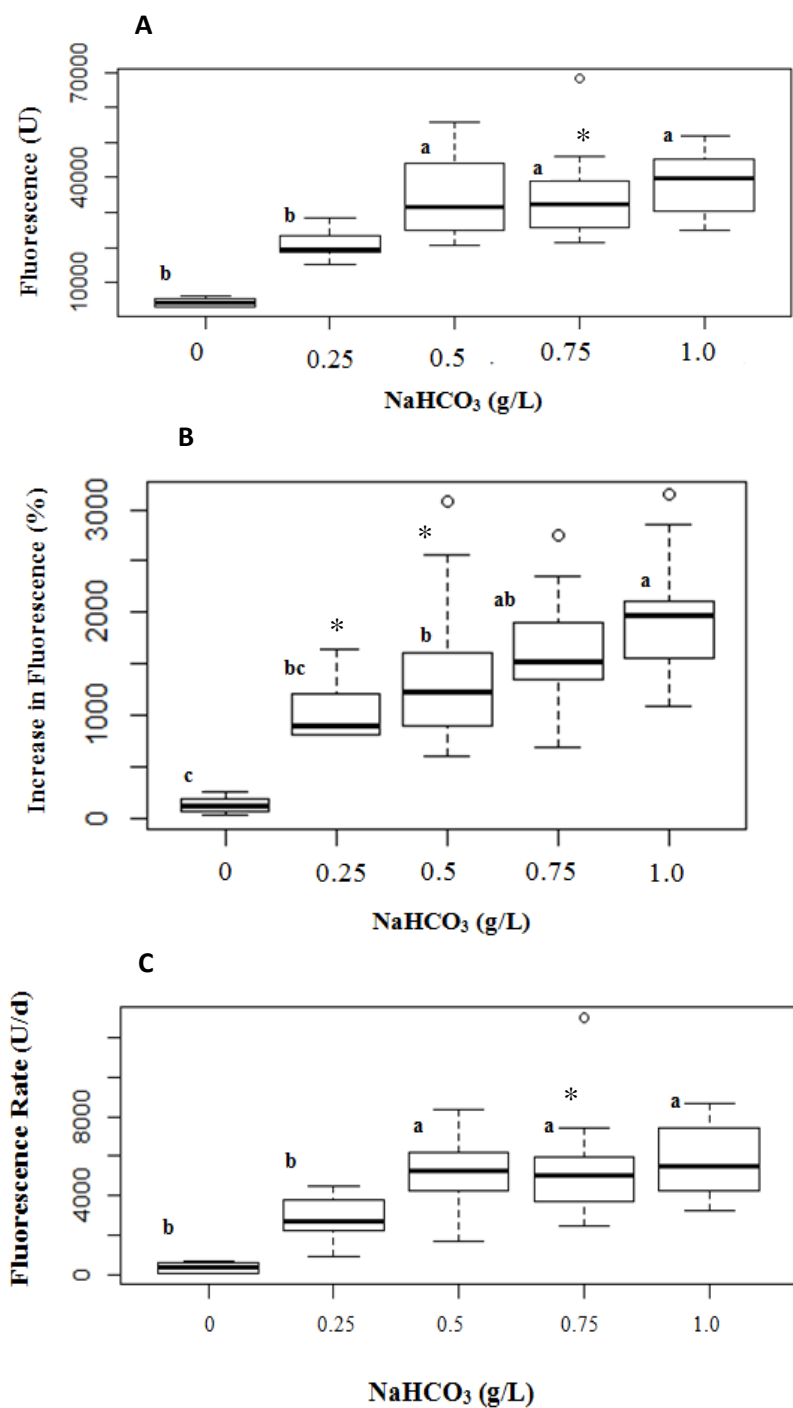


Figure 5.1: Batch trials comparing various NaHCO₃ concentrations for growth of *Anabaena 7120* as measured by fluorescence.

(A) Maximum viability; (B) Percent increase in viability; (C) Rate of viability increase per day.

Trials typically lasted 5-7 days. Different lower case letters indicate a significant difference occurred between two treatments; Asterisk: dataset was not normally distributed; Circles: outliers; Bold center line: median value; Line above bold center line: 3rd quartile; Line below bold center line: 1st quartile; Bar above the box: Maximum value; Bar below the box: minimum value.

5.3.3 Fed-batch trials optimizing NaHCO₃ concentrations in a sealed test tube system

To determine if daily supplementation with NaHCO₃ would increase growth, 0, 8.0×10^{-4} , 2.4×10^{-3} , and 4.8×10^{-3} g NaHCO₃ were added daily during a growth trial to the sealed test tubes that initially contained 0.5 g/L NaHCO₃. These small addition levels were used because excess NaHCO₃ can cause culture alkalinity (Vera et al., 2014; Stegman et al., 2014; Gandhi, 2012). Cyanobacterial growth was measured via the fluorescence assay previously described using the 3 growth parameters previously described.

One-way ANOVAs were performed to determine if statistical differences occurred among the means at a confidence level of 0.95. Duncan's new multiple range tests (MRT) were used for *post hoc* tests of significant differences between means. Figure 5.2 shows the results for the fed-batch trials comparing various NaHCO₃ daily additions for three growth parameters. The 8.0×10^{-4} and 2.4×10^{-3} g/d NaHCO₃ treatments yielded statistically similar growth to the 4.8×10^{-3} g/d NaHCO₃ treatment in all three parameters measured. The 0 g/d NaHCO₃ treatment yielded significantly less growth in all three parameters measured than the 4.8×10^{-3} g/d NaHCO₃ treatment. While these differences were statistically significant, they didn't result in very large improvements in growth. Moreover, daily injection of NaHCO₃ would introduce another variable into chemical inhibition trials with volatile chemicals, as NaHCO₃ supplementation might alter the chemical-to-biomass sorption dynamics. Thus, it was determined that no NaHCO₃ should be added in a fed-batch manner to cyanobacterial cultures in sealed test-tube trials.

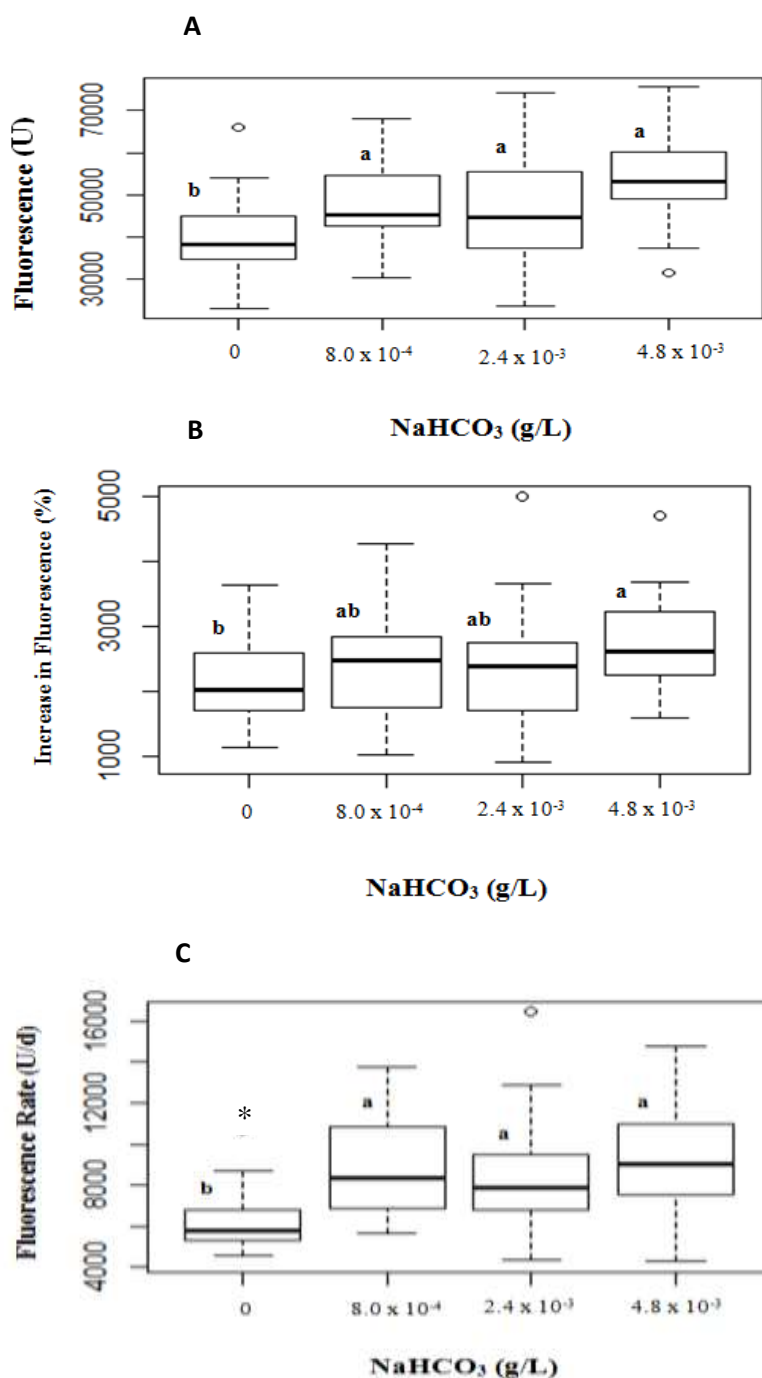


Figure 5.2: Fed-batch trials comparing addition of various NaHCO₃ levels during incubation of Anabaena 7120 as measured by fluorescence.

(A) Maximum viability; (B) Percent increase in viability; (C) Rate of viability increase per day.

Trials typically lasted 3-6 days. Different lower case letters indicate a significant difference occurred between two treatments; Asterisk: dataset was not normally distributed; Circles: outliers; Bold center line: median value; Line above bold center line: 3rd quartile; Line below bold center line: 1st quartile; Bar above the box: Maximum value; Bar below the box: minimum value.

5.3.4 Evaluating the efficacy of the sealed environment using a chemical inhibition test

To determine if the sealed environment test tubes, optimized for filamentous cyanobacterial growth in this study, were necessary to maintain volatile chemical titers in solution we compared this approach to a more traditional approach of opening test tubes to remove samples. The opened tubes were opened daily for five seconds to standardize the test. Sealed and opened test tubes that were not spiked with limonene served as controls and were compared with sealed and opened tubes spiked with 0.04 g/L of the highly volatile hydrocarbon limonene. The three growth parameters derived from the fluorescence assay data were used as the basis for comparison. One-way ANOVAs were performed to determine if statistical differences occurred among the means at a confidence level of 0.95. MRTs were used for *post hoc* tests of significant differences between means.

Figure 5.3 shows the results of cyanobacterial growth in sealed and opened test tubes during a chemical inhibition test with limonene. In Figure 5.3, identical letters indicate no statistical differences occurred among treatments, and different letters indicate a statistically significant difference did occur. Sealed and opened test tubes that were not spiked with limonene showed statistically similar growth results. On the other hand, cultures spiked with 0.04 g/L limonene showed significantly lower growth, and cyanobacterial cultures in sealed test tubes spiked with limonene had significantly less growth than the limonene-spiked test tubes that were opened daily for sampling. Opening the test tubes evidently allowed the highly volatile limonene to evaporate out of culture, decreasing the titer in solution, and allowing for increased cyanobacterial growth. This

study showed that the culture conditions optimized for the sealed environment in this study is the desired protocol for conducting cyanobacterial research with volatile chemicals.

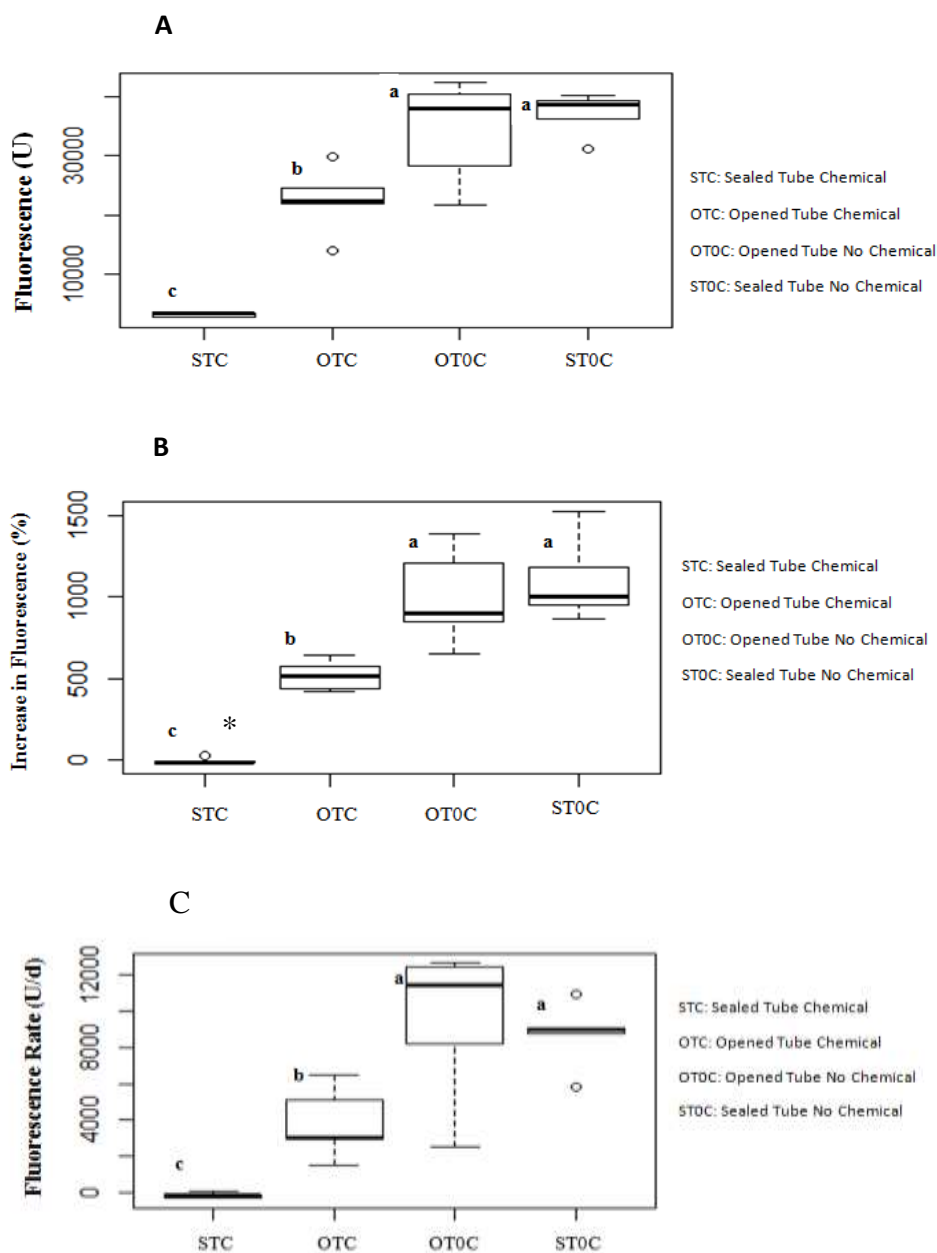


Figure 5.3: Comparison of cyanobacterial growth in sealed test tubes vs. opened test tubes during a chemical inhibition test with 0.04 g/L limonene as measured by fluorescence.

(A) Maximum viability; (B) Percent increase in viability; (C) Rate of viability increase per day.

Trials typically lasted 5-9 days. Different lower case letters indicate a significant difference occurred between two treatments; Asterisk: dataset was not normally distributed; Circles: outliers; Bold center line: median value; Line above bold center line: 3rd quartile; Line below bold center line: 1st quartile; Bar above the box: Maximum value; Bar below the box: minimum value.

5.4 Conclusions

The purpose of this study was to optimize conditions for filamentous cyanobacterial growth in a sealed environment. Growth in a sealed environment is necessary in order to assess the tolerance of strains to volatile chemicals, as well as to develop mutants with increased tolerance via constant exposure to the chemicals. Several research groups are engineering cyanobacteria to produce high-value chemicals and next-generation biofuels directly from CO₂ and sunlight. If the tolerance of the cyanobacteria to these products can be improved, the process will have a better chance of becoming economically feasible.

While, there have been previous chemical inhibition studies with algae (Nagai et al., 2013; Blaise and Vasseur, 2005; Eisentraeger et al., 2003), typically in a microplate format, this methodology is not applicable when working with volatile chemicals. Without a sealed environment, the chemicals would not stay in the aqueous phase. There have also been algal chemical inhibition tests in sealed flasks in medium supplemented with NaHCO₃ to facilitate algal growth in a sealed environment (Mayer et al., 2000; Herman et al., 1990). However, to the best of our knowledge no studies have investigated cyanobacterial chemical inhibition tests in a sealed environment.

First, we established that growth of *Anabaena* sp. 7120 in a sealed environment was optimized when an initial concentration of 0.5 g/L NaHCO₃ was used as the carbon source, and that fed-batch additions of supplemental NaHCO₃ during incubation only provided modest improvements to growth. We also showed that a sealed incubation environment was needed to prevent volatilization of chemicals such as limonene during trials assessing toxicity of the chemicals. This study also showed that a fluorescence

assay using SYTO[®] 9 was preferred over classical methods of monitoring growth in trials where cell densities are low and/or sample volume is limited. The fluorescence assay also has the potential for high-throughput screening due to the microplate format.

Next-generation biofuels and high-value chemical production from cyanobacteria holds much potential to lessen the current burden on fossil fuels. Many of these chemicals are highly volatile and toxic to cyanobacteria. Thus, a means to perform chemical inhibition tests in a sealed environment will be important to determine cyanobacterial tolerance to volatile chemicals, and for developing mutants with increased tolerance to volatile chemicals via selective pressure through constant exposure to the chemical.

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Chapter 6 - Increasing the tolerance of filamentous cyanobacteria to next-generation biofuels via directed evolution

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Abstract

Renewable biofuels can lessen our reliance on fossil fuels. Cyanobacteria are being investigated for the production of biofuels directly from carbon dioxide, thus eliminating the steps of biomass production, harvest, logistics, and conversion required for 1st and 2nd generation biofuels. This study determined the initial tolerance of *Anabaena* sp. PCC 7120, *Anabaena variabilis* ATCC 29413, and *Nostoc punctiforme* ATCC 29133 to four potential biofuels: farnesene, myrcene, linalool, and limonene. These cyanobacteria were then subjected to 3 rounds of directed evolution where the strains were exposed to increasing titers of each chemical. This led to a library of 12 putative mutants with higher tolerance to individual chemicals. These mutants were assessed for growth performance at chemical titers higher than the wildtype strains could tolerate. Power analysis was used to determine the necessary sample size to achieve statistical power of ≥ 0.95 . Student's t-tests were performed to determine if the putative mutants had significantly improved growth parameters compared to the wildtype, thereby

establishing whether an inheritable genetic change or possibly an epigenetic change had occurred. Three mutants were confirmed as having higher tolerance to individual chemicals. This work serves as proof-of-concept that directed evolution is a valid methodology to increase the tolerance of filamentous cyanobacteria to biofuels.

6.1 Introduction

Replacements for petroleum-derived fuels and chemicals are becoming increasingly necessary due to the adverse effects of fossil fuel-generated greenhouse gases, including ozone depletion, global warming, and smog formation (Von Blottnitz and Curran, 2007). The extensive use of fossil fuels has led to pollution, global climate change, and detrimental effects on many organisms (Chen et al., 2011). One solution to this problem is to explore the utilization of cyanobacteria to manufacture next-generation biofuels.

Cyanobacteria are photosynthetic prokaryotes present in a wide variety of ecosystems (Hasunuma et al., 2013; Katoh, 2012; Moreno et al., 1998). They have morphologies ranging from unicellular to filamentous, and utilize the same type of photosynthesis as higher plants (Lindblad et al., 2012). Cyanobacteria have garnered significant attention from industrial microbiologists due to their potential to be genetically engineered to produce high-value chemicals and next-generation biofuels (Gu et al., 2012; Halfmann et al., 2014a; Halfmann et al., 2014b; Kiyota et al., 2014; Lan and Liao, 2011) from CO₂ and solar energy (Machado and Atsumi, 2012). The strains of filamentous cyanobacteria utilized in this study also have the ability to fix atmospheric nitrogen in specialized cells termed heterocysts, located along the filament. Thus, these strains have an advantage in biofuel production, as supplying nitrogen is a major cost in

large scale biofuel strategies (Halfmann et al., 2014b; Ortiz-Marquez et al., 2012; Peccia et al., 2013).

Next-generation biofuels derived from cyanobacteria are considered to be a technically viable alternative energy resource devoid of the major drawbacks associated with 1st and 2nd generation biofuels (Brennan and Owende, 2010). At this time, cyanobacteria appear to be the only current biorenewable resource capable of meeting the global demand for transportation fuels (Chisti, 2007; Schenk et al., 2008; Singh et al., 2011a). While there are several companies with the capability to produce fuel from photosynthetic organisms (Chisti, 2013), the development of cyanobacteria for mass production of fuels and chemicals is still in its infancy (Parmar et al., 2011).

Biofuels and high-value chemicals are often toxic to the microbe that produces them (Chubukov et al., 2015; Dunlop, 2011; Jarboe et al., 2011; Kim et al., 2015; Zhang et al., 2011). Thus for commercial deployment of cyanobacterial fuel/chemical production, it will be necessary to develop strains able to withstand these toxic effects. One strategy to achieve this goal is use of directed evolution, a process in which a microbe is grown under a selective pressure that forces rapid evolution to tolerate that pressure. Directed evolution has been used to improve production of a range of microbial products (Labrou, 2010). The process of directed enzyme evolution begins with creation of a library of strains with mutated genes. Strains that show improvement in the desired property are identified by screening, and are subjected to further cycles of mutation and screening to further enhance performance (Kuchner and Arnold, 1997; Umeno et al., 2005).

In this study we sought to enhance the tolerance of three species of cyanobacteria to four biofuels which are toxic to cyanobacteria (Fig. 6.1). Farnesene is a sesquiterpene with commercial applications in lubricants, cosmetics, fragrances, and biofuels (Buijs et al., 2013; Halfmann et al., 2014a). Myrcene, a monoterpene, has applications as a starting material for high-value compounds such as geraniol/linalool, and (-)-menthol (Kim et al., 2015). Linalool, an energy dense long-chain alcohol has the potential to become a ‘drop-in’ biofuel (Gu et al., 2012). It is also used in perfumes, cosmetics, and flavoring agents (Beier et al., 2014). Limonene, a cyclic monoterpene, has potential as a biodiesel and a biofuel (Halfmann et al., 2014b). The objectives of this study were to: 1) create a library of putative mutant strains of biofuel tolerant filamentous cyanobacteria via directed evolution, and 2) screen the putative mutants in a spiked culture medium to determine if an inheritable genetic or epigenetic change occurred.

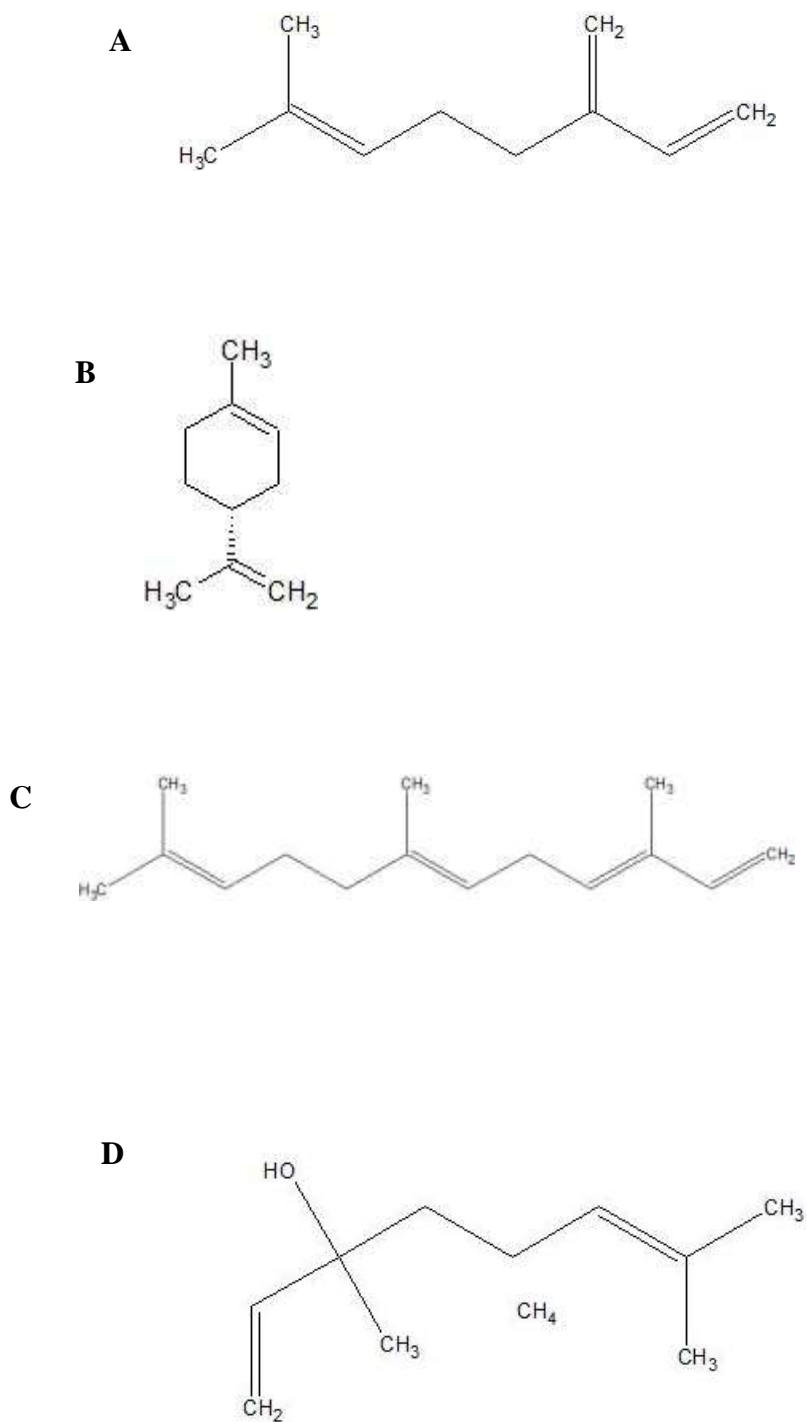


Figure 6.1: Molecular structure of the chemicals utilized in this study.
Myrcene (A), limonene (B), farnesene (C), and linalool (D).

(Structures drawn with ACD/ChemSketch Freeware)

6.2 Materials and methods

2.1 Microbial strains, maintenance, and culture conditions

The filamentous, diazotrophic cyanobacterial strains *Anabaena* sp. PCC 7120 (herein referred to as *Anabaena* sp. 7120), *Anabaena variabilis* ATCC 29413, and *Nostoc punctiforme* ATCC 29133 were obtained from their respective culture collections. For long term storage, strains were frozen at -80°C in 5% v/v methanol. For short term maintenance, the cyanobacteria were grown on BG11 agar (1.5% agar) (Allen and Stanier, 1968) at pH 7.1, incubated at room temperature under constant illumination of 24 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and then stored at room temperature. Light intensity was measured with a Heavy Duty Light Meter with PC Interface (Extech Instruments, Waltham MA, USA).

Inoculum for the experiments described below was grown in 250 ml Erlenmeyer flasks containing 100 ml of sterile BG11 media at pH 7.1 supplemented with 20 mM HEPES buffer. Flasks were stoppered with a foam stopper and then covered with aluminum foil. The flasks were incubated in a Lab-Line® Incubator-Shaker (Lab-Line® Instruments, Melrose Park, IL, USA) at 30°C and 100 rpm under constant illumination of 19 $\mu\text{mol m}^{-2} \text{s}^{-1}$ using fluorescent lights.

In the experiments described below, cyanobacteria were grown in either 20 or 27 ml, sealed test tubes. The sealed test tube system was needed to prevent evaporative loss of the volatile chemicals being tested. In directed evolution trials, cyanobacteria were grown in 20 ml screw capped test tubes. Tubes were filled with ~20 ml BG11 with 20 mM HEPES buffer and 0.5 g/L NaHCO₃ for a carbon source. Cyanobacteria are capable of taking up HCO₃⁻ via transport across the plasma membrane into the cytosol. There

CO₂ is derived from HCO₃⁻ via carbonic anhydrase, which maintains a steady state flux to ribulose-1,5-bisphosphate carboxylase oxygenase for photosynthesis (White et al., 2013). It has been determined by our research group that 0.5 g/L NaHCO₃ is the optimal concentration for cultivating filamentous cyanobacteria in a sealed environment (Johnson et al., 2016d). This method was adapted from previous studies that used a sealed environment for chemical inhibition tests with algae (Herman et al., 1990; Mayer et al., 2000). In mutant screening trials, cyanobacteria were grown in 27 ml screw capped test tubes that had an open top cap sealed with a PTFE/silicone septa to allow inoculation and sampling via a syringe and needle. Tubes were filled with ~27 ml BG11 with 20 mM HEPES buffer and 0.5 g/L NaHCO₃. After inoculation, tubes were incubated at room temperature under constant illumination of approximately 24 μmol m⁻² s⁻¹ while rotating at 8 rpm in a Thermo Fisher Scientific™ Labquake™ Tube Rotator (Thermo Fisher Scientific™, Waltham, MA, USA).

2.2 Determination of wildtype cyanobacteria tolerance to biofuels

Tolerance of the wildtype strains of *Anabaena* sp. 7120, *N. punctiforme*, and *A. variabilis* to farnesene, linalool, myrcene, and limonene was established by determining the titer of the chemical that each strain could survive after a 3 d incubation period. An initial inoculum of a mid-log culture was transferred to test tubes to achieve an initial OD₇₀₀ of 0.5. The test tubes were spiked with specific titers of the biofuel of interest. For each trial, a positive control containing the cyanobacterial strain and no chemical was used. The test tubes were incubated under the conditions previously described.

After 3 d, cultures were centrifuged and washed repeatedly to remove any residual biofuel. This protocol involved: 1) centrifuging at 4,000 rpm at room temperature for 10 min, 2) re-suspending cell pellets in 1 ml BG11 and transferring to a 1.5 ml centrifuge tube, 3) centrifuging at 6,000 rpm at room temperature for 5 min, 4) re-suspending cell pellets in 1 ml BG11, 5) centrifuging at 6,000 rpm at room temperature for 5 minutes, and 6) re-suspending in 1 ml BG11. This cell suspension was then spread onto BG11 plates containing 1.5% agar, and incubated at 30°C with constant illumination at 30 $\mu\text{mol}/\text{m}^2/\text{sec}$ until colonies were present (~1-2 weeks). The highest titer of chemical that the strain could survive was determined to be the initial tolerance level.

2.3 Mutant library creation via directed evolution

To create a library of putative mutants that potentially had increased biofuel tolerance, filamentous cyanobacteria strains were exposed to serially increasing titers of the biofuel of interest. Inoculum was mid-log cultures that were transferred into test tubes containing BG11 medium to achieve an OD_{700} of 0.5. The test tubes were spiked with specific titers of the biofuel, and were incubated under the conditions previously described. After 3 d, the cultures were centrifuged and washed as previously described. Cell suspensions were then inoculated onto BG11 plates containing 1.5% agar and incubated as described previously until colonies were present (~1-2 weeks). This represented one round of directed evolution.

Colonies that survived the highest titer of biofuel were selected from plates, and were then inoculated into 250 ml Erlenmeyer flasks containing 100 ml BG11 without the biofuel to allow for recovery. This was also to ensure that if a genetic change occurred

that led to increased biofuel tolerance, that the genetic change was stable and constant selective pressure wasn't needed. Cultures were incubated under the conditions previously described until they reached the optical density necessary to inoculate the next round of trials. In these trials, even higher titers of the biofuel were exposed to the cultures, and after incubation the cultures were again spread on plates to select the most tolerant survivors. This process was repeated a third time with even higher biofuel titers, and the cyanobacterial strains exhibiting maximal biofuel tolerance were frozen for long term storage. One isolate was selected for each species that survived the highest titer of each biofuel. Thus, with 3 cyanobacterial species and 4 biofuels, 12 putative mutants were selected.

Nomenclature for the putative mutants was based off the parent strain and the titer of biofuel tolerated. Strains were annotated as follows: 'A7120' for *Anabaena* sp. 7120, 'AV' for *Anabaena variabilis*, and 'NP' for *Nostoc punctiforme*. Abbreviations for biofuels were as follows: 'lina' for linalool, 'farn' for farnesene, 'limon' for limonene, and 'myr' for myrcene. Thus, A7120(0.32t).farn was an *Anabaena* sp. 7120 strain that was isolated from a test tube culture spiked with 0.32 g/L farnesene.

2.4 Screening mutants for increased tolerance to biofuels

To determine if the putative mutants in the library had an inheritable genetic or epigenetic change, their growth was compared to the wildtype strain in BG11 spiked with the biofuel of interest. A 1% inoculum (270 μ l) of a mid-log culture was transferred to 27 ml test tubes containing ~27 ml BG11 and 0.5 g/L NaHCO₃. A smaller initial inoculum was used so a standard microbial growth curve could be achieved. Test tubes were spiked

with 10% of the maximum biofuel titer that the putative mutant survived in the library creation study. For example, A7120(0.32t).farn and the wildtype control were inoculated into BG11 spiked with 0.032 g/L farnesene. On a daily basis, 100 µl samples were collected via a 1 ml syringe and a 21-gauge needle. Fluorescence was measured as described below. Fluorescence correlates with viability, thus an increase in fluorescence indicates an increase in viable number of cells. Maximum viability reached during a trial (U), percent change in viability from the initial time point, and rate of change in viability during the trial (U/d) were calculated. The trials continued for 1 d after the maximum viability was reached. This typically occurred during day 17-24 of the trial.

2.5 Analytical methods

Viability was determined in cyanobacterial cultures via fluorescence from SYTO[®] 9 (Life Technologies[™], Carlsbad, CA, USA). SYTO[®] 9 is a fluorescent dye capable of penetrating most cellular membranes and causing all cells containing nucleic acids to fluoresce green (Lee and Rhee, 2001; Shi et al., 2007). Typically, SYTO[®] 9 is used in conjunction with propidium iodide (PI) as a dual-stain assay in which SYTO[®] 9 causes viable cells to fluoresce in the green spectrum and PI causes non-viable cells to fluoresce in the red spectrum. This assay has previously been used in unicellular cyanobacteria (Zhu and Xu, 2013), however our research group determined that PI binds to both viable and non-viable cells in filamentous cyanobacteria and cannot accurately quantitate non-viable cells in the strains used in this study (Johnson et al., 2015). We later determined that SYTO[®] 9 does stain viable filamentous cyanobacteria cells as expected and was used to monitor growth in this study (Johnson et al., 2016a). The amount of fluorescence directly correlates with the amount of viable cells in the culture. This fluorescence

viability assay has been shown to be a superior method of monitoring viability of filamentous cyanobacteria at low biomass titers when compared to optical density and chlorophyll *a* content (Johnson et al., 2016a). Low biomass titers are recommended for chemical toxicity testing (Mayer et al., 1997; Nyholm and Peterson, 1997; Peterson and Nyholm, 1993). At high biomass levels the chemical can bind to the biomass altering the titer exposed to cells (Mayer et al., 2000).

For viability measurements via the fluorescence assay, 100 μ l samples were transferred to a 96-well plate. SYTO[®] 9 was added to each well at a concentration of 5 μ M, and fluorescence was measured by a Synergy 2 Multi-Mode Microplate Reader (BioTek[®], Winooski, VT, USA). To measure green fluorescence intensity units (U) from SYTO[®] 9 stained cells, fluorescence filters with excitation wavelength 485 ± 20 nm; emission wavelength 528 ± 20 nm were used.

2.6 Statistical analyses

To determine if significant differences existed between the biofuel tolerance of a putative mutant and the corresponding wildtype strain, two-sample one-tailed Student's *t*-tests (assuming unequal variances) were performed on the three growth parameters calculated from the fluorescence assay. Values were expressed as means $\pm \sigma$, and $p < 0.05$ was considered statistically significant. One-tailed *t*-tests were used rather than two-tailed *t*-tests as we were only concerned if the putative mutant strain performed better than its respective wildtype strain ($\mu_1 > \mu_2$). Any other outcome was not important as we were only interested in developing cyanobacterial strains with increased tolerance to biofuels.

To determine the sample size needed to achieve statistical power of ≥ 0.95 , G*Power statistical power analysis program was used to conduct *a priori* power analysis (Faul et al., 2007). After 20 trials, if statistical power of ≥ 0.95 was not achieved, then it was concluded that no significant difference among the means existed. All sample sizes compared were of equal size. The Shapiro-Wilk univariate normality test was used to test the normality of each data set (Ghasemi and Zahediasl, 2012). Normality was performed with a confidence level of 0.95. Statistical analyses were performed using Microsoft[®] Excel along with the Simetar[®] add-in. Simetar[®] is a risk analysis software that has been used extensively in business models and prospective businesses that can perform various statistical tests (Richardson and Johnson, 2015).

6.3 Results and discussion

3.1 Determination of wildtype cyanobacterial tolerance to biofuels

Long-chain organic chemicals, such as the potential biofuels tested herein, are generally toxic to microbes. These chemicals can damage the cell's membrane, resulting in leakage of metabolites and ions, which impacts the cells ability to maintain cellular functions (Dunlop, 2011; Segura et al., 2012; Turner and Dunlop, 2014). We first sought to establish the tolerance of the wildtype cyanobacterial strains used in this study to the biofuels of interest. Table 6.1 shows the biofuel titers that the filamentous cyanobacteria strains survived after 3 d exposure. Limonene and myrcene were the most toxic, as none of the species survived 3 d exposure to 0.03 g/L. Thus the initial tolerance was annotated <0.03 g/L. Farnesene was less toxic, as all 3 species were able to tolerate 0.1 g/L. Linalool was the least toxic, with *Anabaena* 7120 having a tolerance of 0.4 g/L, while *A. variabilis* and *N. punctiforme* tolerated 0.45 g/L.

Table 6.1: Biofuel tolerance of wildtype cyanobacteria compared to tolerance following three rounds of directed evolution.

		Tolerance Level (g/L)					
<u>Chemical</u>	<u>Strain</u>	<u>Wildtype</u>	<u>Trial 1</u>	<u>Trial 2</u>	<u>Trial 3</u>	<u>% Increase from Wildtype</u>	
Farnesene	<i>Anabaena</i> 7120	0.1	0.2	0.23	0.32	220	
	<i>A. variabilis</i>	0.1	0.25	0.34	0.43	330	
	<i>N. punctiforme</i>	0.1	0.15	0.21	0.21	110	
Linalool	<i>Anabaena</i> 7120	0.4	0.4	0.4	0.46	15	
	<i>A. variabilis</i>	0.45	0.54	0.63	0.72	60	
	<i>N. punctiforme</i>	0.45	0.54	0.54	0.54	20	
Limonene	<i>Anabaena</i> 7120	<0.03	0.12	0.21	0.36	1,100	
	<i>A. variabilis</i>	<0.03	0.12	0.21	0.36	1,100	
	<i>N. punctiforme</i>	<0.03	No growth	No growth	0.12	300	
Myrcene	<i>Anabaena</i> 7120	<0.03	0.12	0.27	0.42	1,300	
	<i>A. variabilis</i>	<0.03	0.12	0.21	0.36	1,100	
	<i>N. punctiforme</i>	<0.03	No growth	No growth	0.06	100	

Linalool toxicity to microbes is well established (Bagamboula et al., 2004; Queiroga et al., 2007; Schmidt et al., 2005), and it is used as an antimicrobial agent (generally as a component in plant oils) in certain applications such as food preservation or as an anti-leishmanial agent (Busatta et al., 2007; Busatta et al., 2008; Maria do

Socorro et al., 2003). Oregano essential oil containing linalool has an average minimum inhibitory concentration (MIC) of ~0.45 g/L for various Gram positive and negative bacteria (Busatta et al., 2007) Marjoram essential oil containing linalool has an average MIC of ~1.2 g/L for various Gram positive and negative bacteria (Busatta et al., 2008). Pretreatment of mouse peritoneal macrophages with 0.015 g/L plant oil containing linalool reduced by 50% the interaction between macrophages and *Leishmania amazonensis* (Maria do Socorro et al., 2003). Alcohols, such as linalool, possess bactericidal activity against vegetative cells (Dorman and Deans, 2000), with the mechanism of action being damage to the cell membrane (Yomano et al., 1998). It is also well established that hydrocarbons such as limonene, myrcene, and farnesene are toxic to microbes (Dunlop, 2011; Ramos et al., 2002; Sikkema et al., 1995). The mechanism of toxicity for hydrocarbons is that they accumulate in the membrane lipid bilayer which affects the structural and functional properties of the membrane (Sikkema et al., 1995). However, there are some strains of cyanobacteria (e.g., *Anabaena ambigua* and *Nostoc muscorum*) that can tolerate and utilize hydrocarbons as a carbon source (Akoijam et al., 2015; Samanthakamani and Thangaraju, 2015). This conclusion was reached by growing the strains in various concentration of sterilized hydrocarbon sludge and measuring photosynthetic oxygen evolution (Akoijam et al., 2015), or by determining if cyanobacterial colonies can grow on solid medium with 1% petroleum effluent (Samanthakamani and Thangaraju, 2015).

3.2 Mutant library creation via directed evolution

The first step in a directed evolution protocol is to create a library of mutant strains (Kuchner and Arnold, 1997; Umeno et al., 2005). The range of biofuel titers used

to increase cyanobacterial tolerance in each of the 3 trials is shown in Table 6.2. The results of the three sequential directed evolution trials, in which the strains were exposed to progressively higher biofuel titers, are shown in Table 6.1. Throughout these trials, *N. punctiforme* was the weakest performer, and we have previously observed that *N. punctiforme* is less able to handle stressful conditions than *A. variabilis* and *Anabaena* 7120. When grown in the presence of limonene and myrcene, *N. punctiforme* formed small pellets of aggregated cells, leaving the culture fluid transparent. Only a slight amount of growth was evident following trial 3, with tolerance only increasing to 0.06 g/L for myrcene and 0.12 g/L for limonene. In the case of linalool, *N. punctiforme* tolerance increased from 0.45 g/L in the wildtype to 0.54 g/L after the first round of directed evolution. However, no further improvements were noted thereafter. For farnesene, the tolerance of *N. punctiforme* improved from 0.1 g/L in the wildtype to 0.21 g/L after the second round of directed evolution.

Table 6.2: Biofuel titers used in cyanobacteria directed evolution trials.

(Bolded values indicate highest titer cyanobacteria were isolated from after 3 d incubation; No bolded data indicates no growth occurred).

<u>Chemical</u>	<u>Strain</u>	<u>Wildtype tolerance (g/L)</u>	<u>Trial 1 Titters (g/L)</u>	<u>Trial 2 Titters (g/L)</u>	<u>Trial 3 Titters (g/L)</u>
Farnesene	<i>Anabaena</i> 7120	0.1	0.1, 0.15, 0.2 , 0.25	0.2, 0.23 , 0.26, 0.29	0.23, 0.26, 0.29, 0.32
	<i>A. variabilis</i>	0.1	0.1, 0.15, 0.2, 0.25	0.25, 0.28, 0.31, 0.34	0.34, 0.37, 0.4, 0.43
	<i>N. punctiforme</i>	0.1	0.1, 0.15 , 0.2, 0.25	0.15, 0.18, 0.21 , 0.24	0.21 , 0.24, 0.27, 0.3
Linalool	<i>Anabaena</i> 7120	0.4	0.4 , 0.43, 0.46, 0.49	0.4 , 0.43, 0.46, 0.49	0.4, 0.43, 0.46 , 0.49
	<i>A. variabilis</i>	0.45	0.45, 0.48, 0.51, 0.54	0.54, 0.57, 0.6, 0.63	0.63, 0.66, 0.69, 0.72
	<i>N. punctiforme</i>	0.45	0.45, 0.48, 0.51, 0.54	0.54 , 0.57, 0.6, 0.63	0.54 , 0.57, 0.6, 0.63
Limonene	<i>Anabaena</i> 7120	<0.03	0.03, 0.06, 0.09, 0.12	0.12, 0.15, 0.18, 0.21	0.21, 0.26, 0.31, 0.36
	<i>A. variabilis</i>	<0.03	0.03, 0.06, 0.09, 0.12	0.12, 0.15, 0.18, 0.21	0.21, 0.26, 0.31, 0.36
	<i>N. punctiforme</i>	<0.03	0.03, 0.06, 0.09, 0.12	0.03, 0.06, 0.09, 0.12	0.03, 0.06, 0.09, 0.12
Myrcene	<i>Anabaena</i> 7120	<0.03	0.03, 0.06, 0.09, 0.12	0.12, 0.17, 0.22, 0.27	0.27, 0.32, 0.37, 0.42
	<i>A. variabilis</i>	<0.03	0.03, 0.06, 0.09, 0.12	0.12, 0.15, 0.18, 0.21	0.21, 0.26, 0.31, 0.36
	<i>N. punctiforme</i>	<0.03	0.03, 0.06, 0.09, 0.12	0.03, 0.06, 0.09, 0.12	0.03, 0.06 , 0.09, 0.12

In response to stress, *Nostoc* spp. can produce gliding filaments termed hormogonia (Meeks et al., 2001). Risser et al. (2014) associated hormogonia formation with a cell aggregation phenotype by deleting genes responsible for hormogonia formation and observing that hormogonia-deficient mutants could not grow into aggregated colonies. Presumably, the aggregation phenotype we observed with *N. punctiforme* was

due to the stress induced by limonene and myrcene. It is possible that when the cells within a filament disperse to form hormogonia, they become less able to tolerate the chemicals used in this study.

While *A. variabilis* is also capable of hormogonia formation (Takaichi et al., 2006), we did not observe the aggregated phenotype upon exposure to the biofuels tested herein. *Anabaena* 7120 is not capable of hormogonia formation (Cohen et al., 1994). *A. variabilis* and *Anabaena* 7120 showed higher tolerance to all four biofuels, and showed continual improvement throughout the three cycles of directed evolution. *A. variabilis* developed the highest tolerance to farnesene (0.43 g/L) and linalool (0.72 g/L), while *Anabaena* sp. 7120 developed the highest tolerance to myrcene (0.42 g/L). The strains developed the same tolerance to limonene (0.36 g/L).

There has been a considerable amount of research investigating the toxicity of the four chemicals used in this study. The MIC of linalool to methicillin-resistant *Staphylococcus aureus* (MRSA) is > 0.0128 g/L (Kook et al., 2013). Linalool has a MIC value of 0.386 g/L for *Microcystis* NIES-102 (Ozaki et al., 2008). This was established by determining the concentration of linalool that reduced absorbance (of chlorophyll α) by 50% compared to the control culture. Hellier et al., (2013) determined that 0.0002 g/L linalool was toxic to *Synechocystis* sp. PCC 6803 in 24 h, by noting that this titer caused cells to bleach. The data reported in Table 6.1 shows that the three cyanobacteria tested herein were more tolerant to linalool, and that even higher tolerance was achieved through directed evolution.

Various studies have determined the MIC of myrcene, limonene, and farnesene, either as individual chemicals or combined in plant oils (Aggarwal et al., 2002; Delaquis

et al., 2002; Negi et al., 1999). Limonene (12 g/L) has been shown to gradually reduce the growth of the freshwater cyanobacterium *Microcystis aeruginosa* FACHB-905 (Hu et al., 2014). Myrcene has a lowest-complete-inhibition concentration (LCIC) value of >0.0136 g/L for the cyanobacterium *Oscillatoria perornata* and the algae *Selenastrum capricornatum* (Schrader and Harries, 2001). *Synechocystis* sp. PCC 6803 was able to tolerate farnesene for 24 h at 0.010 g/L, which was the highest titer tested (Hellier et al., 2013). While there are numerous studies that elucidate the toxicity of the chemicals used in this study, direct comparisons are not possible due to the differences in methodologies and microorganisms studied. Also, none of the previous studies utilized filamentous cyanobacteria which were used in this study. In 9 of the 12 directed evolution trials to create the mutant library, tolerance to the specific chemical continued to increase from trial 1 to trial 3. Thus it is possible further directed evolution trials could have led to even higher tolerance, and this will be the focus of future work.

3.3 Screening mutants for increased tolerance to biofuels

To determine if the 12 isolates developed in the directed evolution study had inheritable genetic or epigenetic changes that increased biofuel tolerance, they were screened in comparison to the corresponding wildtype strains. Hence, each putative mutant and corresponding wildtype strain were inoculated into BG11 medium that was spiked with 10% of the maximum chemical concentration that the putative mutant had tolerated. The results of this mutant screen are shown in Table 6.3.

Table 6.3: Comparison of putative mutants vs. wildtype strains using two-sample one-tailed Student's t-tests assuming unequal variances.

<i>Anabaena</i> 7120	Maximum Viability (U)	Change in Viability (%)	Rate of Change in Viability (U/d)
A7120(0.32t).farn	+	+	+
A7120(0.46t).lina	§	§	§
A7120(0.36t).limon	*	*	*
A7120(0.42t).myr	*	*	*
<i>A. variabilis</i>			
AV(0.43t).farn	*	*	*
AV(0.72t).lina	+	+	+
AV(0.36t).limon	*	*	*
AV(0.36t).myr	§	§	§
<i>N. punctiforme</i>			
NP(0.21t).farn	*	*	*
NP(0.54t).lina	+	§	+
NP(0.06t).myr	§	§	§

+: Putative mutant performed significantly better than the wildtype

*: Putative mutant did not perform statistically better than the wildtype

§: Insufficient statistical power achieved after 20 trials, thus it was determined no statistical difference exists

Of the 12 mutants screened, 3 were confirmed to be mutants via the statistical analyses that were previously described. Each parent strain of filamentous cyanobacteria yielded one mutant. Exposure to linalool yielded 2 mutants, while farnesene exposure resulted in one mutant. The two chemicals with the highest toxicity (myrcene and limonene) yielded no mutants. AV(0.43t).farn and AV(0.36t).limon were quite difficult to recover from being frozen at -80°C, and once recovered did not grow well even in BG11 not spiked with biofuels. This suggests that the genetic changes that occurred during the 3 d of biofuel exposure weakened the strains.

The statistical information used to compare the confirmed putative mutants to their respective wildtype strain is shown in Table 6.4. Generally, if a significant increase occurred in one growth parameter for a putative mutant compared to the wildtype, then it occurred for all 3 parameters. The only time this was not the case was the % change in viability of NP(0.54t).lina compared to wildtype *N. punctiforme*. After 20 trials, the necessary statistical power was not achieved thus it was concluded that no statistical difference existed in this growth parameter. As the putative mutant performed statistically better in the other two growth parameters measured, it was determined that an inheritable genetic or epigenetic change did occur.

Table 6.4: Statistical information used for the comparison of confirmed mutants vs. wildtype strains.

	Maximum Viability (U)		Change in Viability (%)		Rate of Change in Viability (U/d)	
	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
n = 6						
A7120(0.32t).farn	5635.0	1426.0	139.5	87.7	234.5	190.7
Anabaena sp. 7120	2843.8	813.8	33.0	63.1	34.2	71.6
n = 4						
AV(0.72t).lina	57260.5	16720.1	2061.0	293.5	3459.1	1638.8
A. variabilis	24990.5	18551.9	76.9*	29.8	1221.3	1085.8
n = 17						
NP(0.54t).lina	22253.9*	9702.7	§	§	3669.6*	2924.2
N. punctiforme	10103.24*	9149.9	§	§	527.6*	730.4

§: Insufficient statistical power achieved after 20 trials, thus it was determined no statistical difference exists

*: Dataset was not normally distributed

The 3 confirmed mutants were observed via light microscopy (400x magnification) to determine if any morphological changes were apparent in the mutant strains when exposed to medium supplemented with the biofuel of interest as well as in cultivation medium not spiked with the biofuel. In a study by (Torres et al., 2005), transmission electron microscopy (TEM) was used to determine the morphological

effects in *Anabaena* 7120 associated with hexadecane and dodecane. This study showed that photosynthetic and cellular membranes were altered by dodecane, but the same effect was not observed with hexadecane treatment. Our research group hypothesized that cellular membrane morphology would be altered in the mutant strains when exposed to the chemicals of interest due to the damage in cellular membranes that is caused by similar chemicals (Dunlop, 2011; Segura et al., 2012; Turner and Dunlop, 2014). However, no apparent morphological changes were observed using light microscopy (data not shown). Potentially morphological changes could be observed with TEM, however our research group does not have access to this instrument, thus this was considered outside the scope of this study.

All datasets from the confirmed mutants were normally distributed with the exception of the maximum viability and rate of change in viability for NP(0.54t).lina. Wildtype strains that were not normally distributed were the maximum viability and rate of change in viability for *N. punctiforme* with linalool, and percent change in viability for *A. variabilis* with linalool. This was problematic as Student's t-tests assume normal distribution. An alternative would be to use the Wilcoxon-Mann-Whitney test, however the variances of the datasets are unequal, thus a t-test assuming unequal variances is preferred (Skovlund and Fenstad, 2001). One possible reason for the non-normally distributed data for *N. punctiforme* cultures was the aggregated phenotype previously discussed. Aggregated cells in cultures makes sampling difficult as the cells aren't evenly dispersed. Methods for cell separation (i.e. sonication) were not used, as dispersing the filaments could have affected the ability of the microorganism to survive the biofuel exposure testing.

The robustness of the statistical analyses used in this study enabled us to conclude the three mutants: A7120(0.32t).farn, AV(0.72t).lina, and NP(0.54t).lina have an inheritable genetic or epigenetic change that led to an increased tolerance to the chemical of interest compared to the wildtype. It is hypothesized that the phenotypic changes that led to increased biofuel tolerance could be due to altered efflux pumps. Efflux pumps are cellular membrane protein complexes that protect the cell from biofuel toxicity by exporting the biofuel molecules outside of the cell (Dunlop, 2011; Dunlop et al., 2011; Dunlop et al., 2010; Paulsen et al., 1996; Turner and Dunlop, 2014).

Another mechanism that could be responsible for the increased tolerance is activation of hydrocarbon degradation proteins. There are several examples of cyanobacterial strains capable of degrading hydrocarbons (Cerniglia et al., 1980; Raghukumar et al., 2001). For example, *Anabaena* and *Nostoc* spp. (not the species used in this study) have been shown to be capable of oxidizing naphthalene (Cerniglia et al., 1980). However, typically cyanobacteria only contribute indirectly to hydrocarbon degradation (Harayama et al., 2004). While Cohen, (2000) observed cyanobacteria mats to cause efficient degradation of crude oil, cyanobacteria strains isolated from the mat were not capable of crude oil degradation. The authors concluded that aerobic heterotrophic bacteria in the cyanobacterial mats were responsible for oil degradation.

It is unlikely that hydrocarbon degradation would significantly decrease the production of the chemicals investigated in this study. Two of the chemicals (limonene and farnesene) have previously been produced by genetically engineered strains of filamentous cyanobacteria, and to collect the chemical, air was sparged into the culture vessel to volatilize the chemicals into the headspace (Halfmann, et al., 2014a, b). Thus

the chemicals would probably not be in solution long enough for the bacteria to metabolize them. If hydrocarbon metabolism did exist in industrial strains of cyanobacteria and the responsible gene or genes were known, then the gene could be down-regulated or knocked-out to prevent metabolism from occurring.

The genomes of *Anabaena* 7120 (Kaneko, et al., 2001), *A. variabilis* ATCC 29413 (Chen et al., 2009), and *N. punctiforme* ATCC 29133 (Schütz et al., 2004) have all been sequenced. Ideally, we would be able to determine where the genetic change in the mutated strains have occurred, however a sequenced genome does not mean all genes have been annotated. For example, when *Anabaena* 7120 was sequenced, 27% of the predicted products were hypothetical genes and 28% lacked significant similarity to genes for known and predicted proteins in the public DNA databases (Kaneko et al., 2001). Furthermore, the random mutations induced by directed evolution may be epigenetic or multigenic in nature, thus sequencing would not be conclusive. It is not certain the gene or genes responsible for the phenotypic change would be identified if the genomes were sequenced and is therefore not recommended with the mutated strains used developed in this study.

6.4 Conclusions

This study showed that directed evolution can be used to increase the tolerance of filamentous cyanobacteria to long-chain alcohols and hydrocarbons that can be used as biofuels and industrial chemicals. Mutants developed to tolerate high titers of these biofuels and chemicals could then be genetically engineered to produce the same chemical, as has been shown with wildtype cyanobacteria (Gu et al., 2012; Halfmann et al., 2014a; Halfmann et al., 2014b; Kiyota et al., 2014; Lan and Liao, 2011). Presumably,

an increase in biofuel tolerance would also lead to increased productivity if the microbe has an enhanced ability to export biofuel from the cell (Alper et al., 2006; Dunlop et al., 2011; Tomas et al., 2003). However, there are examples where increased tolerance had no effect or a decreased effect on productivity (Atsumi et al., 2010; Baer et al., 1987; Zhao et al., 2003). As the need for renewable biofuels increases, enhancing microbial tolerance to the biofuel they produce is essential in order to develop an economically feasible production system.

Acknowledgments

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Chapter 7 - Determining the optimal nitrogen source for large-scale cultivation of filamentous cyanobacteria

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Abstract

As the world's population continues to increase and the adverse effects of anthropomorphic CO₂ intensify, it is becoming increasingly important to develop biofuels and chemicals from sustainable resources. Filamentous cyanobacteria, including *Anabaena* sp. PCC 7120, have emerged as a promising source of renewable chemicals and biofuels due to their minimal nutrient requirements and the relative ease with which they can be genetically engineered to produce a diversity of products. This study evaluated the effects of several nitrogen sources on the growth of *Anabaena* sp. PCC 7120, and then performed an environmental comparative study on a theoretical large-scale production process to down select to the best nitrogen source. Sodium nitrate and ammonium chloride yielded 65% more growth compared to the other nitrogen sources

evaluated. Ammonium chloride yielded marginal savings of \$22,318 annually, compared to sodium nitrate over a 27-year lifespan of a proposed chemical production facility utilizing filamentous cyanobacteria. Sodium nitrate had substantially greater negative impacts in every environmental category compared to ammonium chloride. For example, sodium nitrate had a ~3-fold greater negative impact in human health, ecosystem quality, and resources categories. Thus we concluded that ammonium chloride is the preferred nitrogen source in large scale processes involving filamentous cyanobacteria.

7.1 Introduction

It continues to be important that we develop renewable, sustainable processes to produce chemicals and fuels to decrease the world's dependence on fossil fuels. The adverse environmental impacts of fossil fuel use, especially climate changes attributed to increasing CO₂ levels, and the geopolitical issues involved with control of these resources and their transport are the main driving forces behind the desire to create a bioeconomy based on sustainable raw materials (Petrovic, 2015). One option for producing renewable fuels and chemicals is photoautotrophic organisms such as microalgae and cyanobacteria. Many researchers consider these microorganisms to be the most feasible option to sustainably and economically meet the global demand for chemicals and transportation fuels (Brennan and Owende, 2010; Chisti, 2007; Schenk et al., 2008; Singh et al., 2011a).

Filamentous photoautotrophic cyanobacteria have garnered a lot of attention from industrial microbiologists due to their ability to produce high-value chemicals and next-generation biofuels (Gu et al., 2012; Halfmann et al., 2014a; Halfmann et al., 2014b) from CO₂ and solar energy (Machado and Atsumi, 2012). Also of interest is the capability of some strains of filamentous cyanobacteria to fix atmospheric nitrogen via specialized

cells termed heterocysts. This would eliminate the need to add combined nitrogen to the cultivation medium, which would reduce costs. However, nitrogen fixation is an energy demanding process, requiring 8 electrons and at least 16 adenosine triphosphate (ATP) per mole of N₂ fixed (Stal, 2003). Also, heterocysts do not form until the cells have been nitrogen starved for approximately 20 h (Horn, 2008). This occurs because the nitrogen-fixation (*nif*) genes are expressed 18-24 hours after nitrogen deprivation (Kumar et al., 2010). Thus, even though some strains of filamentous cyanobacteria are capable of fixing atmospheric nitrogen, supplementing the medium with combined nitrogen may be preferred from an industrial standpoint.

Table 7.1 shows that previously, *Anabaena* sp. PCC 7120 (hereafter *Anabaena* 7120) has been cultivated in medium without combined nitrogen and several combined nitrogen sources, including: sodium nitrate, ammonium nitrate, and urea. Figure 7.1 shows that combined nitrogen sources are taken up through various permeases and reduced into ammonium. These reactions require energy, which is the reason most photoautotrophs prefer ammonium as a source of nitrogen (Muro-Pastor and Florencio, 2003). Ammonium, whether taken up directly from the medium, or from the metabolism of other nitrogen sources, is then incorporated into carbon skeletons through the sequential operation of two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT) in the GS-GOGAT pathway (Muro-Pastor and Florencio, 2003; Moreno-Vivián and Flores, 2006). Nitrogen in glutamine or glutamate is then distributed to the other nitrogen-containing organic compounds (Flores and Herrero, 2005).

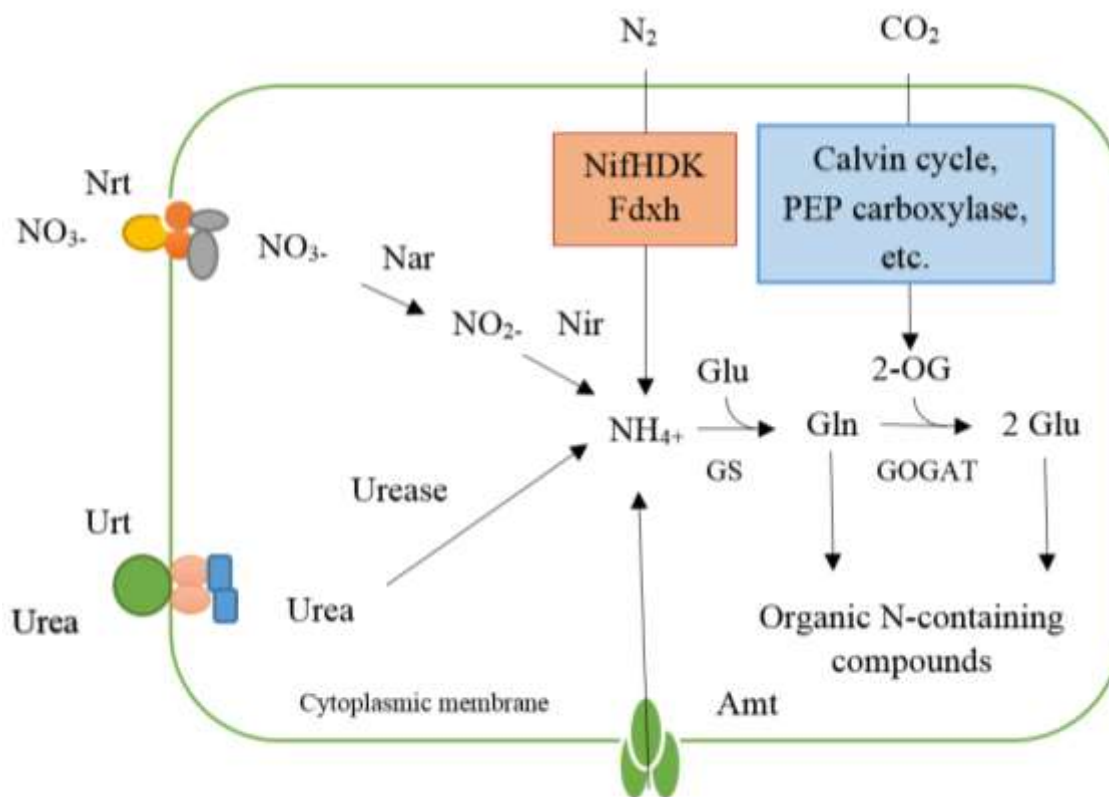


Figure 7.1: Primary nitrogen assimilation pathways in cyanobacteria.
(after Flores and Herrero, 2005).

A common laboratory medium used to cultivate cyanobacteria is BG11 supplemented with sodium nitrate at 248 mg N L^{-1} (Horn, 2008; Allen and Stanier, 1968; Laurent et al., 2005). However, other studies report that ammonium is preferred over nitrate as a nitrogen source for cyanobacteria (Flores and Herrero, 2005; Flores et al., 2005; Boussiba and Gibson, 1991; Ohashi et al., 2011; Herrero et al., 2001). Researchers have used BG11 supplemented with ammonium chloride at $14\text{-}69 \text{ mg N L}^{-1}$ rather than sodium nitrate at 248 mg N L^{-1} for cultivating *Anabaena* 7120 (Merino-Puerto et al., 2010; Picossi et al., 2005; Sato et al., 2004; Laurent et al., 2005).

Table 1 shows several studies that evaluated different nitrogen sources for the growth of various cyanobacterial strains. Standard BG11 contains sodium nitrogen at a concentration of 247.03 mg N L⁻¹. When used to cultivate *Anabaena* 7120, Wang and Liu, (2003) reported a maximum cell density of OD₇₅₀ of ~1.2, and Picossi et al., (2005) reported a maximum growth rate (μ) of 0.87 d⁻¹. Burat et al., (2014) and Merino-Puerto et al., (2013) reported a μ of 0.79 d⁻¹ and Pernil et al., (2008) reported a μ of 0.66 d⁻¹. Several studies have evaluated the growth (μ) of *Anabaena* 7120 in BG11 with no combined nitrogen source (BG11₀) and the results ranged from 0.35 – 0.892 d⁻¹ (Burat et al., 2014; Merino-Puerto et al., 2013; Pernil et al., 2008; Picossi et al., 2005; Picossi et al., 2015; Plominsky et al., 2015; Valladares et al., 2003).

Ammonium chloride at concentrations of 14-69 mg N L⁻¹ has been used by several researchers to cultivate *Anabaena* 7120 (Laurent et al., 2005; Merino-Puerto et al., 2010; Picossi et al., 2005; Sato et al., 2004). Growth rates are not reported in these studies as that was not the focus of the research. For example, Sato et al., (2004) used ammonium chloride in BG11 as a means to repress genes that are activated in the absence of ammonium. Other studies used 28.0 or 56.0 mg N L⁻¹ and observed a μ of 0.55 – 0.690 d⁻¹ (Burat et al., 2014; Merino-Puerto et al., 2013; Plominsky et al., 2015). Wang and Liu, (2003) tested a much higher level of ammonium chloride (247.03 mg N L⁻¹) and observed a ~6-fold decrease in growth (OD₇₅₀) compared to the same concentration of sodium nitrate. The same study found that 247.03 mg N L⁻¹ urea led to a ~12-fold decrease in growth compared to the same concentration of sodium nitrate. Wang and Liu, (2003) thus concluded that ammonium chloride and urea were inhibitory to *Anabaena* 7120 at high titers.

Table 7.1: Studies evaluating nitrogen supplementation for growth of cyanobacteria.

Cyanobacteria species/strain	Basal Medium	Nitrogen type	Nitrogen concentration	Maximum cell density or growth rate reported	Reference
<i>Anabaena</i> 7120	BG11	NaNO ₃	247.03 mg N L ⁻¹	OD ₇₅₀ : ~1.2	Wang and Liu 2003
<i>Anabaena</i> 7120	BG11	NaNO ₃	247.03 mg N L ⁻¹	μ: 0.87 d ⁻¹	Picossi et al. 2005
<i>Anabaena</i> 7120	BG11	NaNO ₃	247.03 mg N L ⁻¹	μ: 0.79 d ⁻¹	Burat et al. 2014; Merino-Puerto et al. 2013
<i>Anabaena</i> 7120	BG11	NaNO ₃	247.03 mg N L ⁻¹	μ: 0.66 d ⁻¹	Pernil et al. 2008
<i>Anabaena</i> 7120	BG11	N ₂	N/A	μ: 0.5 d ⁻¹	Picossi et al. 2005
<i>Anabaena</i> 7120	BG11	N ₂	N/A	μ: 0.892 d ⁻¹	Picossi et al. 2015
<i>Anabaena</i> 7120	BG11	N ₂	N/A	μ: 0.35 d ⁻¹	Valladares et al. 2003
<i>Anabaena</i> 7120	BG11	N ₂	N/A	μ: 0.67 d ⁻¹	Burat et al. 2014
<i>Anabaena</i> 7120	BG11	N ₂	N/A	μ: 0.565 d ⁻¹	Merino-Puerto et al. 2013
<i>Anabaena</i> 7120	BG11	N ₂	N/A	μ: 0.49 d ⁻¹	Pernil et al. 2008
<i>Anabaena</i> 7120	BG11	N ₂	N/A	μ: 0.700 d ⁻¹	Plominsky et al. 2015
<i>Anabaena</i> 7120	BG11	NH ₄ Cl	247.03 mg N L ⁻¹	OD ₇₅₀ : ~0.2	Wang and Liu 2003
<i>Anabaena</i> 7120	BG11	NH ₄ Cl	56.0 mg N L ⁻¹	μ: 0.55 d ⁻¹	Burat et al. 2014
<i>Anabaena</i> 7120	BG11	NH ₄ Cl	56.0 mg N L ⁻¹	μ: 0.625 d ⁻¹	Merino-Puerto et al. 2013
<i>Anabaena</i> 7120	BG11	NH ₄ Cl	28.0 mg N L ⁻¹	μ: 0.690 d ⁻¹	Plominsky et al. 2015
<i>Anabaena</i> 7120	BG11	NH ₄ Cl	14-69 mg N L ⁻¹	No growth rates reported	Merino-Puerto et al. 2010; Picossi et al. 2005; Sato et al. 2004; Laurent et al. 2005
<i>Anabaena</i> 7120	BG11	CH ₄ N ₂ O	247.03 mg N L ⁻¹	OD ₇₅₀ : ~0.1	Wang and Liu 2003
<i>Anabaena circinalis</i>	ASM-1	Ammonium (form not specified)	0.04 mg N L ⁻¹	μ: 0.14 d ⁻¹	Velzeboer et al. 2001
<i>Arthrospira platensis</i>	Paoletti et al. 1975	NH ₄ NO ₃	630 mg N L ⁻¹	Cell density: 4844 mg L ⁻¹	Martinez et al. 2010
<i>Arthrospira platensis</i>	Schlösser et al. 1982	CH ₄ N ₂ O	1.00 kg m ⁻³	Cell density: 2960 g m ⁻³	Bezerra et al. 2013

Nitrogen supplementation research has also been conducted on other cyanobacterial strains (Table 7.1). Velzeboer et al, (2001) evaluated a very low level of an undefined ammonium source (0.04 mg N L^{-1}) and obtained a specific growth rate of 0.14 d^{-1} . In trials with *Arthrospira platensis*, biomass and nitrogen-to-cell conversion factors were higher at concentrations of ammonium nitrate and urea compared to identical concentrations of sodium nitrate (Bezerra et al., 2013; Stanca and Popovici, 1996). However, to the best of our knowledge, a comprehensive evaluation of various nitrogen sources (including N_2 gas) has not been conducted for the growth of *Anabaena* 7120. The objectives of this study were to: 1) compare the effects of nitrogen supplementation on the growth, heterocyst frequency, and filament morphology of *Anabaena* 7120, 2) perform an environmental comparative study on the best nitrogen source for a theoretical large-scale production facility, and 3) determine which nitrogen source is preferred for the large-scale cultivation of filamentous cyanobacteria. The nitrogen sources evaluated in this study were: sodium nitrate, ammonium chloride, ammonium nitrate, urea, and atmospheric nitrogen. Nitrite was not investigated in this study as it is toxic to many animals, including humans (Cockburn et al., 2013; Archer, 2012), and thus would not be preferred in large-scale processes. This study will be of value to researchers interested in the scaling-up of any industrial process involving filamentous cyanobacteria.

7.2 Materials and methods

7.2.1 Microbial strains, maintenance, and culture conditions

The filamentous, diazotrophic cyanobacterial strain *Anabaena* 7120, a model specie for filamentous cyanobacteria (Bryant, 2006; Rippka et al., 1979), was obtained

from the Pasteur Culture Collection of Cyanobacteria (Paris, France). For long term storage, the strain was frozen at -80°C in 5% v/v methanol. For short term maintenance, the strain was grown on BG11 agar (1.5% agar) (Allen and Stanier, 1968) at pH 7.1 and incubated at room temperature under constant illumination of $24\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$, and then stored at room temperature. Light intensities were measured with a Heavy Duty Light Meter with PC Interface (Extech[®] Instruments, Waltham MA, USA).

In the experiments described below, cyanobacterial cultures were grown in 250 ml Erlenmeyer flasks containing 100 ml of various cultivation media at pH 7.1 supplemented with 20 mM HEPES buffer. All variations of cultivation media were BG11 (Allen and Stanier, 1968) with different nitrogen sources (Table 7.2). To make the media, the chemicals were mixed thoroughly and pH was adjusted to 7.1. Then the media was autoclaved and once cooled, HEPES was added. Flasks were stoppered with a foam stopper and the opening covered with aluminum foil prior to autoclaving. To provide inoculum for the experiments, a seed culture of *Anabaena* 7120 was cultivated in a 250 ml Erlenmeyer flask containing 100 ml BG11₀ (standard BG11 without NaNO_3) at pH 7.1 supplemented with 20 mM HEPES buffer. The flasks were incubated in a Lab-Line[®] Incubator-Shaker (Lab-Line[®] Instruments, Melrose Park, IL, USA) at 30°C and 100 rpm under constant illumination of $19\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ using fluorescent lights.

7.2.2 Evaluation of nitrogen sources on growth of *Anabaena* 7120

To determine the effects of different nitrogen sources on the growth of *Anabaena* 7120, a 1% (1 ml) inoculum of the seed culture, grown to mid-log phase, was transferred into 100 ml of BG11 medium, either supplemented with different nitrogen sources or not supplemented with any fixed nitrogen source (BG11₀) (Table 7.2). The 250 ml

Erlenmeyer flasks were incubated as previously described until 1 d after the maximum viability was reached. The pH was maintained at 7.00-7.20 throughout incubation by adjusting as necessary with 3 M HCl or 10 M NaOH. For daily viability determinations, 100 μ l samples were transferred to a microplate. For daily heterocyst frequency determinations via microscopy, 100 μ l samples were transferred to a 1.5 ml microcentrifuge tube. At the conclusion of each trial to observe filament morphology via microscopy, 100 μ l samples were transferred to a 1.5 ml microcentrifuge tube.

Table 7.2: Nitrogen sources evaluated for growth of *Anabaena* 7120.

Medium	N source	Concentration of N source in medium *
BG11	NaNO ₃	1.50 g/L
BG11 ₀	N ₂	N/A
BG11 _U	CH ₄ N ₂ O	0.53 g/L
BG11 _{AN}	(NH ₄)(NO ₃)	0.71 g/L
BG11 _{AC}	NH ₄ Cl	0.94 g/L

*The concentration of each nitrogen source in the cultivation medium was 248 mg N L⁻¹ nitrogen

7.2.3 Comparative study of sodium nitrate and ammonium chloride for large-scale *Anabaena 7120* production

The current bulk prices of sodium nitrate (N source in BG11) and ammonium chloride (N source in BG11_{AC}) were used, along with cell production data, to compare the economics of using these nitrogen sources in a commercial production facility. Life-cycle analysis (LCA) modeling was also performed using the SimaPro 8 software package to compare the environmental impacts of using different nitrogen sources. Inventory data were collected from various databases provided in the software (Finkbeiner et al., 2006), including: Ecoinvent v3 LCI database, US Input Output library, and LCA Food and Industry data v2. SimaPro 8 contains a number of impact assessment methods, which are used to calculate impact assessment results.

For the life cycle impact assessment (LCIA), Eco-indicator 99 was employed. Eco-indicator 99 is an endpoint method based on a weighing procedure which uses the damage-oriented approach. Also, Eco-indicator 99 is capable of expressing the environmental impact as a single value. The advantage of using Eco-indicator 99 is that the standard unit given in all the categories is point (Pt) or millipoint (mPt) which makes the comparison of different components and products possible. Eco-indicator 99 evaluates three different cultural perspectives or “Archetypes” including Hierarchist (H), Individualist (I) as well as Egalitarian (E) (Acero et al., 2014; Goedkoop et al., 2008).

Generally, in life cycle studies, the emissions and impacts to the environment are expressed in different impact categories including: acidification, ozone layer depletion, ecotoxicity, and resource extraction. Due to the difficulty of assigning meaningful

weighing factors for these impact categories, the different types of damage that are caused by these impact categories were weighed. Therefore, the seriousness of three damage categories was considered including: Damage to Human Health (DHH), Damage to Ecosystem Quality (DEQ), and Damage to Resources (DR) (Acero et al., 2014; Goedkoop et al., 2008).

7.2.5 Analytical methods

Classical methods of quantifying filamentous cyanobacteria are not effective due to their filamentous morphology (Johnson et al., 2015; Johnson et al., 2016a). Thus, cyanobacterial viability was determined via fluorescence from SYTO[®] 9 (Life Technologies[™], Carlsbad, CA, USA) (Johnson et al., 2016a). SYTO[®] 9 is a membrane permeant fluorescent dye capable of crossing cellular membranes and causing cells containing nucleic acids to fluoresce in the green spectrum (Lee and Rhee, 2001; Shi et al., 2007). The amount of fluorescence directly correlates with the amount of viable cells in the culture. This assay has been shown to be capable of monitoring the viability of *Anabaena* 7120 and has a strong correlation to chlorophyll and absorbance measurements (Johnson et al., 2016a,d). For viability measurements via the fluorescence assay, 100 μ l samples were transferred to a 96-well microplate. SYTO[®] 9 was added to each well at a concentration of 5 μ M, and fluorescence was measured by a Synergy 2 Multi-Mode Microplate Reader (BioTek[®], Winooski, VT, USA). To measure green fluorescence intensity units (U) from SYTO[®] 9 stained cells, fluorescence filters with excitation wavelength 485 ± 20 nm; emission wavelength 528 ± 20 nm were used.

To prepare a slide for microscopy, one drop of a cyanobacterial culture was transferred to a microscope slide via a transfer pipet and a cover slip was placed on top of the culture. To observe heterocysts and determine their frequency, the Leica ATC™ 2000 compound microscope was used to observe the culture at 400x total magnification. Heterocyst frequency was determined by counting the number of vegetative cells per heterocyst for 10 heterocysts and the data was reported as the mean \pm standard deviation. If there were insufficient cyanobacterial cells to count 10 heterocysts, the data was annotated as too few to count (TFTC). To observe cyanobacterial filament morphology, a microscope slide was prepared as previously described and the BX53 upright compound microscope was used to observe the culture at 400x total magnification. Photomicrographs were taken with an Olympus® DP70 digital microscope camera (Olympus®, Tokyo, Japan).

7.2.6 Statistical analyses

The Shapiro-Wilk univariate normality test was used to test the normality of each dataset (Ghasemi and Zahediasl, 2012). Normality was performed at a confidence level of 0.95 using Microsoft® Excel along with the Simetar® add-in. Simetar® is a risk analysis software that been used extensively in commercial/business models (Richardson and Johnson, 2015). To ensure the sample size of each treatment was adequate to achieve statistical power of ≥ 0.95 , G*Power statistical power analysis program was used to conduct *post hoc* power analysis (Faul et al., 2007).

To determine if significant differences existed among the treatments (nitrogen sources) on *Anabaena* 7120 growth, one-way analysis of variance (ANOVA) was performed using the R statistical software program and the packages ‘reshape2’ and

'agricolae' written by H. Wickham and F. de Mendiburu, respectively (R Core Team 2013). The ANOVA table was also calculated with Simetar[®] to ensure calculations were accurate. If the ANOVA determined significant differences among treatments, then the Duncan's new multiple range test (MRT) was performed to determine among which treatments the statistical differences occurred. All sample sizes compared were of equal size (n = 5).

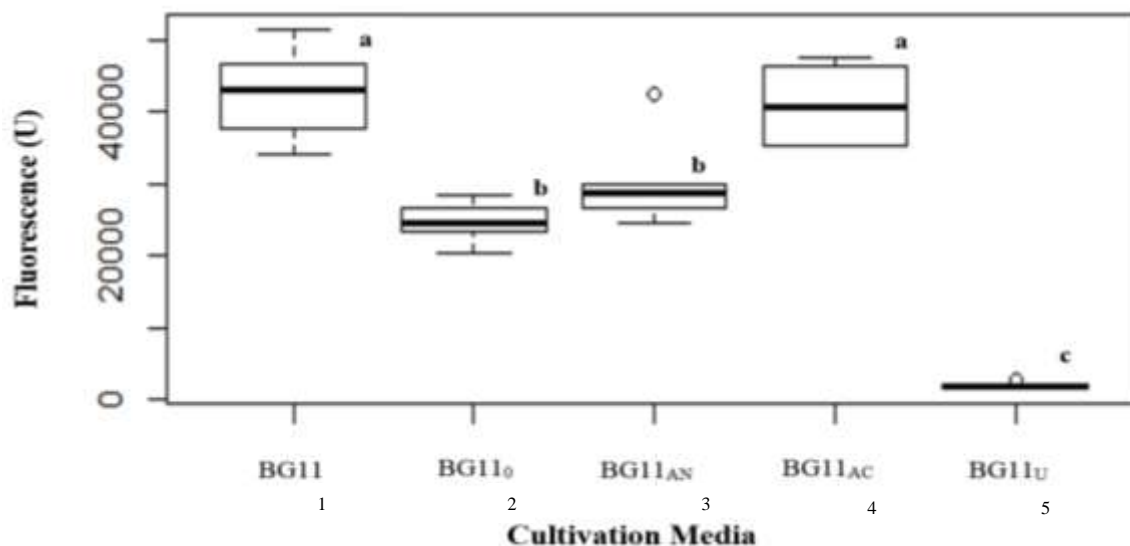
7.3 Results and Discussion

7.3.1 Evaluation of nitrogen sources on growth of *Anabaena 7120*

Supplying nitrogen to support cell growth and metabolism in large-scale cyanobacteria production systems is an important factor, as the manner in which nitrogen is supplied is an essential driver of economic feasibility and environmental sustainability (Peccia et al., 2013). Diazotrophic, filamentous strains of cyanobacteria such as *Anabaena 7120* may be considered industrially favorable compared to non-diazotrophic strains due to their ability to fix atmospheric N₂. However, this is an energy intensive process for the cell (Stal, 2003), and may cause a decrease in productivity due to the amount of ATP required for nitrogen fixation. Alternatively, cyanobacteria can be provided with fixed forms of nitrogen to maximize cell growth and productivity. However, there are obvious differences between the performance of various types of fixed nitrogen sources, as well as the costs and environmental footprint of their manufacture.

Anabaena 7120 was cultivated in BG11 containing different nitrogen sources to compare the effects on cell growth and viability, heterocyst frequency, and filament

morphology. Growth was measured as viability as determined via a fluorescence assay. As the amount of fluorescence directly correlates with the amount of viable cells in the culture, we can make comparisons of the effect of nitrogen sources on the growth of *Anabaena* 7120. For example, if one culture yields 1000 U and a second culture yields 10,000 U, then we can conclude there is a 10-fold increase in viable cells in the second culture. Heterocyst frequency and filament morphology were observed microscopically. The effect of the different nitrogen sources on the growth (viability) of *Anabaena* 7120 can be seen in Figure 7.2. All datasets used for statistical analyses were normally distributed. BG11 (sodium nitrate) and BG11_{AC} (ammonium chloride) yielded statistically similar growth (~41,000 U), and were significantly higher than the other treatments. BG11_U (urea) resulted in the least growth (~1900 U), while BG11₀ (no fixed nitrogen) and BG11_{AN} (ammonium nitrate) were intermediate (~27,000 U).



- 1: Sodium nitrate
- 2: No fixed nitrogen source
- 3: Ammonium nitrate
- 4: Ammonium chloride
- 5: Urea

Figure 7.2: Effect of different nitrogen sources on the maximum viability (U) of *Anabaena 7120* during a growth trial.

(Trials lasted 1 d after maximum viability was reached, typically 10-18 d) (Different letters denoted on the figures indicate statistical differences among the treatments as determined by the MRT).

We anticipated that sodium nitrate would yield optimal growth, as it has been the standard cyanobacteria cultivation medium for ~50 years. (Allen and Stanier, 1968) However we did not expect that ammonium chloride, at the high concentration tested, would also result in rapid growth. While several studies have used 4-69 mg N L⁻¹ ammonium chloride to cultivate *Anabaena 7120* (Merino-Puerto, et al. 2010; Picossi et al., 2005; Sato et al., 2004; Laurent et al., 2005), Wang and Liu, (2013), concluded that 247.03 mg N L⁻¹ ammonium chloride was inhibitory to *Anabaena 7120*. A potential explanation for the improved results we obtained on ammonium chloride could be related

to the fact that we controlled pH on a daily basis. Ammonium uptake in cyanobacteria is affected by the pH-dependent equilibrium between NH_3 and NH_4^+ . NH_3 can diffuse through membranes in alkaline conditions while NH_4^+ cannot (Drath et al., 2008). As cyanobacteria culture conditions are generally alkaline (pH 9-10) at the end of the exponential growth phase (Castenholz, 1988), these conditions could hinder the microbe's ability to uptake NH_4^+ . Thus, in Wang and Liu's trials (2013), if the pH became alkaline, the cultures supplemented with ammonium chloride may have suffered from ammonium toxicity. Another contradictory reference about the effects of ammonium chloride is in a review article by Markou and Georgakakis, (2011) where they state that Boussiba and Gibson, (1991) concluded that growth of an *Anabaena* sp. was completely inhibited by 139 mg N L^{-1} ammonium chloride. However, that study actually used ammonia rather than ammonium chloride.

BG11_{AN} and BG11₀ yielded statistically similar growth of *Anabaena* 7120 in this study; less than that of BG11 and BG11_{AC}, but more growth than BG11_U. While previous studies have utilized ammonium nitrate to cultivate *A. platensis* (Cruz-Martínez et al., 2015; Martinez et al., 2010), to the best of our knowledge it has not been used with *Anabaena* 7120. Therefore, the results cannot be compared. From an industrial standpoint, it would have been preferable if BG11 without a fixed nitrogen source yielded the most growth. However, that was not the case, as the energetic cost of fixing atmospheric nitrogen presumably led to a decrease in growth. Therefore, even though there was a savings by not having to supplement the growth medium with fixed nitrogen, the subsequent decrease in cell productivity may offset this benefit. BG11_U yielded minimal growth in this study. Researchers have successfully used urea (1.00 kg m^{-3} or

373 mg N L⁻¹ to cultivate *A. platensis* (Bezerra et al., 2013; Stanca and Popovici, 1996). However, it has been shown that 247.03 mg N L⁻¹ urea decreased the growth of *Anabaena* 7120 when compared to 247.03 mg N L⁻¹ sodium nitrate (Wang and Liu, 2013), similar to what we observed in this study.

Heterocyst frequency was used to assess if and when fixed nitrogen sources became limiting during the trials. The appearance of heterocysts typically occurs ~20 h after depletion of fixed nitrogen (Horn, 2008). Inoculum cultures were cultivated in BG11₀ to ensure no carryover of fixed nitrogen into the treatments, and the average initial heterocyst frequency for all trials was 17.2 ± 3.8 vegetative cells per heterocyst. Generally, *Anabaena* 7120 cell densities were too low in the first 3 days to obtain cell or heterocyst counts. By day 4, BG11_{AN}, BG11_{AC}, and BG11 yielded enough growth to determine heterocyst frequency. None of these cultures developed heterocysts during the 10-18 d trials, which indicated that *Anabaena* 7120 was not limited by fixed nitrogen. BG11_U did not yield enough growth to determine heterocyst frequency, and these trials only lasted 6-8 d. Cyanobacteria cultivated in BG11₀ had an initial heterocyst frequency of 13.0 ± 2.4 vegetative cells per heterocyst, and this value gradually increased throughout the trial. The average frequency at the end of the trial was 26.9 ± 3.7 vegetative cells per heterocyst, which concurs with previous studies that observed heterocysts occur in *Anabaena* 7120 every 10-20 vegetative cells (Ehira and Ohmori, 2006; Baier et al., 2004; Buikema and Haselkorn, 1991), and that the intervals between heterocysts becomes greater in older cultures (Liang et al., 1992).

Photomicrographs (Fig. 7.3) were taken of all of the cyanobacterial cultures at the conclusion of each trial to allow for observations and comparisons of the filament

morphology in media supplemented with different nitrogen sources. Photomicrographs were not taken of cultures in BG11_U because there was minimal growth and cells were not present at the conclusion of the trials. Generally, *Anabaena* 7120 filaments in BG11 and BG11₀ contained more than 100 cells. Ideally, we could have quantitated the exact number of cells in the filament but it is quite difficult to view all of the cells in a filament on the same plane so that they can be counted accurately. Also, filaments tend to clump together, adding to the difficulty of counting the total number of cells in a filament.

Anabaena 7120 filaments cultivated in BG11_{AN} and BG11_{AC} usually contained no more than 20 cells. By the end of the trials, the filaments were beginning to deteriorate and cell lysis was apparent. While the filament length of cultures in BG11_{AC} was less than the filament length of cultures cultivated in BG11, the maximal growth achieved during trials was statistically similar. Older, poorly growing cyanobacteria cultures tend to have shorter filaments (Bauer et al., 1995). Thus, even though similar maximal growth was achieved in the two different cultivation media, the conditions may have been less hospitable for cyanobacterial growth in BG11_{AC} at the conclusion of the trials. As the BG11_{AN} and BG11_{AC} cultures had similar filament morphologies, which were distinctly different than BG11 and BG11₀ cultures, it is assumed the filament deterioration was due to ammonium. The reason for filament deterioration may be attributed to the ability of *Anabaena* 7120 to uptake NH_4^+ as the culture becomes older. As mentioned earlier, NH_4^+ uptake is affected by the pH-dependent equilibrium between NH_3 and NH_4^+ (Drath et al., 2008). If this capability was hindered in old cultures, ammonium toxicity could occur.

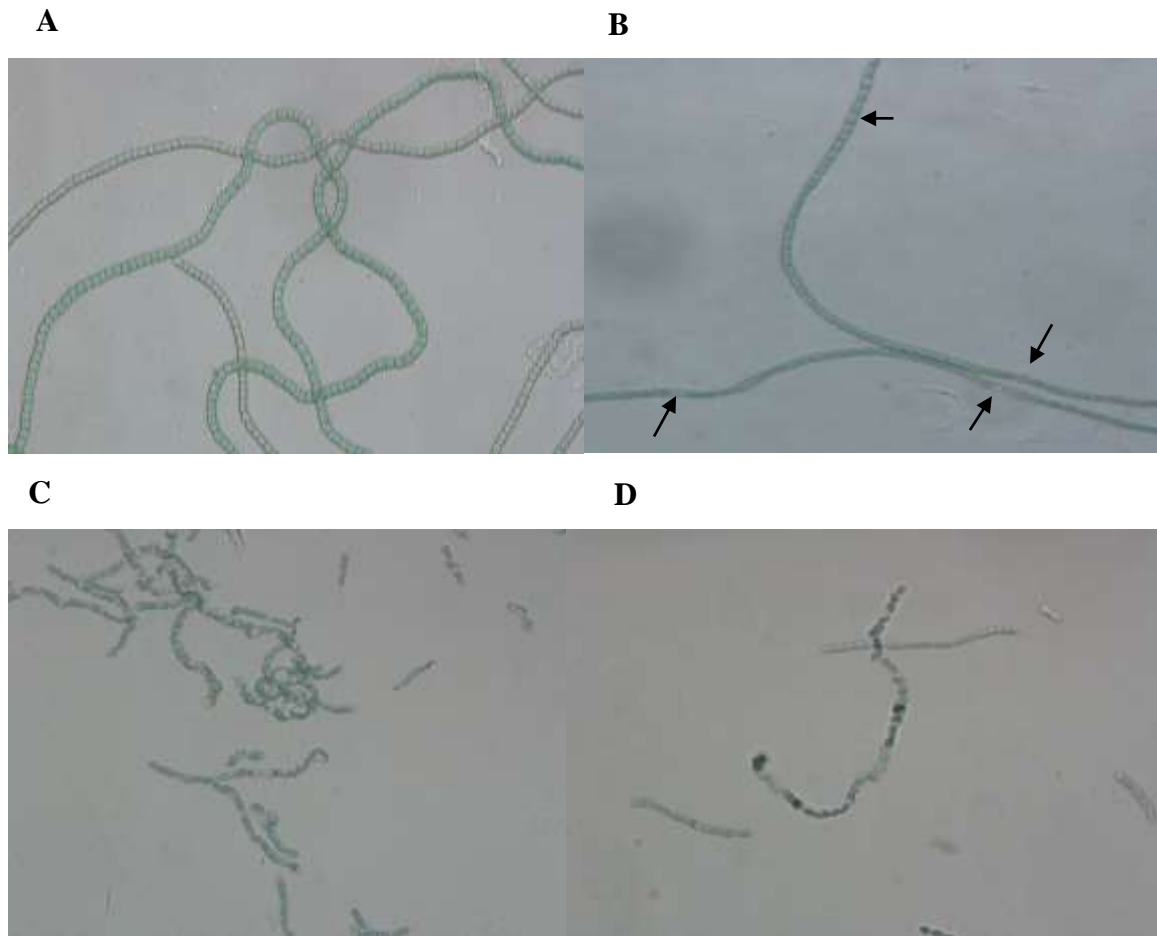


Figure 7.3: Photomicrographs (400x magnification) of *Anabaena 7120* cultures cultivated in different nitrogen sources. (A: BG11; B: BG11₀; C: BG11_{AC}; D: BG11_{AN}) (Arrows indicate heterocysts).

7.3.2 Comparative study of sodium nitrate and ammonium chloride for large-scale *Anabaena 7120* production

In this study, BG11 supplemented with sodium nitrate or ammonium chloride yielded significantly higher growth of *Anabaena 7120* than the other nitrogen sources investigated. We then assessed the economic and environmental impacts of using these nitrogen sources to cultivate *Anabaena 7120* in a large-scale, industrial process. For this

exercise, a theoretical limonene production facility that utilizes *Anabaena* 7120 to produce 1 million liters of limonene annually, covers 10,544 m² of land, and processes 53 million L/yr of BG11 was considered (Johnson et al., 2016b). Based on the nitrogen use rates previously described, this facility would require 79.5 tons/yr of sodium nitrate, at a cost of \$28,620, using a price of \$360/ton (the current price range is \$320-400/ton) (Shanxi Leixin Chemical Co., Ltd.). If ammonium chloride was used instead, the annual requirement would be 49.82 tons at an annual cost of \$6,302, based on a price of \$126.50/ton (the current price range is 110-143 \$/ton) (Dahua Group Dalian Guanlin International Trade Co., Ltd.). Thus, using ammonium chloride instead of sodium nitrate would result in an annual savings of \$22,318. Considering a lifespan of 27 years for the facility, the total savings would be \$602,586. This is a relatively small amount saved over the course of 27 years, thus an environmental assessment was also performed.

Several methods can be used to assess the environmental impacts associated with a material or process. Life cycle analysis (LCA) is a method which is commonly applied to evaluate the environmental impacts of a process by quantifying energy and resource demands, as well as resulting emissions. An LCA also provides valuable information to identify the “priority” areas for improvements, where actions would have the greatest effect on reducing the corresponding environmental burdens. Generally, an LCA consists of four steps including: 1) Goal and scope, 2) Life cycle inventory, 3) Life cycle impact assessment, and 4) Interpretation.

The functional unit is a key element in LCA that must be defined clearly to allow meaningful comparison between various scenarios (Mathews, 2008; Goedkoop et al., 2008). The functional unit is a measure of function of the system and provides a reference

to relate inputs and outputs to a common measure of function in order to compare different essential systems. Defining the functional unit can be complicated, thus it should be comparable and logical so it can be referenced throughout the LCA study (Baumann and Tillman, 2004; Goedkoop et al., 2010; Guinée, 2002). In the LCA conducted herein, the goal was to gain insight and compare the environmental profiles and emissions associated with using BG11 or BG11_{AN} for large scale production of *Anabaena* 7120. Therefore, the functional unit in this study was defined as 1 m³ of cultivation medium. In an LCA when two or more alternative routes (in this study, two different chemicals) are being studied, the economy-environment system boundary should be specifically determined for each option (Guinée, 2002). In this study, the economy-environment system boundary was handled consistently over the various product systems, excluding infrastructure processes. As this is a comparative analysis which aims to compare the effects of using ammonium chloride or sodium nitrate, the results are independent of the defined boundaries that are normally used in an LCA. In this method, the effect of infrastructures will not be considered, and the chemicals are considered to be supplied from the supply chain.

Figures 7.4A and 7.4B show the comparison of damage assessments associated with 1 m³ of BG11 using sodium nitrate or ammonium chloride as the nitrogen source. Figure 7.4A shows the damage assessment categories as a percentage (independent of the boundaries). The damage from sodium nitrate is considered to be 100%, and when switching to ammonium chloride the change in percentage is related to the change in damage. For example, if sodium nitrate was replaced with ammonium chloride in the facility, it would result in a 70% reduction in damage to human health. Figure 7.4B

shows the normalized data, and again, this data is independent of the boundaries. To express the life cycle impact of a chemical in these categories, we ‘translated’ the impacts into millipoints (mPt). mPt is a dimensionless Eco-indicator value established in the Eco-99 system, that reflects the ecological costs related to a system or chemical. The mPt are related to each chemical and mPt for each category are defined differently. The highest mPt score is associated with the worst environmental performance (Asdrubali et al. 2015).

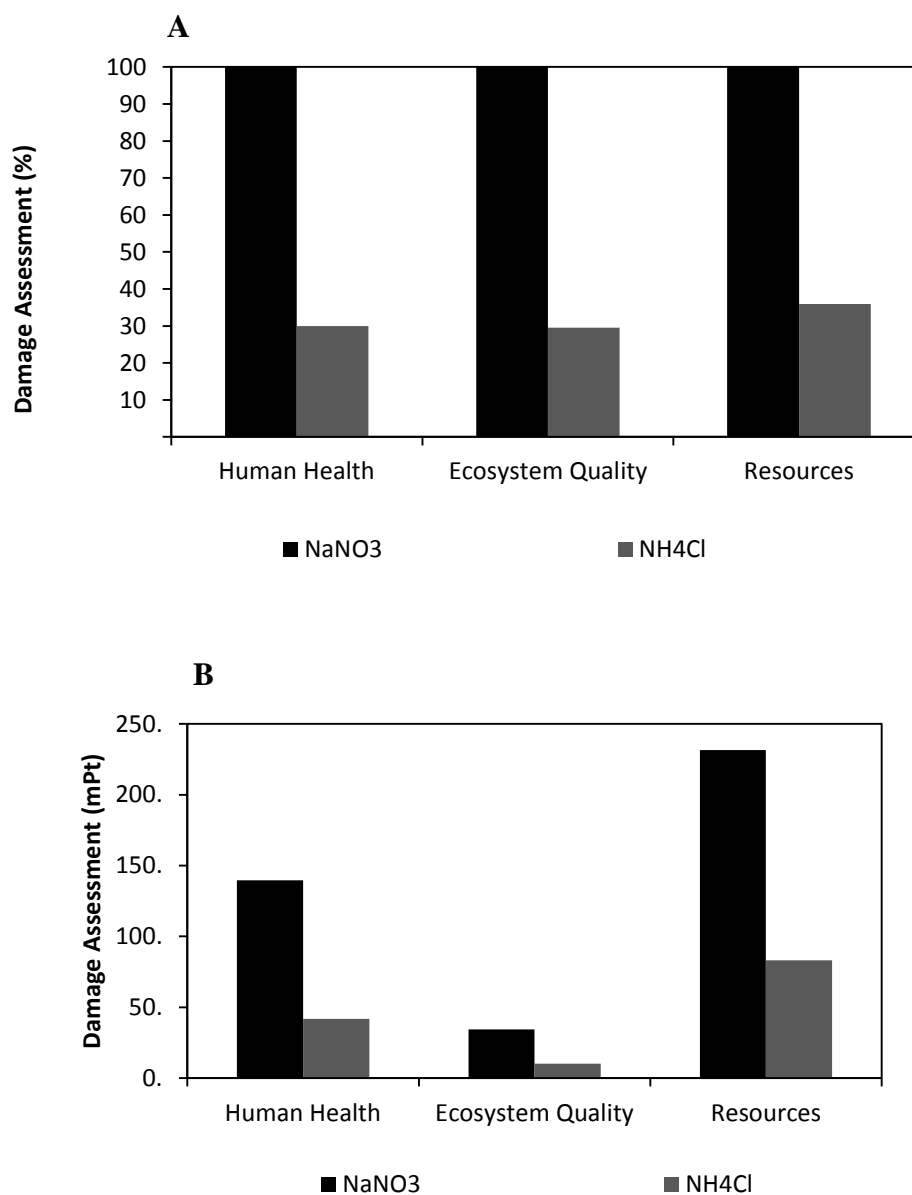


Figure 7.4: Comparison of damage assessments associated with 1 m³ of BG11 supplemented with sodium nitrate or ammonium chloride. (4A: damage assessment, 4B: normalization). (Method used: Eco-indicator 99 (H) LCA Food V2.03 / Europe EI 99 H/H. Infrastructure prices were excluded).

Damage to human health (DHH) represents the number of years of living disabled and the number of years of life lost. It also represents disability adjusted life years (DALY) and represents the impacts of factors such as climate change (disease + displacement), ozone layer depletion (cancer + cataracts), radiation effects (cancer),

respiratory effects, and cancer. DALY is a concept considered for defining the impacts on human health. Damage to Ecosystem Quality (DEQ) is expressed as the loss of species in a specific area during a specific time period. Damage to resources (DR) indicates the surplus energy required for the extractions of minerals and fossil fuels. In these calculations, production of the chemicals as well as the final emissions would have environmental impacts and are considered in both the production phase and the disposal (emission) phase.

In the human health, ecosystems quality, and resources categories the negative impacts of sodium nitrate use were much higher than that of ammonium chloride, ~3.3-fold higher in each category (Fig. 7.4A). When the data was normalized for the same category of impact, sodium nitrate still had a much higher negative impact than ammonium chloride in all 3 categories (Fig. 7.4B). In each category, the impact was at least two-fold higher for sodium nitrate.

The characterization of environmental impacts for using BG11 with sodium nitrate or ammonium chloride is shown in Figure 7.5. This characterization is generally performed based on the endpoint level (damage) and includes fate analysis, exposure, effects analysis, and damage analysis. Characterization is typically applied for categories including: emissions, acidification/eutrophication, land use, and resource depletion. Characterization of emissions includes carcinogens, respiratory organics and inorganics, climate change, radiation, ozone layer, and ecotoxicity. The carcinogenic effect is related to emissions of carcinogenic substances released into the air, water, and soil. Respiratory organics represent the emissions of organic substances into the air which cause

respiratory effects, while respiratory inorganics result from winter smog caused by the emissions of dust, sulphur, and nitrogen.

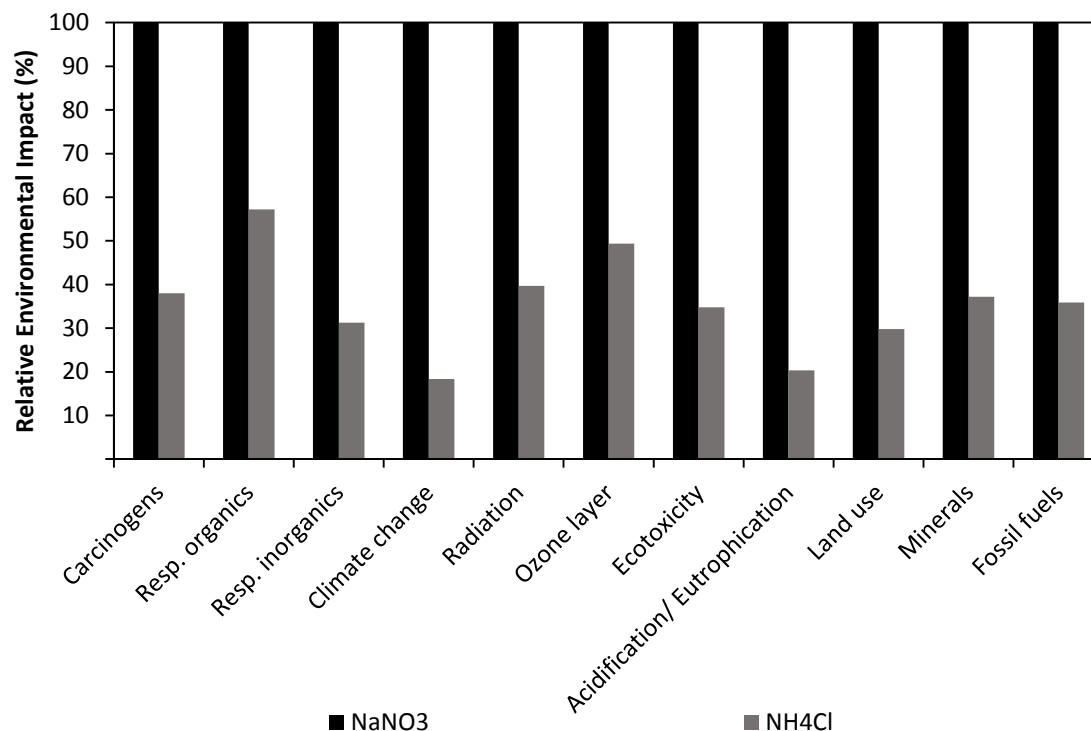


Figure 7.5: Characterization of relative impacts associated with 1 m³ of BG11 supplemented with sodium nitrate or ammonium chloride.

(Method used: Eco-indicator 99 (H) LCA Food V2.03 / Europe EI 99 H/H. Infrastructure prices were excluded).

The climate change category reflects the increase of diseases and death caused by climate change. The radiation category reflects the level of exposure from ionizing radiation. The ozone layer category reflects the increase in UV irradiation resulting from emissions of ozone-depleting substances into the air. The damage to ecosystem quality category is expressed in terms of ecotoxicity and it is the result of emissions of ecotoxic substances into the air, water, and soil. In the emission category, damage is expressed as DALY/kg emissions, excluding the ecotoxicity category which is expressed as potentially

affected fraction (PAF) \times m² \times year/kg emissions. The emission effects associated with BG11 using sodium nitrate as a nitrogen source are much more severe than that of BG11 using ammonium chloride as a nitrogen source in every category calculated.

Another factor expressed in characterization diagrams is acidification/eutrophication. Acidic gases react with water in the atmosphere which results in acid-containing precipitation. Acidification causes ecosystem impairment of varying degrees depending on the nature of the ecosystems. Gases that cause acid precipitation include: ammonia (NH₃), nitrogen oxides (NO_x), and sulphur oxides (SO_x). Acidification potential is expressed using the reference unit, kg SO₂ equivalents. Eutrophication reflects the accumulation of nutrients in aquatic systems and can be expressed in terms of kg PO₄³⁻ equivalents or Kg N equivalents, depending on the model. In the land use category, the impact of a material or service on the land due to agriculture, anthropogenic settlement, and resource extractions are reflected. The consumption of non-biological resources such as fossil fuels, minerals, metals, etc. are considered in the depletion of abiotic resources category. This depletion can be measured in terms of kg of antimony, kg of minerals, or MJ of fossil fuels (Acero et al., 2014; Goedkoop et al., 2008).

Generally, every emission effect category was at least 2-fold higher for sodium nitrate and in the case of the climate change and acidification/eutrophication categories the effect was approximately five-fold higher for sodium nitrate than ammonium chloride. Figure 7.5 provides strong evidence that using sodium nitrate as the nitrogen source in BG11 would have a much greater negative impact on the environment

compared to using ammonium chloride in large scale processes to cultivate *Anabaena* 7120.

Figure 7.6 shows the single score comparison of 1 m³ of BG11 with sodium nitrate or ammonium chloride. The overall environmental effect of using BG11 with sodium nitrate is ~400 mPt compared to ~150 mPt when using BG11 with ammonium chloride. Sodium nitrate had at least a 2-fold higher negative impact than ammonium chloride in each of three categories measured (resources, ecosystems quality, and human health). The effect on resources constitutes over half of the entire calculated effect for both nitrogen sources. The effect on ecosystems quality was the smallest compared to the other effects measured for both nitrogen sources. The effect on human health was more than the effect on ecosystems quality, but less than resources for both nitrogen sources.

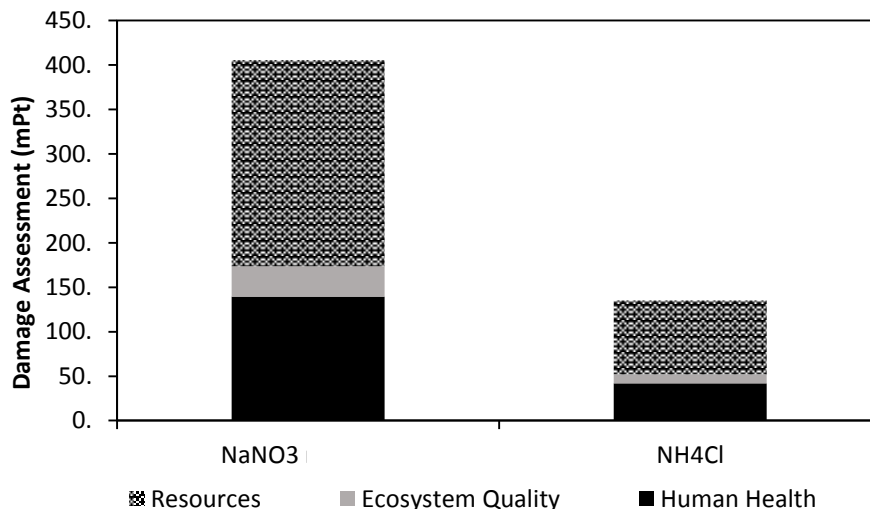


Figure 7.6: Single score comparison of 1 m³ of BG11 supplemented with sodium nitrate or ammonium chloride.

(Method used: Eco-indicator 99 (H) LCA Food V2.03 / Europe EI 99 H/H. Infrastructure prices were excluded).

The different environmental impacts derived from using ammonium chloride rather than sodium nitrate in large-scale processes can be attributed to 2 reasons. The first reason is that ~37% more sodium nitrate is required to prepare 1 m³ of BG11 compared to ammonium chloride. The second reason is based on the differences between the inherent environmental impacts attributed to production and disposal of the two chemicals. Factors that affect total emissions include: the chemicals used in the manufacturing process, functions and chemistry of release phenomena for the chemicals, applied extraction and purification technologies, supply chains, energetic balances, and other factors. In other words, the environmental profile of these chemicals represent impacts resulting from all relevant process steps over the entire supply chain of the chemicals.

We concluded that the overall environmental profile of ammonium chloride was superior to sodium nitrate for all categories presented in Figure 7.5. Some of these differences were marginal. For example, the environmental impact of respiratory organics from ammonium chloride was determined to be ~8% less than sodium nitrate. However, for other categories the differences were much larger. The environmental impact of using ammonium chloride would be ~70% less on climate change and ~68% less on acidification, compared to sodium nitrate. Impacts on climate change and acidification could be attributed to the differences in solubility of these two chemicals, which is 84 g/100g_w for sodium chloride and 35 g/100g_w for ammonium chloride at 15°C, 1 atmospheric pressure (Patnaik 2003).

Considering the entire LCA, it is clear that using sodium nitrate would have a much greater detrimental impact on the environment than using ammonium chloride to

cultivate *Anabaena* 7120 in a large scale process. There is also an estimated savings of \$602,586 associated with using ammonium chloride rather than ammonium nitrate over a 27-year lifespan of a proposed production facility. Since BG11 and BG11_{AN} yielded statistically similar amounts of growth of *Anabaena* 7120, it would be preferred to use ammonium chloride rather than sodium nitrate.

7.4 Conclusions

Cyanobacteria are prime candidates for industrial production of chemicals and fuels due to their metabolic diversity (Ruffing, 2011). Filamentous cyanobacteria, in particular, have emerged as a promising platform for generating next-generation biofuels and high-value chemicals. For any large scale process involving the cultivation of filamentous cyanobacteria to become economically feasible the cost of cultivation medium must be minimized. As there is not a clear consensus in the literature regarding the optimal nitrogen source for cultivating filamentous cyanobacteria, the purpose of this study was to elucidate a nitrogen source or sources that would be preferred in large scale processes. This was accomplished by evaluating BG11 supplemented with different nitrogen sources to determine which source would be preferred for large scale production of *Anabaena* 7120. BG11 supplemented with ammonium chloride and BG11 supplemented with sodium nitrate yielded statistically higher growth of *Anabaena* 7120 than the other nitrogen sources evaluated. While BG11 supplemented with ammonium chloride yielded statistically similar growth of *Anabaena* 7120 as BG11 supplemented with sodium nitrate, the environmental impact was significantly less in all categories measured. Thus, we conclude in large scale processes involving the cultivation of

Anabaena 7120, BG11 supplemented with ammonium chloride is the preferred nitrogen source.

Acknowledgements

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Chapter 8 - Producing next-generation biofuels from filamentous cyanobacteria: an economic feasibility analysis

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Abstract

The need for renewable, sustainable sources of biofuels continues to increase as the world's population continues to grow. Using microorganisms as biofuel producers is one area that is being researched extensively for this purpose. *Anabaena* sp. PCC 7120 is a filamentous strain of cyanobacteria capable of fixing atmospheric nitrogen, and has been genetically engineered to produce limonene, a cyclic hydrocarbon which has potential as a next-generation biofuel. This study analyzed the economic feasibility of a

theoretical next-generation production facility that uses genetically engineered *Anabaena* 7120 to produce limonene. The economic feasibility of a limonene production facility was analyzed using the Farm-level Algae Risk Model (FARM). This model is an integrated systems compilation of numerous technoeconomic models that has been used previously in several algal production scenarios. FARM simulated 10 years of operation for the production facility for two scenarios. The 1st scenario used actual productivity data (0.018 mg/L/d) from a genetically engineered strain of filamentous cyanobacteria, while the 2nd scenario used a ‘best case’ assumption that limonene productivity can be increased 100-fold (1.8 mg/L/d). It was determined that the average probability of economic success of the 1st scenario at year 5 was 0%, while the average probability of success of the 2nd scenario was 100%. Assuming no fractional reductions in OPEX and CAPEX, the average net present value (NPV) at year 5 of the 1st scenario was -588 M\$, compared to 392 M\$ for the 2nd scenario. Further analysis determined that a limonene productivity of 1.02 mg/L/d is needed to yield an NPV of 0 dollars at year 5. This study shows strong evidence that a next-generation biofuel production facility utilizing genetically engineered strains of filamentous cyanobacteria could become economically feasible in the future if strains are developed with increased biofuel productivities.

8.1 Introduction

Environmental factors and market forces continue to drive the demand for next-generation biofuels. Adverse environmental impacts of greenhouse gas emissions from fossil fuel use include ozone depletion, global warming, and smog formation (Von Blottnitz and Curran, 2007). The widespread use of fossil fuels has led pollution, global climate change, and detrimental effects on the health of many organisms (Chen et al.,

2011). Production of next-generation biofuels using genetically engineered microorganisms, such as photosynthetic cyanobacteria, is one area being researched extensively to decrease dependency on fossil fuels. Cyanobacteria are prime candidates for this application due to their natural metabolic diversity (Ruffing, 2011). Cyanobacteria are present in diverse habitats, ranging from polar regions to the tropics, (Katoh, 2012; Moreno, et al. 1998; Hasunuma, et al. 2013), and have existed on Earth for at least 2.7 billion years (Badger and Price, 2003). They have morphologies ranging from unicellular to filamentous, and utilize the same photosynthetic process as higher plants (Lindblad et al., 2012). Many filamentous strains of cyanobacteria capable of fixing atmospheric nitrogen have emerged as promising platforms in which to engineer production of fuels and chemicals (Schoepp et al., 2014). Thus far, researchers have engineered filamentous cyanobacteria to produce high-value chemicals and potential next-generation biofuels such as limonene (Halfmann et al., 2014b), farnesene (Halfmann et al., 2014a), and linalool (Gu et al., 2012).

While next-generation biofuel production from filamentous cyanobacteria is technically feasible, a thorough literature review has yielded no projections regarding economic viability. However, many studies on algal production of high-value chemicals and next-generation biofuels are available, and could serve as a model for comparison (Richardson et al., 2014a; Richardson and Johnson, 2015; Richardson et al., 2014b; Richardson et al., 2010; Richardson and Johnson, 2014; Richardson et al., 2012; Richardson and Johnson, 2013). Process-model based technoeconomic analyses are commonly used to compare alternative processes and products (Caspeta and Nielsen, 2013; Hermann and Patel, 2007; Klein-Marcuschamer et al., 2011; Patel et al., 2006). An

economic feasibility analysis of a next-generation biofuel production process from filamentous cyanobacteria would provide a valuable comparison to well-established algal models, while at the same time identifying bottlenecks in the process. This will elucidate targets for future research to move towards economically feasible production of next-generation biofuels from filamentous cyanobacteria.

Several programs capable of simulating processes for technoeconomic analyses are available. These include: Aspen Plus[®] (Davis et al., 2011), HOMER[®] (Zoulias and Lymberopoulos, 2007), and Cadsim Plus[®] (Benali et al., 2014). The Farm-level Algae Risk Model (FARM), formerly known as the Algae Income Simulation Model (AISIM), is a Monte Carlo firm level economic simulation model that goes beyond a technoeconomic analysis. The FARM is designed to simulate the annual cultivation, harvesting, extraction, and financial/economic activities of an algae farm (Richardson and Johnson, 2012). The model is an integrated systems compilation of several technoeconomic models for different phases of a commercial algae farm, and also includes the financial, marketing, and income tax aspects of a business (Richardson and Johnson, 2015). The simulation results provide estimates of the probability of economic success for different pathways, and the sensitivity of production costs to changes in capital and operating expenses (Richardson and Johnson, 2015). It is a preferred model for this study as it was designed specifically for processes involving photoautotrophic microorganisms. The similarities between algae and cyanobacteria allows for economic feasibility analyses with filamentous cyanobacteria using FARM.

This study addressed the economic feasibility of a next-generation biofuel process from filamentous cyanobacteria. Multiple algal farm scenarios have been modeled using

FARM for the National Alliance for Biofuels and Bioproducts (NAABB) (Richardson and Johnson, 2015). Of these, a scenario using a genetically modified algae grown in an Algae Raceway Integrated Design (ARID) cultivation system, harvested with an electrocoagulation (EC) harvesting system, and converted into biofuel using the Hydrothermal Liquefaction-Catalytic Hydrothermal Gasification (HTL-CHG) extraction method was the most promising (Richardson and Johnson, 2015). We hypothesized that a system producing 4th generation biofuels directly from engineered filamentous cyanobacteria would have a higher probability of success than 3rd generation biofuels produced from algae, since downstream processes to convert oil into biofuels would not be needed. The highest-volume application for the engineered metabolism of microorganisms is production of transportation fuels (Keasling, 2010), and at this time it appears that cyanobacteria are the sole renewable resource capable of meeting the global demand of transportation fuels (Chisti, 2007; Schenk et al., 2008; Singh et al., 2011a). This strongly suggests that cyanobacteria hold great potential for future next-generation biofuel production.

This study analyzed two scenarios with different limonene productivities. The 1st scenario used actual limonene production data from an engineered strain of filamentous cyanobacteria (0.018 mg/L/h) (Halfmann et al., 2014b). The 2nd scenario used a ‘best-case’ scenario which assumed a 100-fold increase in productivity to 1.8 mg/L/h. This productivity was calculated based on a claim from Joule Unlimited, Inc. that cyanobacteria can be engineered to produce 10 mg/L/h ethanol (Green et al., 2015). Ethanol’s molecular weight is 46 g/mole, while ethylene is 28 g/mole. Thus, the equivalent hydrocarbon productivity of 10 mg/L/h ethanol would be 6 mg/L/h, and we

chose a more conservative productivity of 1.8 mg/L/h. The results of this financial/economic analysis will provide evidence regarding economic feasibility.

8.2 The Farm-level Algae Risk Model (FARM)

FARM requires the Simetar[®] add-in for Microsoft[®] Excel to incorporate risk. Simetar[®] has been used extensively for risk analysis in business models and prospective businesses (Richardson et al., 2006). FARM can be considered as an integrated systems compilation of numerous technoeconomic and financial models for different phases on an algae farm (Richardson and Johnson, 2013). FARM has been used in various scenarios (Richardson and Johnson 2014, 2015; Richardson et al., 2014a; Richardson et al., 2014b; Richardson et al., 2012) to determine economic feasibility of specific systems or technologies related to algal farming. Richardson et al., (Richardson and Johnson, 2015) provides an overview of the functionality of FARM.

8.3 Process description and assumptions

Figure 8.1 illustrates the proposed limonene production process which is comprised of photobioreactors (PBRs), a gas cleaning unit, a gas stripping and limonene recovery unit, a solid fraction recovery unit, an anaerobic digestion unit, and a wastewater treatment unit (Richardson et al., 2014b; Cheng et al., 2009; Kovalcik, 2013; Li et al., 2011). Table 8.1 presents the stream components of the process for limonene production in the 2nd scenario. In this economic feasibility analysis, data regarding cyanobacteria cultivation and growth were obtained from a recent study in which a strain of filamentous cyanobacteria had been genetically engineered to produce limonene (Halfmann et al., 2014b). For the cultivation phase, model data from the NAABB and

National Renewable Energy Laboratory (NREL) were updated, modified, and used in FARM (Davis et al., 2012; Richardson and Johnson, 2015; DOE, 2015). The number of PBRs and harvesting units were selected based on the desired throughput and required annual limonene production of the proposed system. The information on operating and capital expenses for several technologies used in this model was provided by the NAABB specific economic model (FARM) of algae crude oil production at a commercial size scale. For harvesting, dewatering and drying, data was obtained from a study by Gebreslassie et al., 2013.

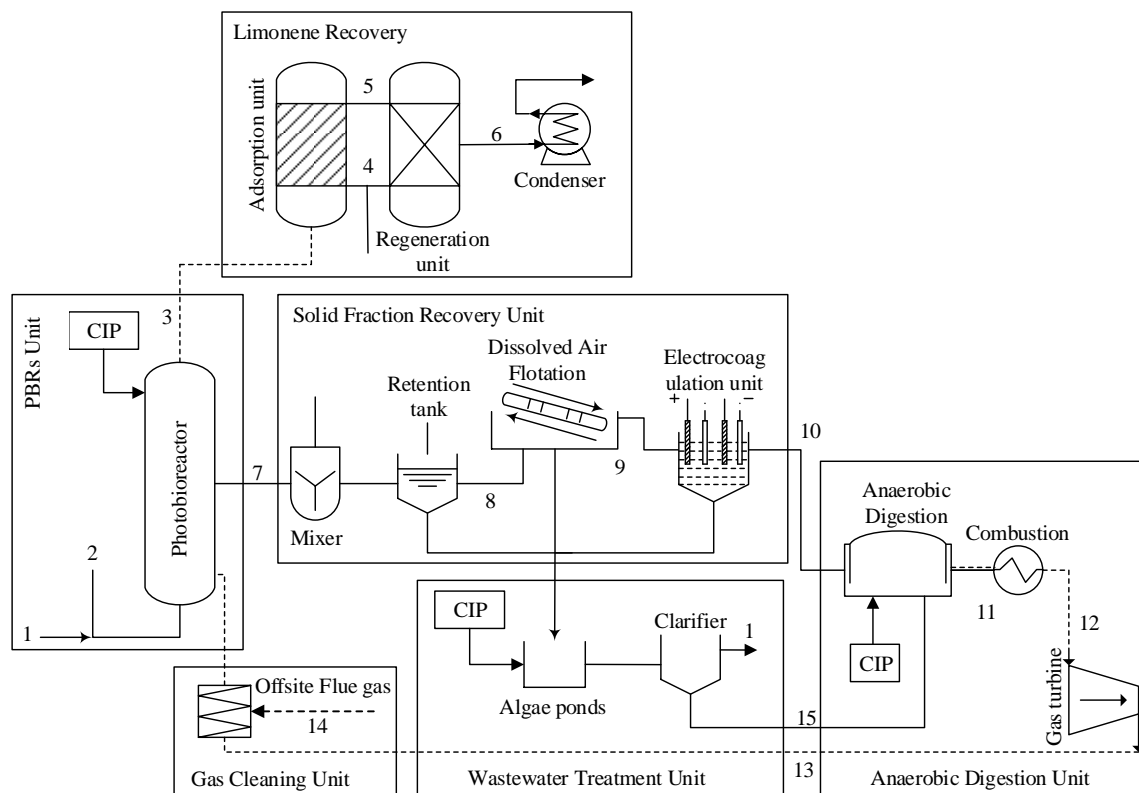


Figure 8.1: Process flow diagram for limonene production.

Numbers in Figure 8.1 are used to indicate different streams in the process flow diagram. Solid lines indicate a liquid stream and dashed lines indicate a gas stream. Stream #1 is water which will be fed to the PBRs. Stream #2 is the nutritional stream where the chemicals needed for BG11 (Table 8.2) will be mixed with the water and used in the PBRs for cultivation. Stream #3 is a gaseous exhaust containing limonene that is sent to the adsorption unit. Stream #4 consists of the microwave regenerated activated carbon (AC) along with a 10% (w/w) of fresh activated carbon make up. Stream #5 consists of the spent AC that will undergo regeneration, limonene, and water. Stream #6 is limonene rich gaseous stream, resulting from microwave regeneration of spent AC. Streams #7-9 are the biomass + cultivation medium that concentrate the biomass. From the DAF unit, water is sent to algal ponds where it undergoes wastewater treatment and is recycled back into the system. Stream #10 is the biomass + liquid (160 g L^{-1}) that undergoes anaerobic digestion. Stream #11 is the produced biogas and biomass residues from anaerobic digestion that undergo combustion. Stream #12 is the gas produced from combustion that goes to the gas turbine. The thermal energy generated during combustion is used to create electricity in the gas turbine. Stream #13 is the gas that goes to the gas cleaning unit. Stream #14 is offsite flue gas that is used in the process. Stream #15 is the biomass residues that goes from the bottom of the clarifier to the anaerobic digestion unit.

Table 8.1: Stream components in the process presented in Figure 8.1 for the 2nd scenario.
In the 1st scenario, all values are identical except for limonene, which is decreased 100-fold.

Input and Outputs	1	2	3 ^b	4	5	6	7	8	9	10	11 ^{b,g}	12,13 ^{b,h}	14 ^b	15 ^b
	(ML batch ⁻¹)	(ton batch ⁻¹)	(m ³ s ⁻¹)	(kg batch ⁻¹)	(ton batch ⁻¹)	(kg h ⁻¹)	(ton day ⁻¹)	(ton day ⁻¹)	(ton day ⁻¹)	(ton day ⁻¹)	(m ³ batch ⁻¹)	(m ³ batch ⁻¹)	(m ³ s ⁻¹)	(L d ⁻¹)
water	2.348	43.52	0.181 ^c	0	11.469	45.5	159.44	94.663 ^f	9.566	5.979	358.8	5237.8	0	5979
CO ₂	0	0	0.033	0	0	0	0	0	0	0	1865.5	6744.5	0.39	0
Limonene	0	0	0.007	0	38.230	106	0	0	0	0	0	0	0	0
Solid biomass	0	0	0	0	0	0	0.956	0.956	0.956	0.956	0	0	0	0
Nitrogen	0	0	3.263	0	0	0	0	0	0	0	71.8	39032	2.67	0
Activated carbon	0	0	0	8080 ^d	0.404 ^e	0	0	0	0	0	0	0	0	0
Methane	0	0	0	0	0	0	0	0	0	0	4879	0	0	0
Other	0	4.35 ^a	0	0	0	0	0	0	0	0	0	0	0.21	0
Total	2.35	47.88	3.48	8080	50.103	151.5	160.39	96.619	10.523	6.936	7175	51014	3.26	5979

a: Nutrients used are shown in Table 8.2. b: represents gas streams. c: gas considered to be saturated contains 27.7 g_w/kg_{air}. d: Activated carbon in the adsorber- life time of carbon is considered to be 5 years. e: make up activated carbon. f: evaporation of water was considered to be 1 ton.day⁻¹. g: composition of the produced biogas after AD was considered as 68% CH₄, 26 % CO₂, 1% N₂, 0% O₂, 5 % H₂O h: composition of the flue gas was considered as N₂ 82%, CO₂ 12%, O₂ 5.5%.

8.3.1 Cyanobacteria cultivation

The strain utilized in the production system is a genetically engineered *Anabaena* sp. PCC 7120 (herein referred to as *Anabaena* 7120) capable of limonene production (Halfmann et al., 2014b). The required nutrients, growth rates, productivity and limonene production yield data for the cultivation phase were obtained from recent studies on this engineered strain (Halfmann et al., 2014c; Halfmann et al., 2014b). *Anabaena* 7120 is capable of fixing atmospheric nitrogen, which could be an advantage in biofuel production since combined nitrogen is a major cost in large scale biofuel strategies (Halfmann et al., 2014b; Ortiz-Marquez et al., 2012; Peccia et al., 2013). Currently the engineered strain contains the limonene synthase gene on a plasmid, thus requiring addition of an antibiotic (100 µg/ml neomycin) to the cultivation medium to ensure plasmid retention. The use of antibiotics is undesirable in industrial applications due to the additional costs of the antibiotics and the metabolic burden associated with retaining plasmids (Ueki et al., 2014; Mairhofer et al., 2013). For the purpose of this study, it is thus assumed that the gene can be integrated into the chromosome without any change in limonene production, thereby eliminating the need for antibiotics.

The PBRs used in this study are assumed to be tubular plastic cylinders with a 38 cm diameter and a height of ~2 m. There are 10,544 PBRs in the facility, and the volume of each reactor is 0.227 m³. The cycle time of each PBR is 15 d. Based on energy costs and current productivity, Schenk et al., (2008) state that PBR costs should not exceed \$15 m⁻², thus this number was used in this analysis. The PBRs will be cooled using a sprinkler system that sprays water on the PBR surface (Richardson et al., 2012; Davis et al., 2011).

Nutrient demands for cyanobacteria cultivation will be met using BG11 medium. The chemical composition of BG11 is shown in Table 8.2.

Table 8.2: Chemical composition of BG11.

Chemical	Concentration (g/L)
NaNO ₃	1.5
MgSO ₄ ·7H ₂ O	7.5×10^{-2}
CaCl ₂ ·2H ₂ O	3.7×10^{-2}
Citric acid	6×10^{-3}
Ferric ammonium citrate (18% Fe)	6×10^{-3}
Na ₂ CO ₃	4×10^{-2}
H ₃ BO ₃	2.86×10^{-3}
MnCl ₂ ·4H ₂ O	1.81×10^{-3}
ZnSO ₄ ·7H ₂ O	2.22×10^{-4}
Na ₂ MoO ₄ ·2H ₂ O	3.9×10^{-4}
CuSO ₄ ·5H ₂ O	7.9×10^{-5}
Co(NO ₃) ₂ ·6H ₂ O	4.94×10^{-5}

Cyanobacteria production systems require a CO₂ supply for a carbon source, as well as to control pH, since pH is linked to the CO₂ concentration (Kunjapur and Eldridge, 2010). The CO₂ supply must be precisely controlled so that carbon does not become limiting, yet does not reach a concentration that becomes inhibitory due to high CO₂ partial pressures (Sobczuk et al., 2000; Kunjapur and Eldridge, 2010) or low pH. A

mixture of 5% CO₂ and 95% air will be sparged into the PBRs at a rate of 0.25 L/L/min (Johnson et al., 2016d). The CO₂-enriched air flow will also provide gas exchange by stripping out excess dissolved oxygen, as well as the produced limonene. The sparged gas will also create turbulence sufficient to keep cells in suspension.

8.3.2 Limonene recovery

Limonene is a cyclic monoterpene that is a promising biodiesel and next-generation biofuel candidate due to its immiscibility with water and low freezing point (Halfmann et al., 2014b; Hellier et al., 2013). It is also a biodegradable solvent that can replace products such as acetone in organic solvents (Pourbafrani et al., 2013). Limonene was chosen as the product for this system due to its high-value and the fact that production data from genetically engineered cyanobacteria is available (Halfmann et al., 2014b). This production system can also be used for other high-value volatile organic compounds (VOCs).

A cost-effective means of product recovery is essential for any bioprocess. Limonene recovery from dilute fermentation streams via gas-stripping may be economically favorable (Kiyota et al., 2014). Gas stripping is a relatively simple process in which a gas is continuously bubbled through culture fluid to volatilize and carry out the chemical (as well as some water vapor), which is subsequently condensed (Xue et al., 2016). The challenge with applying gas stripping to cyanobacteria cultures is that the gas stream exiting the PBR would be very dilute. The temperature required to condense limonene from a vapor stream is directly correlated with the limonene concentration. Thus, a dilute limonene vapor would require exceptionally cold condensation temperatures (-15°C). The added costs of cooling would negate the benefits of gas

stripping. To overcome this challenge, the gas stripping vapor could be passed into an absorber unit to concentrate the chemical first, rather than utilize direct condensation (Figure 8.1). A smaller volume of gas can subsequently be used to displace the chemical from the absorber unit, resulting in a higher concentration in the secondary vapor. This allows for cold water to be used to condense the product (Cao et al., 2015).

Activated carbon (AC) has been widely employed to adsorb volatile organic compounds (benzene, toluene, formaldehyde, n-hexane, limonene, and ethanol) from gas streams that are generated from a wide range of industries (Subrenat and Le Cloirec, 2004). AC has a large surface area, high porosity, a variety of pore sizes and structures, and rapid adsorption capabilities. Thus, AC may provide an economically favorable option for recovering and concentrating limonene via gas stripping. To collect limonene produced from genetically engineered cyanobacteria, a column packed with AC will be connected to the outlet of the PBRs. The gas-stripped limonene vapor will enter the column and be adsorbed by the porous AC. After the AC column is saturated, hot air will be passed through the column to desorb limonene, and this limonene-enriched air will be passed through a cold water condenser to condense limonene and water vapor, which will then phase separate. (Price and Schmidt, 1998). The regenerated AC column can then be placed back into operation.

8.3.3 Solid Fraction Recovery

A successful low-energy and low-cost method of harvesting cell biomass is essential for the proposed system. The harvesting method must have low capital, operating, and maintenance costs. The design of the harvesting unit should provide ease

of operation and integration with cultivation facilities to limit pumping and power requirements. In addition, it should have low environmental impacts and low carbon and water footprints (Udom et al., 2013). The harvesting strategy used in this study was based on the most promising of several NAABB developed technologies modeled by Richardson and Johnson, (2015).

Biomass will be harvested using a retention tank that concentrates the cyanobacteria to 10 g/L via autoflocculation (Davis et al., 2011). Autoflocculation occurs due to high pH in cyanobacteria cultures caused by photosynthesis, in which precipitation of Ca, Mg, and phosphate ions form a chemical flocculating agent that results in cell settling (Benemann and Oswald, 1996). This will be followed by dissolved air flotation (DAF) which thickens the material to 100 g/L, and electrocoagulation (EC) which concentrates the slurry to 160 g/L (Gebreslassie et al., 2013; Kovalcik, 2013). DAF involves dissolving air at a high pressure in a saturator via a pump and releasing at a high pressure in the flotation cell. The sudden reduction in pressure of the water stream causes the formation of bubbles which creates the driving force on the biomass to move upward (Wadhvani et al., 2015). EC works by generating coagulating ions via electro-oxidation of an anode *in situ* when direct current is employed (Han et al., 2015) In this study, the capacity of the EC unit was determined based on Kovalcik et al., (2013). This combination of solid fraction recovery steps was chosen as presumably it will minimize downstream processing costs (Grima et al. 2003).

8.3.4 Anaerobic Digestion

In anaerobic digestion (AD) units, organic biomass is biologically converted to biogas and residual sludge, which can be used as fertilizer. The organic matter is partially degraded by different anaerobic microorganisms by a succession of biological reactions: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Weiland, 2010; Benemann and Oswald, 1996). Advantages of using AD in an industrial process include: considerable reduction in the organic and pollution load, improved economic profile, improved energy recovery, and odor suppression (Holm-Nielsen et al., 2009). The chemical composition and the physical characteristics of the produced biogas are highly dependent on the substrate and the organic matter load. Typically, it is a mixture of methane and CO₂ and may contain other gases including: N₂, O₂, H₂O, H₂S and NH₃ (Benemann and Oswald, 1996; Holm-Nielsen et al., 2009).

Anabaena spp. contain approximately 5% lipids (Griffiths and Harrison, 2009), 45% proteins (Han et al., 2016) and 31% carbohydrates (Fawzy and Issa, 2015) which makes it an attractive substrate for AD. The theoretical methane yield is defined as liters of methane per gram of volatile solids (VS) and is expressed as L CH₄ per g VS. It can be calculated based on the stoichiometric conversion of organic matter to methane and CO₂ (Kaparaju et al., 2009). Lipids have a higher theoretical methane yield (1.0 L CH₄ per g VS) compared to proteins and carbohydrates (0.85 L CH₄ per g VS and 0.42 CH₄ per g VS, respectively (Zamalloa et al., 2011). However, methane yields for algal biomass are lower (0.09 – 0.34 CH₄ per g VS) due to the resistance of algal cell membrane components to digestibility, and it is assumed cyanobacteria will have similar characteristics. Thus, the retention times required for digestibility in the AD would be

high (~20 – 30 days). Typically, the AD units will produce a sulfur-free high energy content biogas (up to 68%), which is non-corrosive and suitable for engine generators (Zamalloa et al., 2011; Chisti, 2008b). In the theoretical production facility evaluated in this study, the pre-concentrated biomass from the solid fraction recovery unit is fed to a high-rate AD (Zamalloa et al., 2011) to produce biogas, which can be further converted to electrical and thermal energy. It is also assumed that during the combustion process, the steam produced in the combustor will be fed into an energy recovery system, which will generate electricity via a steam turbine. The high protein content of *Anabaena* spp. suggests that the biomass could also be used as a single cell protein, which has been done previously with microalgae strains (Becker, 2007). This is an area that could be explored and potentially used in future modeling studies.

8.3.5 Wastewater treatment and gas cleaning units

A wastewater treatment unit, based on conventional municipal wastewater treatment (MWWT) facilities was considered in this study with some minor modifications. In our system, the flow rate is much lower than that of conventional MWWTs (Lundquist et al., 2010). Also, in the primary treatment stage of a municipal wastewater facility, a primary clarifier is employed as a sedimentation basin for continuous removal of solid fractions. In this study, the primary clarification unit was omitted, assuming that primary solids will be fractionated in high rate algal wastewater ponds (HRAPs) (Craggs et al., 2012). HRAPs are wastewater treatment lagoons that use heterotrophic bacteria for the aerobic degradation of organic compounds and microalgae for oxygen production (Peccia et al., 2013; Mehrabadi et al., 2016; Hernandez et al.,

2016). In this study, wastewater was assumed to be directly sent to the HRAPs with an average hydraulic retention time of 4 days (Kovalcik, 2013).

The concentration of algae in the ponds can be calculated based on the required oxygen to satisfy five-day biochemical oxygen demand (BOD₅) in the pond. It was assumed that 1.55 g O₂ was produced per gram of algae biomass, and 1.1 grams O₂ is required per gram of BOD₅ removed. Therefore, one gram of algae would remove 1.4 g of BOD₅ (Oswald et al., 1953). The retention time of the clarifier was assumed to be 2 hours and the overflow rate was assumed to be 30 – 50 m³/m²/d (Metcalf, 2003). These solids will be sent to an AD unit for biological conversion, where energy is required for the solids scraper as well as for sump pumps to transfer the solids to the AD unit (Kovalcik, 2013).

In this study, it was assumed that the flue gases from the gas turbine are used as the primary source of CO₂ required for cyanobacterial cultivation. We also assume that the limonene production facility will be constructed next to a coal or natural gas power plant and that the production facility can utilize the flue gases from the plant. The AD units produce a sulfur-free biogas which is burned in the turbine and results in a sulfur-free flue gas stream (Chisti, 2008b). The composition of the flue gas from the gas turbine is 82% N₂, 12% CO₂, and 5.5% O₂ based on 68% methane biogas produced in the AD (Chisti 2008b). Conventional flue gases, such as what will enter the production facility from the neighboring power plant, contain mainly carbon monoxide (CO), nitrogen oxides (NO_x), sulfur dioxide (SO₂), nitrous oxide (N₂O), hydrogen chloride (HCl), and hydrogen fluoride (HF). Thus, a gas cleaning unit was required.

There are different technologies available for gas cleaning including dry scrubbing processes and wet processes. The wet processes, which are usually proposed for hazardous waste and household waste combustion facilities, are costly and energy extensive (IPPC, 2006). In this production facility, a conventional dry gas cleaning unit was used. In this technology, dust and hazardous materials are removed in a fabric filter based on physical sorption or chemistry sorption. The blowing agent, NaHCO_3 , is initially blown into the flue gas stream where pollutants are chemically bonded to the agent (IPPC, 2006). Then, the agent is deposited in a bag filter and the clean gas is sent to the PBRs.

8.3.6 Assumptions

The required land is of great importance when establishing PBR systems. The land needed is dependent on the size and involved technology of the production system. Environmental, geographic, and technological characteristics of the land are important determinants for the suitability of the land. The land used for this production system is considered to be located in the Southwestern USA. (Richardson et al., 2014b). In addition, the land is considered to be non-arable to improve the economics of the system. Based on the selected region, the cost for land is assumed to be very low at \$500/acre (Holtermann and Madlener, 2011; Richardson et al., 2010), and located next to a coal or natural gas power plant. In the event that land this cheap is not available next to a power plant, the change in CAPEX will be minimal due to the small amount of land needed to cultivate cyanobacteria in PBRs. For example, if land for the production facility was purchased at \$5000/acre, the change in CAPEX would only be \$23,310, and the change

in CAPEX would only be 1.6%. Considering the estimated production cost of \$107.9 million of both scenarios at year 5, this number is inconsequential.

Cyanobacterial biomass consists of 40 to 50% carbon (Kumar and Das, 2012), which suggests that 1.5 to 2.0 kg of CO₂ is required for each 1 kg of cyanobacteria produced in the cultivation phase. CO₂ is directly injected into PBRs via internal sparging through 3 mm diameter nozzles located at the bottom of the illuminated reactors. CO₂ expenses from the production facility are assumed to be \$0 due to the neighboring power plant. The production facility is assumed to be operational continuously for ~8000 h/yr (330 d/yr, 24 h/d). Cultivation and harvesting are considered at a continual basis for 24 h/d for plant operation of 330 d/yr. Cyanobacterial productivity is assumed to be 1.25 kg/m³/d based on Davis et al., (2011). Thus, harvesting volume is limited to 37.5 kg/m³/month based on the required annual production. For cyanobacterial cultivation, a lag phase of 3 days was considered in which the microbes adapt to the growth conditions. Afterwards, 10 days of log phase was considered with a limonene production of 0.018 mg/L/d (1st scenario) or 1.8 mg L/d (2nd scenario). Day 14 is considered the beginning of the stationary phase where chemical production typically decreases and cultivation medium is sent for harvesting.

The condenser for recovering limonene is considered to be a refrigerated type due to the high expected VOC contents in the gas stream. For these condensers, the efficiency is expected to be > 90%, thus 90% is assumed for this system. The condenser efficiency and associated costs are taken from a study by Shareef et al., (1995). Fixed-bed units are employed for limonene adsorption from the gas stream. During adsorption, limonene outlet will increase from zero (at the start of the cycle) to the breakthrough concentration

(at the end of the cycle). The efficiency is considered as a function of time, and can be calculated via integration over the length of the absorption cycle. An overall efficiency of 85% is considered in this study for a shorter adsorption cycle. Overall cost estimations for this unit were obtained from Vataavuk et al., (1999). Adsorption and desorption times are important for determining the required AC and can be calculated based on adsorption theory. This is also important for establishing desorption auxiliary equipment and utility requirements (Vataavuk et al., 1999). In this study, one shift at 8 h/d was considered for adsorption and one 16 h/d shift was considered for desorption or standby.

Two different approaches are available for estimating the required amount of AC in adsorbing systems. One approach considers the unsteady-state energy and mass transfer phenomena occurring in the adsorbent bed. In the other approach, the carbon requirement is estimated based on the nature of the VOC and other parameters in the absorbers. In this study, the amount of required carbon is considered based on the former approach. Also, 10% of fresh carbon make up is considered for the system. Microwave regeneration of AC is suggested to reduce downstream expenses for limonene recovery (Coss and Cha, 2000). The adsorber sizing procedure will be determined via the bed surface loading rate. In this study, a standard 4 ft carbon bed depth with a maximum gas loading of rate of 100 cfm per square foot of bed surface is maintained. This will ensure adequate gas contact and sufficient time to reach adsorption equilibrium. Adsorption capacity, which is defined as the amount of target product capable of being adsorbed on a unit weight of activated carbon, is dependent on characteristics of the target product, the temperature of the gas stream, and also on the type of AC in use. Typical adsorption capacities range from 5% to 30% of the weight of the carbon (Shepherd 2001). Based on

a 30% adsorption capacity of the applied AC, the amount of required activated carbon was calculated.

8.4 Results and discussion

FARM simulated 10 years of operation for the production facility using 2 different scenarios. The 1st scenario used actual production data from a limonene producing filamentous cyanobacteria strain (Halfmann et al., 2014b). The 2nd scenario assumed a 100-fold increase in limonene productivity compared to the 1st scenario. The summary statistics for the key output variables (KOVs) from the 1st and 2nd scenario are shown in Tables 8.3 and 8.4, respectively. The fractional mean of production costs, taxes, dividend costs, and non-production costs are shown in Figure 8.2.

Table 8.3: Summary statistics for KOVs used in the 1st scenario.

Iteration (1 st scenario)	Mean	StDev	CV	Min	Max
Production Costs (M\$)	107.933	5.816	5.388	90.578	131.429
Interest (M\$)	35.293	6.785	19.225	20.975	55.412
Dividends (M\$)	0.080	0.000	0.000	0.080	0.080
Principal	0.162	0.000	0.025	0.162	0.162
Replacement	0.029	0.000	0.091	0.029	0.029
Taxes	0.000	0.000	0.000	0.000	0.000
Total Costs (M\$)	143.466	9.035	6.298	119.591	173.614
Prod Cost Fraction	0.754	0.037	4.844	0.638	0.844
Interest Fraction	0.245	0.037	14.942	0.154	0.360
Dividends Fraction	0.001	0.000	6.277	0.000	0.001
Principal Fraction	0.001	0.000	6.277	0.001	0.001
Replace Fraction	0.000	0.000	6.276	0.000	0.000
Taxes Fraction	0.000	0.000	0.000	0.000	0.000
TC (\$/Ton of Biomass) Yr 5	14.335	1.176	8.205	11.713	19.208
Bio Prod Yr 5 (tons)	10242208.358	474725.713	4.635	8203675.851	11224538.020
Rec (M\$) 5	2.247	0.431	19.178	1.612	5.193
NCI (M\$) 5	-140.979	9.044	-6.415	-171.255	-117.520

Table 8.4: Summary statistics for KOVs used in the 2nd scenario.

Iteration (2nd scenario)	Mean	StDev	CV	Min	Max
Production Costs (M\$\$s)	107.918	5.584	5.174	88.346	127.168
Interest (M\$\$s)	0.876	0.156	17.858	0.505	1.361
Dividends (M\$\$s)	17.896	6.776	37.860	7.540	65.255
Principal	0.162	0.000	0.025	0.162	0.162
Replacement	0.029	0.000	0.091	0.029	0.029
Taxes	35.105	15.810	45.036	10.940	145.609
Total Costs (M\$\$s)	161.954	22.934	14.161	121.927	313.310
Prod Cost Fraction	0.676	0.080	11.810	0.324	0.851
Interest Fraction	0.005	0.001	21.176	0.003	0.009
Dividends Fraction	0.108	0.023	21.241	0.057	0.208
Principal Fraction	0.001	0.000	11.868	0.001	0.001
Replace Fraction	0.000	0.000	11.864	0.000	0.000
Taxes Fraction	0.209	0.058	27.603	0.086	0.465
TC (\$/Ton of Biomass) Yr 5	12.675	0.977	7.705	10.701	17.174
Bio Prod Yr 5 (tons)	10236132.713	488529.290	4.773	8258798.428	11002757.950
Rec (M\$\$s) 5	227.566	45.171	19.850	153.018	536.786
NCI (M\$\$s) 5	118.773	45.170	38.031	49.732	434.500

A



B

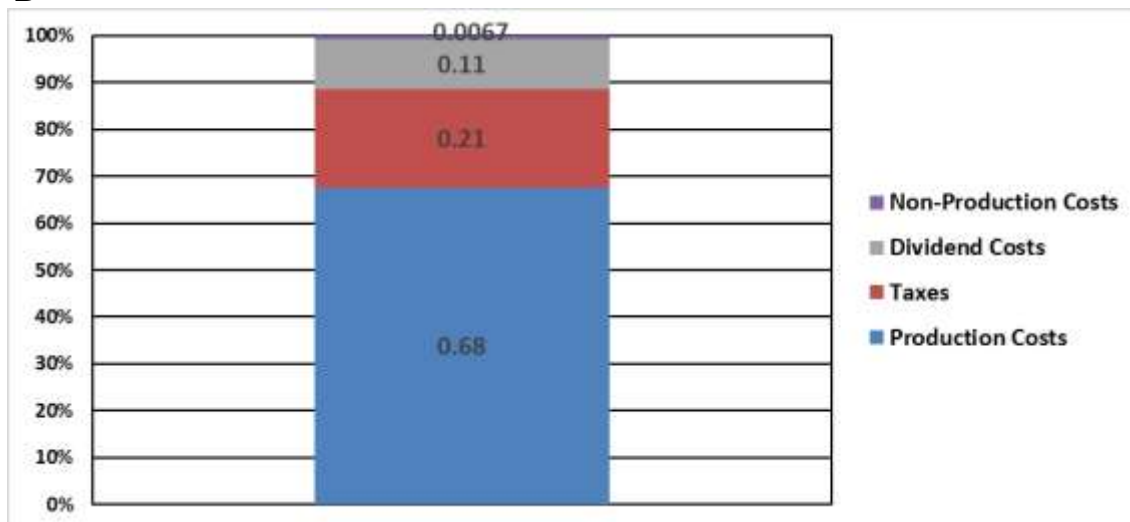


Figure 8.2: Fractional mean of production costs, taxes, dividend costs, and non-production costs.

(A: 1st scenario; B: 2nd scenario).

The mean annual production cost of both scenarios at year 5 was \$107.9 million.

However, in the 1st scenario it constituted 75% of the total OPEX, while in the 2nd scenario it constituted 68% of the total OPEX. In the 1st scenario, the non-production costs constitute 24% of the total OPEX, compared to 0.7% in the 2nd scenario. Non-

production costs consist of principle, replacement of machinery, and interest costs. The reason that the 1st scenario has a larger percentage of the OPEX consisting of non-production costs is due to interest still being paid at year 5 in the 1st scenario. Both scenarios evaluated would produce ~11 billion tons of biomass at year 5. The production cost for the 1st scenario at year 5 would be \$15.10 per L limonene produced, compared to \$0.15 per L limonene produced for the 2nd scenario. The model estimated the commodity price of limonene at year 5 will be \$4.66 per L, which shows why the 2nd scenario is economically feasible and the 1st scenario is not.

The average probability of economic success and NPV at year 5 for the 1st and 2nd scenarios assuming fractional reductions in CAPEX and OPEX is shown in Table 8.4 and 8.5, respectively. Each scenario was simulated for 100 combinations reducing CAPEX and OPEX in 10% increments from 0 to 90% (Richardson and Johnson, 2015). In the 1st scenario, every scenario in the 10 x 10 matrix yields a 0% probability of economic success at year 5. Assuming no fractional reduction in CAPEX or OPEX, the average NPV at year 5 was \$-588 million. Reducing the CAPEX and OPEX by 0.9 would still yield a 0% probability of economic success and the average NPV was \$-48 million. In the 2nd scenario, every scenario in the 10 x 10 matrix yields a 100% probability of economic success at year 5. Assuming no fractional reduction in CAPEX or OPEX, the average NPV at year 5 was \$392 million. Reducing the CAPEX and OPEX by 0.9 increases the average NPV to \$695 million. Reductions in CAPEX have little impact in increasing the NPV of either scenario because the total CAPEX for both scenarios is \$1.5 million. Thus, fractional reductions have little effect on the NPV of each scenario, which is \$-588 million for the 1st scenario and \$392 million for the 2nd scenario when no fractional

reductions are considered. In the model, it was determined that a limonene productivity of 1.02 mg/L/d is needed to yield an NPV of 0 dollars at year 5.

Table 8.5: Average probability of economic success and NPV at year 5 for scenario assuming fractional reductions in CAPEX and OPEX for the 1st scenario.

Probability of Economic Success and Net Present Value (M\$)

ARID Fraction OPEX	Fractional Reductions in the CAPEX									
	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-588	-588	-588	-588	-587	-587	-587	-587	-587	-587
0.1	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-528	-528	-528	-528	-528	-527	-527	-527	-527	-527
0.2	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-468	-468	-468	-468	-468	-468	-467	-467	-467	-467
0.3	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-409	-408	-408	-408	-408	-408	-408	-407	-407	-407
0.4	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-349	-349	-348	-348	-348	-348	-348	-348	-347	-347
0.5	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-289	-289	-289	-288	-288	-288	-288	-288	-288	-287
0.6	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-229	-229	-229	-229	-228	-228	-228	-228	-228	-228
0.7	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-169	-169	-169	-169	-169	-168	-168	-168	-168	-168
0.8	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-109	-109	-109	-109	-109	-109	-108	-108	-108	-108
0.9	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%
	-50	-49	-49	-49	-49	-49	-48	-48	-48	-48

Table 8.6: Average probability of economic success and NPV at year 5 for scenario assuming fractional reductions in CAPEX and OPEX for the 2nd scenario.

Probability of Economic Success and Net Present Value (M\$)										
ARID	Fractional Reductions in the CAPEX									
Fraction OPEX	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	392	392	392	392	392	392	392	393	393	393
0.1	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	425	425	425	426	426	426	426	426	426	426
0.2	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	459	459	459	459	459	459	460	460	460	460
0.3	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	492	492	493	493	493	493	493	493	493	494
0.4	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	526	526	526	526	526	527	527	527	527	527
0.5	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	559	560	560	560	560	560	560	560	561	561
0.6	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	593	593	593	593	594	594	594	594	594	594
0.7	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	627	627	627	627	627	627	627	627	628	628
0.8	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	660	660	660	661	661	661	661	661	661	661
0.9	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%
	694	694	694	694	694	694	694	695	695	695

Comparisons can be made between this model, specifically in the 1st scenario which uses actual production data, and an algal farm scenario that was also modeled using the FARM. The algal farm scenario was considered to be the most promising of the 7 scenarios evaluated for the NAABB (Richardson and Johnson, 2015). It should be noted that this is not an ‘apples to apples’ comparison. The analysis in this study evaluates the best case scenario for many aspects of the operation while the algal study evaluated technologies that were currently available and have been proven at a scaled-up rate, using actual yields verified by field values, not maximum theoretical yields. The cyanobacterial model had a 100% probability of economic success compared to a 0% probability of economic success for the algal model. There are several factors that caused

the cyanobacterial model to have a much greater chance of economic success than the algal model. The total CAPEX for the algal model was \$1,070.3 million compared to \$1.5 million for the cyanobacterial model. The reason for the large difference is the cost of land (\$35.9 million), construction (\$158.98 million), and liners (\$156.9 million) for the algal model, which cultivates the microbe outdoors and needs large amounts of land for cultivation. In the cyanobacterial model, there is an 82-fold increase in liter of product per acre of land compared to the algal model. In the cyanobacteria model, cultivation takes place inside PBRs, thus there was no need for liners and the cost of land combined with construction was only \$0.063 million. The algal model also had a larger OPEX value compared to the cyanobacterial model at year 5 of the simulation, \$261.1 million vs. \$162.0 million, respectively. The major operating cost that causes the substantial difference between the algal scenario and the best case cyanobacterial scenario is interest. In the algal model at year 5, interest was \$102.2 million compared to \$0.5 million in the cyanobacterial model. The majority of OPEX at year 5 of the cyanobacterial model is from costs associated with the EC unit (\$83.0 million).

By altering the limonene productivities in the model, we were able to determine that a productivity of 1.02 mg/L/d is needed to yield an NPV of 0 dollars at year 5. This suggests that in the production facility described in this study, limonene productivity greater than 1.02 mg/L/d would yield an economically feasible production facility. However, if the productivity facility was changed in any way, such as the amount of PBRs used to cultivate cyanobacteria, the productivity needed to obtain an NPV of 0 would also probably change. Since the current limonene productivity of the genetically engineered cyanobacteria strain is only 0.018 mg/L/d, this study strongly suggests that

the production facility described in this study would not be economically feasible at this time.

Filamentous cyanobacteria strain development is an area that needs to be improved to achieve the goal of an economically successful next-generation biofuel facility. Biofuels, such as limonene, are typically toxic to the microbe that produces them (Chubukov et al. 2015; Dunlop et al. 2011; Jarboe et al. 2011; Kim et al. 2015; Zhang et al. 2011). Thus, for a production process to become economically feasible, it will be necessary to develop strains able to tolerate the titers of chemicals that they are producing. This can be done via several mutagenesis techniques, including directed evolution (Johnson et al. 2016c). Developing renewable, sustainable sources of fuel is becoming increasingly important. This study shows strong evidence that a next-generation biofuel production facility utilizing genetically engineered strains of filamentous cyanobacteria could become economically feasible in the future.

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Chapter 9 - Life cycle analysis of next-generation biofuel production from filamentous cyanobacteria

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Abstract

Due to the adverse effects of fossil fuel use, it is becoming increasingly important to produce next-generation biofuels from renewable, sustainable sources. Filamentous strains of cyanobacteria have emerged as promising industrial microorganisms capable of producing a range of biofuels and chemicals. In this study, a life cycle analysis (LCA) was

conducted on a theoretical production facility that uses a genetically engineered strain of filamentous cyanobacteria to produce the cyclic hydrocarbon limonene. The environmental profile, and not the economic feasibility of the process, was therefore the key metric evaluated. In Scenario 1, the cyanobacterium was assumed to produce limonene at a rate of 1.8 mg/L/h, resulting in an annual production of 32,727 L/yr of limonene. In Scenario 2, limonene productivity was set at 55.5 mg/L/h, resulting in annual production of 1,000,000 L/yr. Both scenarios were assumed to produce the same amount of cellular biomass, which was converted by gas turbines into electricity to power the facility. Excess electricity was assumed to be sold to the grid. The major environmental burdens of the facility, which were measured in eco-points and calculated based on the Eco-indicator 99 method, were the cyanobacteria nutrient supply (especially sodium nitrate) and the photobioreactor (PBR) electrical requirements. Scenario 1 was the least energy intensive, with a global warming potential of -16.180 kg CO₂ equivalent/FU, compared to scenario 2 (-0.435 kg CO₂ equivalent/FU) or fossil-derived diesel (0.449 kg CO₂ equivalent/FU). The lower output of limonene in Scenario 1 meant that less energy was required for product recovery, leaving more electricity for sale to the grid. Even though a higher limonene productivity will worsen the environmental profile of the process, this study strongly suggests that the limonene productivity facility described in this study for both scenarios holds great potential as a future sustainable solution for producing next-generation biofuels.

9.1 Introduction

Developing renewable, sustainable sources of biofuels is necessary in order to decrease the environmental burden created by extensive use of fossil fuels. Fossil fuel

reserves are finite and the adverse effects of fossil fuel-generated greenhouse gases are well established (Von Blottnitz and Curran, 2007; Chen et al., 2011; Welkie et al., 2014). Biofuels can be categorized based on the type of feedstock used and/or the type of fuel produced. Each new generation of biofuel has been developed to overcome limitations or disadvantages of prior generations. This categorization has led to 4 generations of biofuels being defined (Dutta et al., 2014). While each biofuel type has its own advantages and disadvantages, together they have begun to decrease the burden of global fossil fuel consumption.

First generation biofuels were developed in the 1970s and 80s and consist of either: 1) ethanol produced via fermentation of sugar (primarily from sugar cane) or hydrolyzed starch (primarily from corn), or 2) biodiesel produced via trans-esterification of vegetable oil (primarily soybean oil or animal fats). The fuel ethanol process is well established and consists of feedstock pretreatment (milling, crushing, and solubilizing in water), saccharification (converting starch into sugars for the corn ethanol process), fermentation, distillation, and co-product recovery (Cardona and Sánchez, 2007; Kwiatkowski et al., 2006). First generation biofuels have three major disadvantages: production costs, market access, and competition for arable land with food crops. Because 1st generation biofuel feedstocks are also used for food, the feedstock usually accounts for more than 33% of total production costs, and this situation is unlikely to change as the world population and food demand continues to rise (Dien et al., 2003). Second generation biofuels are typically defined as ethanol or other biofuels produced from lignocellulosic biomass, which includes a diverse range of by-products, wastes, and dedicated feedstocks (Sims et al., 2010). The sustainability of 2nd generation biofuels is

limited by land availability and competition for land use (Petersen et al., 2015; Deenanath et al., 2012; Mabee et al., 2011; Balat, 2011).

Due to the drawbacks associated with 1st and 2nd generation biofuels, 3rd and 4th generation biofuels have been developed. These are fuels derived from the fixation of CO₂ by photosynthetic algae and cyanobacteria (Singh et al., 2011b), where the photosynthetic organism serves as both the photocatalyst and producer of biofuel (Lindberg et al., 2010). At this time, algae and cyanobacteria appear to be the only sources of biofuel capable of meeting the global demand for transportation fuel (Brennan and Owende, 2010; Chisti, 2007; Schenk et al., 2008; Singh et al., 2011a). While algal oil can potentially be used directly as a fuel, in most cases the oil is subsequently processed through traditional oil refinery or biodiesel technologies into biofuels (Chernova et al., 2012; Chernova et al., 2010; Senko et al., 2012). Therefore, many researchers now suggest that the definition of 3rd generation biofuels be altered to photoautotrophic conversion of CO₂ into oil or algal biomass that is subsequently converted into biofuels (Dutta et al., 2014). This conversion step is a limitation to 3rd generation biofuels that does not exist with 4th generation biofuels. Fourth generation biofuel is the term used for the production of 'drop-in' biofuels directly from genetically engineered algae or cyanobacteria (Chernova et al., 2012; Chernova et al., 2010; Senko et al., 2012).

Heterocyst-forming filamentous cyanobacteria have the ability to fix atmospheric nitrogen, meaning that the cultivation medium does not need a combined nitrogen source, which is a considerable expense. This is one of the reasons that industrial microbiologists have focused on engineering filamentous cyanobacteria to produce next-generation biofuels and high-value chemicals (Schoepp et al., 2014), including limonene (Halfmann

et al., 2014b), farnesene (Halfmann et al., 2014a), myrcene (Gu et al., 2016), and linalool (Gu et al., 2012). While, it is possible to cultivate nitrogen-fixing filamentous strains of cyanobacteria in medium lacking combined nitrogen, the production data used in this study was derived from cyanobacteria cultivated in medium containing sodium nitrate. Thus, sodium nitrate was included in this model.

The aim of this study was to evaluate the environmental profile of a theoretical, next-generation biofuel production facility that uses genetically engineered cyanobacteria to produce limonene. A well-to-pump strategy was applied to define the systems boundaries. Scenario 1 was defined based on a theoretical production facility described by Johnson et al., in which 32,727 L/yr limonene was produced (Johnson et al., 2016a). Scenario 2 was based on a theoretical facility described by Halfmann, et al., which produced 1,000,000 L/yr of limonene (Halfmann et al., 2014b). The only difference in Scenario 1 and Scenario 2 was the total annual limonene production, which was due to different limonene productivities of the engineered cyanobacteria. The environmental profiles of both scenarios were then compared to the conventional production of fossil-based diesel.

9.2 Data and methods

9.2.1 Production systems overview

A process flow diagram of the limonene production process is shown in Figure 9.1, while Table 9.1 lists process inputs and parameters for both scenarios. The process includes photobioreactors (PBRs), clean-in-place units (CIPs), a limonene recovery unit, a biomass harvesting unit, an anaerobic digestion (AD) unit, and a wastewater treatment

unit (Richardson et al., 2014b; Cheng et al., 2009; Kovalcik, 2013; Li et al., 2011). The system requires a CO₂ supply as a carbon source for cyanobacteria and to control culture pH (Kunjapur and Eldridge, 2010). In this system, a CO₂-enriched air flow will be applied for agitation and also to provide gas exchange by stripping out dissolved oxygen and limonene.

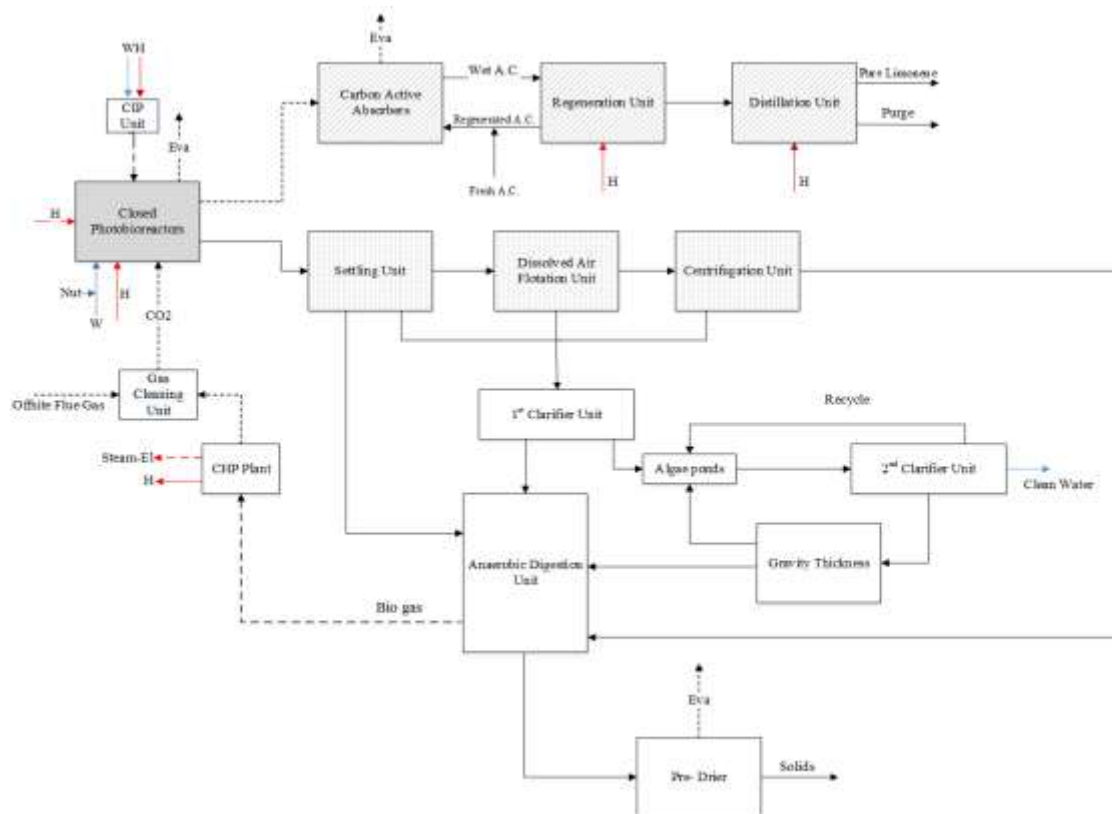


Figure 9.1: Process flow diagram for a cyanobacteria limonene production facility.

H: heat streams (red lines); W: clean water streams (blue lines); S.S: superheated steam; Nut: nutrients required for BG11 medium; Eva: evaporation; AC: activated carbon; Dashed lines: gas streams; Units with dot-pattern: solid fraction recovery unit; Units with diagonal-line pattern: limonene recovery unit.

Table 9.1: Limonene production inputs and parameters for Scenario 1 and Scenario 2.

Process	Value (Scenario 1)	Value (Scenario 2)	Unit	Reference
<i>Adsorption unit</i>				
Limonene production capacity	32433	1000000	L/yr	
Required electricity for condensation	128	3968	kWh/yr	[50]
Electricity for adsorber unit (Adsorption)	7.2	223.2	MWh/yr	
Microwave power consumption	41.6	1289.6	MWh/yr	[72]
<i>Cultivation</i>				
Biomass production	14350	14350	kg biomass/batch ¹	
Required electricity in PBRs ^a	31.9	31.9	MWh/yr	
Log phase days per cultivation cycle	12	12	days	
Nutrients ^b required for cultivation				[58]
Sodium nitrate	87003	87003	kg/yr	
Ferric ammonium citrate	348	348	kg/yr	
Potassium phosphate dibasic anhydrous	2319	2319	kg/yr	
Magnesium sulfate	2119	2119	kg/yr	
Calcium chloride	2085	2085	kg/yr	
Citric acid	349	349	kg/yr	
Sodium carbonate	1160	1160	kg/yr	
Manganese chloride	105	105	kg/yr	
Boric acid	164	164	kg/yr	
EDTA	56	56	kg/yr	
Water consumption for cultivation	2391000	2391000	L/batch	
Energy ^c required in PBR's	283	283	MWh/yr	[70]
Theoretical required CO ₂	87903	87903	kg/yr	
Energy required for pumping CO ₂	12.9	12.9	MWh/yr	[70]
<i>Harvesting</i>				
Cultivation broth ^d	159439	159439	L day ⁻¹	
Electricity required for mixing	20	20	kWh per 1000 m ³ of broth	[51]
Energy required in DAF unit	0.250	0.250	kWh kg ⁻¹ of biomass	[29]
Energy required in EC unit	0.039	0.039	kWh kg ⁻¹ of biomass	[74]
<i>Anaerobic digestion</i>				
Methane, 96 vol%	7175	7175	m ³ /batch	[85]
Total electricity consumption	0.2162	0.2162	kWh/kg algae biomass	[86]
Total electricity produced from biogas	943.338	943.338	MWh/yr	
Total heat produced from biogas	628.892	628.892	MWh/yr	

^a: Based on 1 t/d biomass production; ^b: Considering a 50 kg/yr cut-off; ^c: Energy required for pumping is included; ^d: Biomass concentration in the broth: 6 g/L.

In this study, both scenarios were defined assuming the PBRs can utilize solar light as their primary source. Biogas produced in the AD unit is directed to the gas turbine and electricity produced from biogas is assumed to compensate for the electricity consumption of this process. In Scenario 1, electricity produced by the gas turbine is greater than the electricity consumption of the entire process and the excess electricity produced is assumed to be sold to the grid. In Scenario 2, electricity produced by the gas turbine is slightly less than the electricity consumed in the process. It is also assumed that hot gases from the gas turbine in both scenarios will be sent to a neighboring ethanol plant for energy recovery to be utilized in distillation processes. This is commonly calculated as negative heat consumption in chemical industries.

9.2.2 Cultivation

A genetically engineered, limonene producing strain of *Anabaena* sp. PCC 7120 (herein referred to as *Anabaena* sp. 7120) (Halfmann et al. 2014b), was considered in this system. The PBRs used in this system are tubular bubble columns with a pump used for gas circulation (Sobczuk et al., 2000; Kunjapur and Eldridge, 2010), and will be cleaned via CIP units.

9.2.3 Limonene recovery

From an economic standpoint, limonene recovery from dilute fermentation streams via gas-stripping may be preferred (Kiyota et al., 2014). However, the gas stream exiting the PBRs would be very dilute and present a challenge for limonene recovery by simple condensation. To overcome this challenge, the gas stream is first passed through a column packed with activated carbon (AC) to absorb the limonene. After saturation, the

adsorption column is heated to regenerate the AC and release the limonene into a much smaller volume of gas, which is then condensed via cold water (Cao et al., 2015).

Microwave regeneration of AC is considered in this study as it is believed to reduce downstream expenses for limonene recovery (Coss and Cha, 2000).

9.2.4 Biomass harvesting unit

A low-energy and low-cost method of harvesting cell biomass is essential to establish an economically feasible process. The harvesting unit should be integrated with cultivation facilities to reduce energy demands for the system. It should also have low environmental impacts and low carbon or water footprints (Udom et al., 2013). In this study, solid biomass will be recovered by the technology proposed by Richardson and Johnson, (2013). Cultivation broth will be passed through the mixer and the settling tank that initially concentrates the cyanobacteria via autoflocculation (Davis et al., 2011). The medium will then enter the dissolved air flotation (DAF) unit followed by the electrocoagulation (EC) unit which concentrates the slurry to a final concentration of 160 g/L (Gebreslassie et al., 2013; Kovalcik, 2013). It has been suggested that this combination will minimize downstream costs (Grima et al., 2003).

9.2.5 Anaerobic digestion

In the proposed system, an AD unit is used to manage the large quantities of residual biomass produced and to improve the economic and energetic balance of the system. It is believed that conversion of cyanobacteria biomass into methane will allow for substantial energy recovery in the system (Sialve et al., 2009). Thus, in this process the majority of cyanobacterial biomass residue will undergo anaerobic digestion for

energy recovery. This energy is considered the primary energy source in the system (Chisti, 2008a; Ehimen et al., 2011), and the surplus of energy produced in the system can be sold to the grid.

9.3 LCA methodology

9.3.1 Notable assumptions

LCA modeling was carried out using SimaPro 8 software (Pré Consultants, 2001) This considers all four interrelated steps of an LCA including: “Goal and scope”, “Life cycle inventory”, “Life cycle impact assessment” and “Interpretation” according to ISO 14000 series (ISO 14041-43) (Finkbeiner et al., 2006). Inventory data was collected from different sources, including GREET 2014 (Wang et al., 2014), Ecoinvent 3.2 LCA database (Weidema et al., 2013), technoeconomic reports (Celenza, 1999; DOE, 2015; Kovalcik, 2013; Lee and Palsson, 1994; Shareef et al., 1995; Shepherd, 2001; Vataavuk et al., 1999), and the literature (Allen and Stanier, 1968; Cogne et al., 2005; Davis et al., 2011; Gebreslassie et al., 2013; Halfmann et al., 2014c; Halfmann et al., 2014a; Johnson et al., 2016a; Richardson and Johnson, 2013, 2015). In this study, a well-to-pump strategy was considered to investigate the overall sustainability and net energy balance of the limonene production system. This LCA will also provide baseline information for typical next-generation biofuel processes (Sander and Murthy, 2010). A well-to-pump strategy is often used for LCAs of transportation fuel production processes and it includes processes from the extraction of resources to delivery of fuel at refueling stations. The functional unit (FU) is a measure of function of the system and provides a reference to relate inputs and outputs to a common measure of function in order to compare different systems

(Sander and Murthy, 2010). In this study, a FU of 1 L of limonene was considered. The objective of this study was to perform a comparative study on the environmental profile of the two scenarios, and to determine which one is better from an environmental standpoint. The future use of limonene is not considered, thus this is not considered a complete LCA study and the well-to-pump strategy was used.

9.3.2 Notable assumptions

Table 9.2 provides a list of notable assumptions that were considered in the different boundaries provided in Figure 9.1. The energy required in the process units for Scenario 1 was calculated based on 32,727 liters per year limonene production and a limonene productivity of 1.8 mg/L/h. This productivity was calculated based on a claim from Joule Unlimited, Inc. that cyanobacteria can be engineered to produce 10 mg/L/h ethanol (Green et al., 2015). Ethanol's molecular weight is 46 g/mole, while ethylene is 28 g/mole. Thus, the equivalent hydrocarbon productivity of 10 mg/L/h ethanol would be 6 mg/L/h, and we chose a more conservative value of 1.8 mg/L/h (Johnson et al., 2016a). In Scenario 2, energy required by the process units was calculated based on proposed limonene production by Halfmann et al. of 1,000,000 liters per year limonene and a limonene productivity of 55 mg/L/h (Halfmann et al., 2014b). While it is unlikely that 55 mg/L/h limonene production will ever be accomplished by cyanobacteria, this will allow us to determine the effect of increased limonene production on the environmental profile of the theoretical production facility described in this study. For cyanobacterial cultivation, a lag phase of 3 days was considered in which the microbes become accustomed to the growth medium followed by 10 days of production. Afterwards, the

medium is sent for harvesting and energy recovery. Thus, a growth cycle of 15 days (22 batches per year) was considered for the production system.

Table 9.2: Notable assumptions used in this study.

Process	Assumptions	Reference
Adsorption unit	Annual limonene production: 32,433 L/yr (Scenario 1) and 1,000,000 L/yr (Scenario 2).	[28]
	Condenser efficiency: 90%	[50]
	Adsorption unit efficiency: 85%. Adsorption/Desorption period: 8:8 h/h.	[54]
	Carbon make up: 10%	
	Carbon adsorption capacity: 30 %	[53]
	Carbon bed density: 480.554 kg m ³	
Cultivation	Nutrient demands for cyanobacterial cultivation: BG ₁₁ medium	[58]
	chemical composition of Cyanobacteria: CH _{1.575} O _{0.459} N _{0.173} S _{0.006} P _{0.006}	[57]
	Productivity of strain: 1.25 kg. m ⁻³ day ⁻¹	[40]
	limonene productivity: 1.8 mg.L ⁻¹ day ⁻¹ (Scenario 1) and 55.5 mg.L ⁻¹ day ⁻¹ (Scenario 2)	[25, 56]
	Cyanobacteria growth cycle: 15 days. Log phase 12 days.	
	Wastewater taken after secondary treatment	
	Air enriched with 1% CO ₂	
Harvesting	Biomass concentration send for harvesting: 6 g L ⁻¹	[52, 87]
	Settling tank: Autoflocculation: concentrates the cyanobacteria to 1%	[40]
	Dissolved air flotation (DAF): concentrates the cyanobacteria to 10%. Electrocoagulation unit concentrates the slurry to 16%.	[31, 41]
	Dry biomass density: 1 kg L ⁻¹	[60]
Energy recovery	Anaerobic digester: 0.5 m ³ methane per kg of residual biomass	[85]

9.3.3 System boundary

In LCA studies, a boundary around the system must be defined which is determined by the scope of the study. In this study, 3 different boundaries are defined including the boundary between the product system and the environment system, the boundary between the relevant and irrelevant processes (cut-off), and the boundary between the system under consideration and other product systems (allocation) (Goedkoop et al., 2010; Guinée, 2002). Here, the system boundary is considered from cradle-to-gate as a first order, where only the production of materials and transportations are included. In some LCA studies, satisfactory results can still be obtained when excluding capital goods (Hsu, 2012). Figure 9.1 illustrates the boundaries defined in this study. However, capital goods may contribute up to 30% of the total environmental impacts and in modern data bases (i.e. Ecoinvent and the USA Input-Output data bases) capital goods are included (Goedkoop et al., 2010; Lehtinen et al., 2011).

9.4 Data sources

9.4.1 Data collection and relating data to unit processes

Data for the unit processes were gathered from the Farm-level Algae Risk Model (FARM) and other similar technologies. The FARM, formerly known as the Algae Income Simulation Model (AISIM), is a Monte Carlo firm level simulation model designed to simulate the annual cultivation, harvesting, extraction, and financial/economic activities of an algae farm (Richardson and Johnson, 2012). The similarities between algae and cyanobacteria allows for the model to be used for production facilities which use cyanobacteria. The main difference between an algae and

a cyanobacteria production facility is that algal systems typically produce oil which must subsequently be converted into biofuels, while cyanobacteria can be engineered to directly produce the biofuel which is excreted from the cell and volatilized into the culture headspace with minimal cell disruption. The data structure used in this study is composed of cultivation data, limonene recovery data, harvesting data, and data regarding energy recovery units presented in this system. For this LCA, information is considered for 5 different areas, including: process information, materials information, equipment information, water treatment, and energy content recovery information (Shepherd, 2001).

In this study, data regarding cyanobacterial cultivation and growth were obtained from recent studies in which strains of filamentous cyanobacteria have been genetically engineered to produce limonene (Halfmann et al., 2014b). For the cultivation phase, modified and updated data from the National Alliance for Advanced Biofuels and Bioproducts (NAABB) and the National Renewable Energy Laboratory (NREL), which were integrated into FARM, were the primary sources (Davis et al., 2012; DOE, 2015; Richardson and Johnson, 2015). Other supplementary data on unit operations was adapted from studies on algal biodiesel production and similar technologies, including pyrolysis and water treatment facilities (Chen et al., 2011; Delrue et al., 2012; Fernandes et al., 2015; Khoo et al., 2011). For limonene recovery, data was obtained from different sources for adsorbers (Coss and Cha, 2000; Shepherd, 2001; Vatauvuk et al., 1999) (design factors, capacities and energy demands), a microwave regeneration unit (energy demands) (Price and Schmidt, 1998), and the energy required for condensers (Shareef et al., 1995). For biomass harvesting, dewatering, and drying, supplementary data for energy consumption was obtained for mixing (Celenza, 1999), the DAF unit (Richardson

et al., 2014b; Rubio et al., 2002), and the EC units (Gebreslassie et al., 2013; Murdock and Lacey, 2010).

9.4.2 Software

Previously, FARM has been used to evaluate the annual cultivation, harvesting, extraction, and financial/economic activities of an algae farm (Richardson and Johnson, 2012). Different scenarios were assessed to determine the preferred strategy for algal biofuel production facilities (Richardson and Johnson, 2014, 2015; Richardson et al., 2014b; Richardson et al., 2012), and the most successful scenarios based on FARM were used in this LCA study. LCA software packages used in this study include Simapro 8.0 and GREET 1 2014 (for algal biodiesel and transportation) (Wang, 2014).

9.5 Coproduct allocation and displacement

In this proposed system, allocation is not a challenge as the number of coproducts from the unit operations is limited. In energy recovery units, steam, electricity, and residual biomass from the AD are typical co-products. The produced steam, biogas, and electricity can provide the energy required for the operations and the excess electricity can be sold to the grid. Excess steam was considered to be sold to a neighboring ethanol plant. There are different approaches to distributing the emissions produced from this operation. The allocation of energy and emissions of these products can be determined based on mass, economy, or produced energy. As the produced steam, biogas, and electricity can be substituted for fossil fuels in this study, the allocation based on economic value was utilized. Thus, the energy and emissions from these sources will

replace the energy and emissions associated with fossil fuel energy for the production of steam and electricity in the corresponding operations (González-García et al., 2010).

In this study, 3.815 kg and 0.123 kg dry biomass retentate was considered to be produced per liter of limonene for Scenarios 1 and 2, respectively. It is assumed that the dry biomass retentate has the same economic value as dry wood chips. Conservatively, limonene is considered to have a similar economic value as diesel. Based on the economic value in the United States market (DOE, 2011; EIA, 2016), 64% of the environmental load is allocated for limonene and 36% is allocated for biomass retentate for Scenario 1. In Scenario 2, 98% of the environmental load is allocated for limonene and 2% is allocated for solid residues from anaerobic digestion. Allocation for water replacing fresh water was considered to be 0% as the clean water from the water treatment facility was assumed to replace fresh water for cyanobacteria cultivation. Lastly, flue gas from the incinerator was assumed to replace pure CO₂ for cultivation.

9.6 Results and discussions

9.6.1 Overall energetic analysis

Table 9.3 summarizes the overall energy demand for unit processes, co-product allocations, net energy demand per FU of limonene. Overall energetics of the process can be described as total energy input, net energy input, net energy balance, and net energy ratio as described below:

$$\text{Total energy} = \Sigma \text{ sub-process energy inputs}$$

$$\text{Net energy input} = \text{Total energy input} - \text{by-product allocations}$$

Net energy balance = Net energy input – energy in FU of main product

Net energy ratio = Net energy input / energy in FU of main product

Table 9.3: Energy demand for unit processes, co-product allocations, net energy demand per FU of limonene, and CO₂ emissions allocated for limonene.

	Energy input (kWh/FU)	By-product allocation (kWh/FU)	Net energy input (kWh/FU)
Energy for PBRs	8.726	6.108	2.618
Electricity for PBRs	0.982	0.687	0.295
Energy for pumping CO₂ in PBRs	0.397	0.278	0.119
Electricity for absorption unit	0.222	0.156	0.067
Electricity for microwave AC regeneration	1.283	0.899	0.385
Electricity for condensation unit	0.004	0.003	0.001
Electricity for mixing broth for harvesting	0.033	0.023	0.010
Electricity for DAF unit	2.447	1.713	0.734
Electricity for EC unit	0.382	0.267	0.114
Electricity for anaerobic digestion	2.116	1.481	0.635
Electricity from methane gas turbine	-29.243	-20.470	-8.773
Steam from methane gas turbine	-19.496	-13.647	-5.849
Total	-32.148	-22.503	-9.644

Total energy and net energy input for the FU of limonene for Scenario 1 were calculated as -32.148 and -20.575 kWh, respectively. Considering the limonene heat value of 10.508 kWh/L (Růžička Jr and Domalski, 1993), the net energy balance was calculated as -31.083 kWh/FU. This is much higher than the value reported by Sander

and Murthy for algal biodiesel production (-6.7 kWh/FU) (Sander and Murthy, 2010). The net energy ratio of Scenario 1 was -1.95. Huo et al., (2008), reported a net energy ratio of 0.15 for soybean biodiesel production. These data provide evidence that limonene production from cyanobacteria will be substantially more environmentally favorable than algal and soybean biodiesel production.

Total energy and net energy input for the FU of limonene for Scenario 2 were 0.423 and 0.414 kWh, respectively. The net energy balance was calculated as -10.094 kWh/FU, which is higher than the value reported for algal biodiesel production (-6.7 kWh/FU) by Sander and Murthy, (2010). The net energy ratio of Scenario 2 was 0.04, which is substantially higher than Scenario 1 (-1.95). However, it is still lower than the value reported by Huo et al., (2008) (0.15). This shows that Scenario 2 will consume electricity in the process, but its electricity consumption is lower compared to algal biodiesel production technologies. These data provide evidence that by increasing limonene productivity of cyanobacteria, the overall environmental profile of the system is worsened. The main reason for this is that the downstream processes of limonene are much more energy intensive compared to the downstream processes of the biomass production and by increasing the ratio of limonene to biomass, the overall energy consumption of the system increases.

Figures 9.2 and 9.3 show the environmental load of each process stage based on Eco-indicator 99 for both scenarios (Goedkoop and Spriensma, 2001). For Scenario 1, even though no artificial lighting was assumed, the electricity needed for the PBRs was the most significant individual contributor to the total environmental load. It was responsible for ~42% of the total energy consumption. In this technology, other

downstream processes were considerably less energy intensive compared to processes which already exist in lipid based algal biodiesel production technologies. This superior energetic balance for downstream processes is the result of eliminating the process of oil extraction from the biomass, which is quite energy intensive. Thus, in this technology there is no need for energy intensive oil extraction processes as the limonene will be continuously volatilized into the headspace during cultivation.

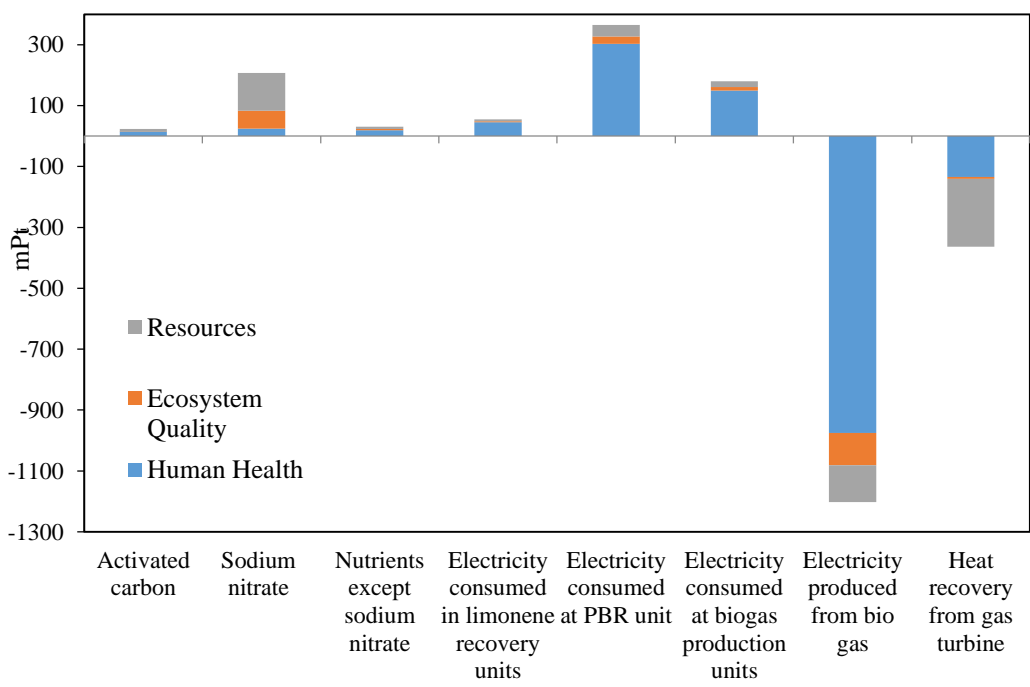


Figure 9.2: Environmental load of each process stage of Scenario 1.
 (Method: Eco-indicator 99 V2.10 / Single score / Excluding infrastructure processes).

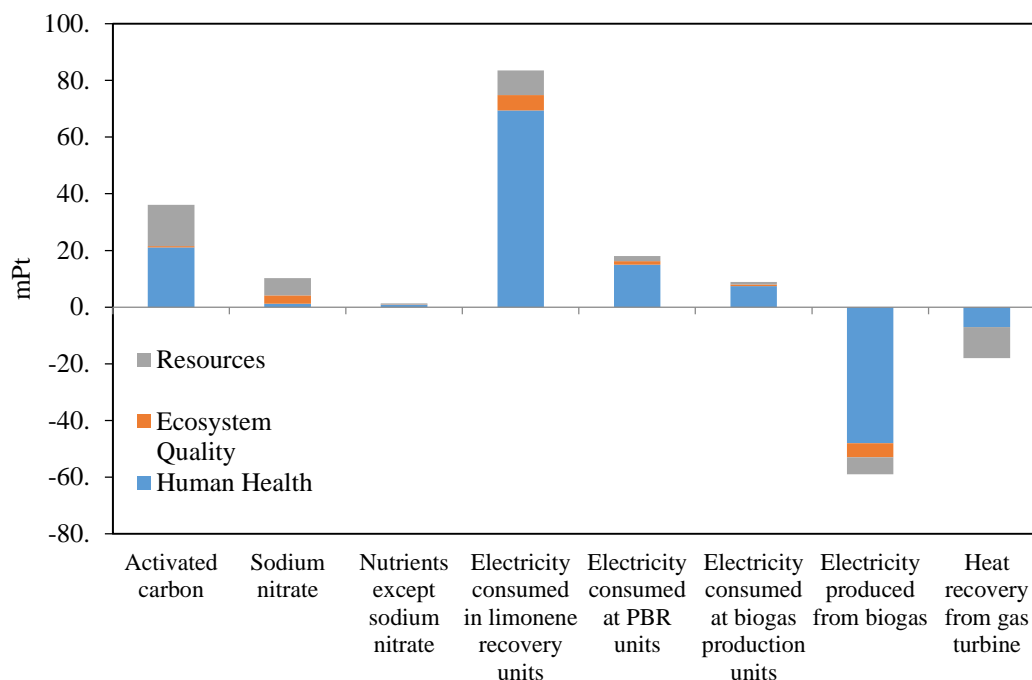


Figure 9.3: Environmental load of each process stage of Scenario 2.
(Method: Eco-indicator 99 V2.10 / Single score / Excluding infrastructure processes.)

Figure 9.3 shows that the negative eco-points (positive environmental load) assigned to the production of electricity and heat from biogas per FU of limonene has been drastically reduced in Scenario 2 compared to Scenario 1. Although the overall heat and electricity production of the facility remained almost identical to Scenario 1, it has been divided by a larger amount of limonene (~31-fold increase), and the environmental load allocated to limonene was increased from 64% to 98%. This translates into a ~20-fold reduction in the energy produced per FU allocated to limonene. This is similar to what occurs to the environmental loads caused by nutrients and electricity consumed by the PBR and biogas production units. “Allocation” of 64% for limonene in Scenario 1 means that 64% of the environmental load per FU of limonene caused by each input-output category was attributed to 1 L of limonene. Using the same method in Scenario 2, ~98% of environmental load per FU was attributed to 1 L of limonene. Although the

environmental load caused by the consumption of AC and electricity for recovery unit per 1 L of limonene was identical for both scenarios, the amount allocated to 1 L of limonene was increased ~1.5-fold in Scenario 2.

Figure 9.4 shows the comparison of various categories of environmental loads on 3 scenarios: Scenario 1, Scenario 2, and diesel from fossil fuels. Each category was normalized to 100%. These results provide evidence that limonene production via both scenarios would be more environmentally favorable than fossil based diesel. Scenario 1 was substantially more environmental friendly than Scenario 2 due to the environmental profile of the system becoming worse due to increased limonene production.

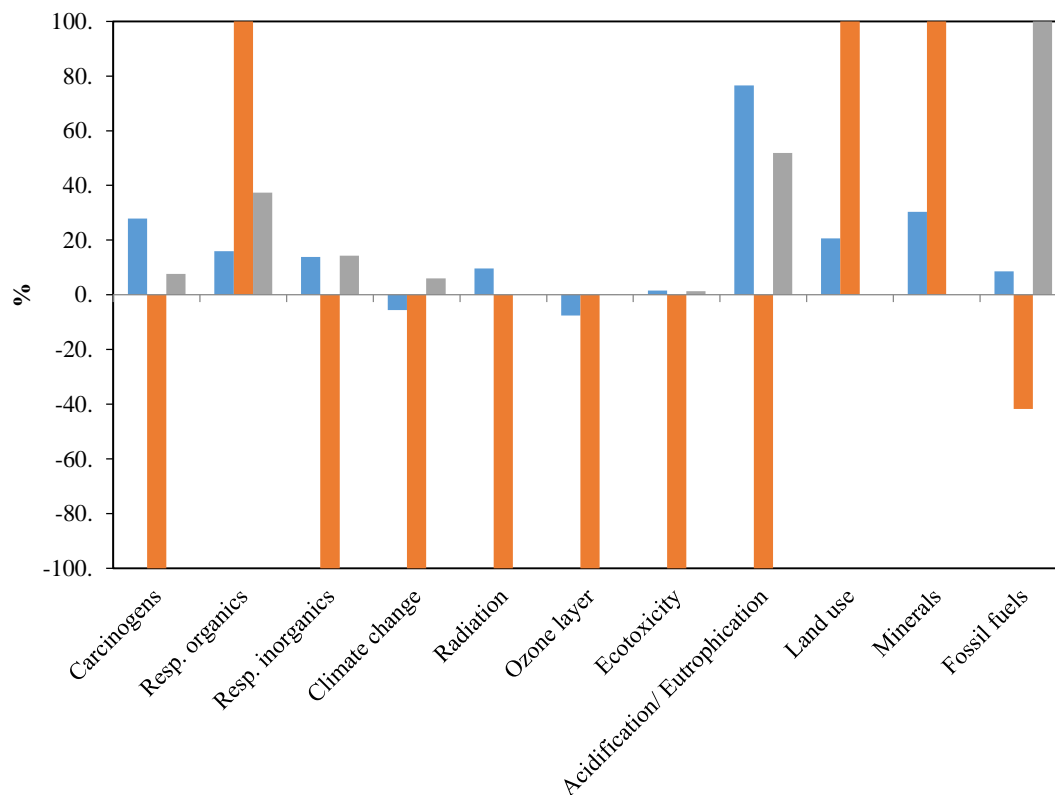


Figure 9.4: Comparison of various categories of environmental loads for production of 1 L of limonene for Scenario 1 and 2 and 1 L 'Diesel, at refinery/L/US'. (Method: Eco-indicator 99 V2.10 / Characterization / Excluding infrastructure processes). (Each category is normalized to 100%. Orange columns represent Scenario 1, blue columns represent Scenario 2 and grey columns for Diesel, at refinery/L/US.)

Single score comparisons of environmental loads for the 3 scenarios is shown in Figure 9.5. This comparison provides more evidence that limonene production in Scenario 1 causes a substantially lower overall environmental load compared to Scenario 2. In Scenario 2, overall electricity consumed by PBRs, the production of biomass, and electricity produced from biomass remains virtually identical to Scenario 1, yet the production of biomass and electricity produced from biomass and electricity produced from biogas per FU has been reduced. Due to the very high energy balance of the AD and

energy recovery unit, reducing the proportion of biomass production of the whole system dramatically affects the energy balance.

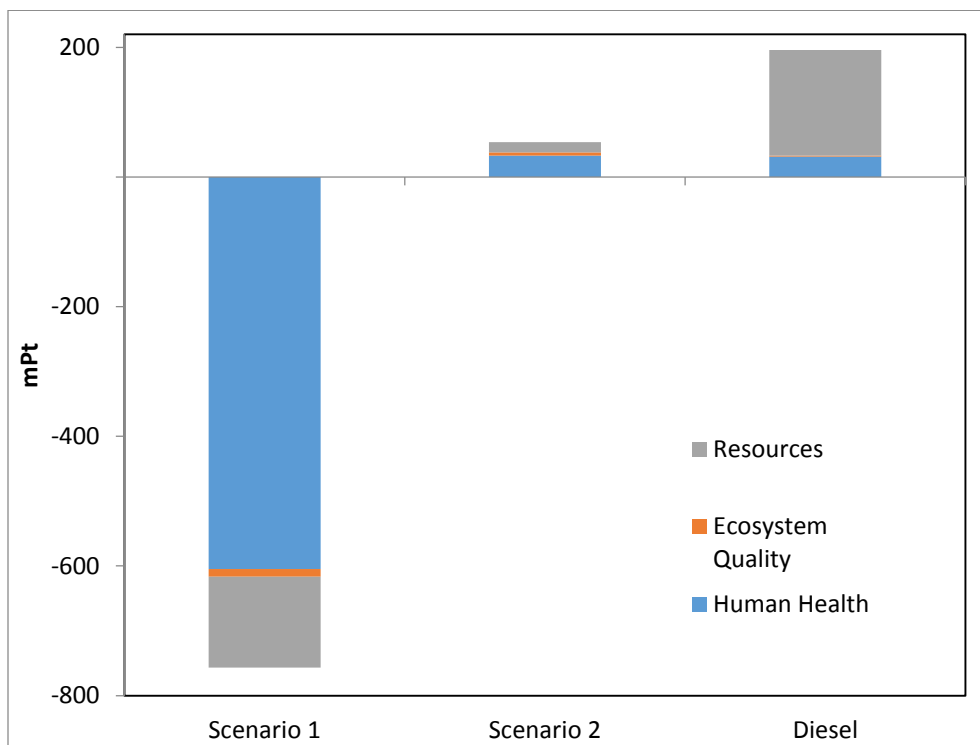


Figure 9.5: Damage assessment comparison of the environmental loads of production of 1 L of limonene for Scenario 1 and 2 and 1 L 'Diesel, at refinery/L/US'.

(Method: Eco-indicator 99 V2.10 / Single score / Excluding infrastructure processes).

Figure 9.6 compares the global warming potential of different scenarios based on Greenhouse Gas Protocol v1.01 (Weidema et al., 2013). Results show CO₂ equivalent emissions allocated to 1 L of limonene in Scenarios 1 and 2 are equal to -16.81 and -0.435 kg/L limonene, respectively. For fossil-based diesel, it is 0.449 kg/L. The negative CO₂ equivalent emissions in Scenarios 1 and 2 indicates that in both scenarios, the amount of prevented greenhouse gas emissions (caused by electricity generation and fixing CO₂ in limonene and biomass) were higher than greenhouse gas emissions caused

by the consumption of electricity and nutrients. This also provides evidence that the production of limonene from Scenarios 1 and 2 is more environmentally favorable than fossil-based diesel. Also, this provides evidence that the global warming potential of Scenario 1 is better than Scenario 2.

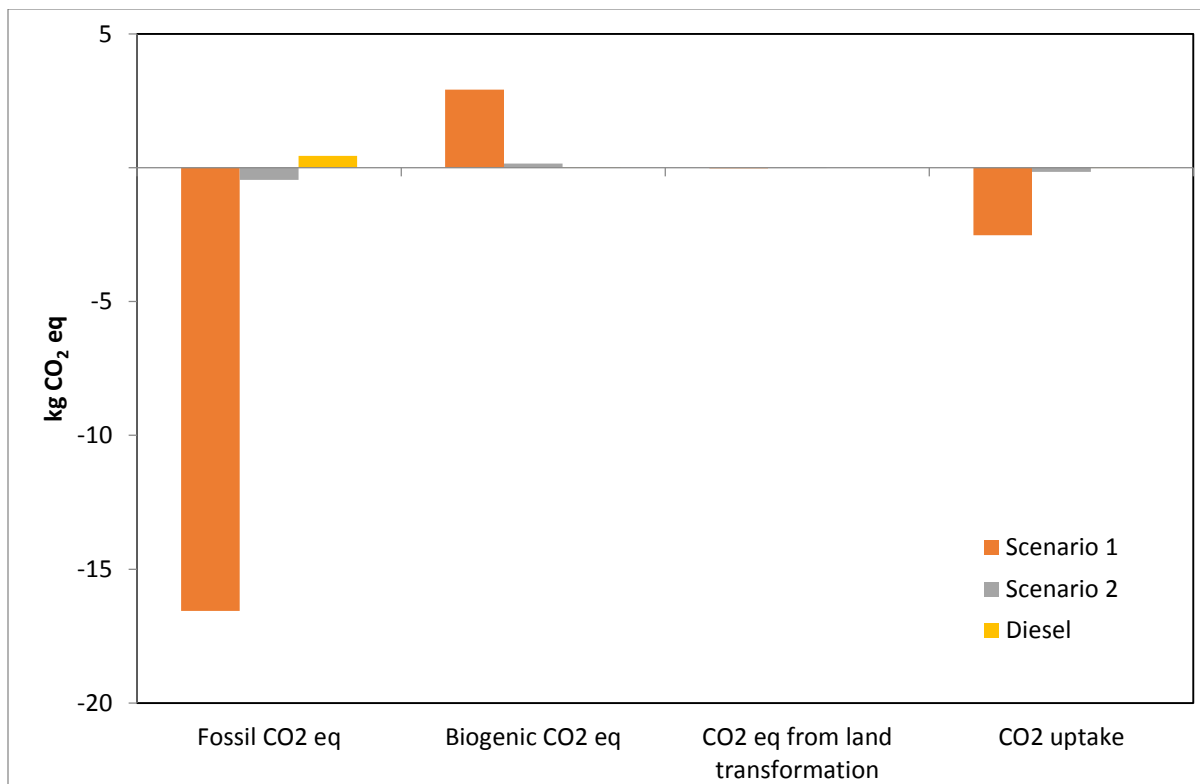


Figure 9.6: Comparison of global warming potential of production of 1 L of limonene for Scenario 1 and 2 and 1 L 'Diesel, at refinery/L/US'. (Method: Greenhouse Gas Protocol V1.01/CO₂ eq).

9.7 Conclusions

This LCA study showed that production of limonene by genetically engineered filamentous cyanobacteria is less energy intensive than both fossil fuel based diesel and 3rd generation biodiesel production. Algal biodiesel production is more energy intensive due to the additional steps of drying biomass, separation of lipids, and conversion of

lipids into biodiesel. Scenario 1 was found to be more environmentally friendly compared to Scenario 2 because Scenario 1 has a higher ratio of biomass to limonene production (produced biomass per 1 L limonene in the system). This biomass can be later converted to biogas and consequently, its energy can be recovered in a gas turbine that produces electricity and steam. This energy can then be substituted for fossil fuel based energy that is required by the system. Thus, produced energy per FU was higher in Scenario 1 than Scenario 2. Also, the surplus electricity and steam produced can be sold to the grid or a neighboring ethanol plant.

Although higher cyanobacterial productivities are more economically favorable for the production of limonene, the results of this study show that higher productivities do not necessarily improve the environmental profile of the process. This occurs because biomass production is substantially more environmental friendly than limonene production. Therefore, by increasing the ratio of limonene to biomass, the overall environmental profile of the system became worse. This study also strongly suggests that a limonene production facility using filamentous cyanobacteria that can use CO₂ emitted from coal-fired power plants and/or ethanol production plants as its carbon source could be a future sustainable solution for producing next-generation biofuels. Finally, this study also shows evidence that as the cyanobacterial productivity of next-generation biofuels, such as limonene are increased, that the result will be a decrease in the environmental profile for the entire process.

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Chapter 10 - Summary and Conclusions

Cyanobacteria are photosynthetic prokaryotes present in diverse habitats, ranging from the tropics to polar regions (Hasunuma et al., 2013; Katoh, 2012; Moreno et al., 1998). Although, they utilize an identical photosynthesis process as higher plants (Lindblad, et al., 2012), cyanobacteria are capable of significantly higher photosynthetic and growth rates (Hasunuma et al., 2013) as they are inherently more efficient solar collectors (Dismukes et al., 2008). Cyanobacteria hold a great deal of potential as industrial microbes due to their potential to be genetically engineered to produce high-value chemicals and next-generation biofuels (Gu et al., 2012; Halfmann et al., 2014a; Halfmann et al., 2014b; Kiyota et al., 2014; Lan and Liao, 2011) from CO₂ and solar energy (Machado and Atsumi, 2012).

The first objective in this project was to develop a method of monitoring growth of filamentous cyanobacteria. Because of their filamentous morphology, standard methods of quantifying viability are not possible. Thus, a dual-fluorescence assay based upon the LIVE/DEAD® BacLight™ Bacterial Viability Kit was evaluated for its ability to quantify the percent viability of filamentous cyanobacteria using a microplate reader in a high-throughput 96-well plate format. It was determined via the microplate reader, as well as confocal and wide-field epi-fluorescence microscopy, that the assay did not work properly with filamentous cyanobacteria. This was due to the non-viable cell indicator, propidium iodide (PI), binding non-specifically to both non-viable and viable cells (Chapter III). While PI did not work as expected with filamentous cyanobacteria, other

fluorochromes were identified that could potentially be used to selectively stain non-viable cells.

In Chapter IV, a dual-stain assay using SYTO[®] 9 and SYTOX[®] Blue was investigated for its ability to accurately quantify viable and non-viable filamentous cyanobacterial cells. A strong correlation again existed between SYTO[®] 9 and viable cells. However, SYTOX[®] Blue did not work as a non-viable cell indicator due to non-specific binding in both viable and non-viable cells, similar to what was observed with PI in Chapter III. Autofluorescence from light harvesting pigments was also evaluated as a viable cell indicator of filamentous cyanobacteria. It was determined that this method was not practical because cyanobacteria pigments have several emission peaks that can't be captured by a single emission filter. Also, some light harvesting pigments continued to fluoresce after the cell became non-viable. SYTO[®] 9 as a viable cell indicator in filamentous cyanobacteria was compared to absorbance and chlorophyll α content in a chemical inhibition testing protocol. At the low cell densities required for chemical inhibition tests, SYTO[®] 9 was superior to absorbance and chlorophyll α content in quantifying viability. It was then concluded that fluorescence from SYTO[®] 9 is an accurate, reliable indicator of viability of filamentous cyanobacteria and can be used in a high-throughput manner via a microplate reader.

Many next-generation biofuels are toxic to cells, thus developing cyanobacteria strains with increased tolerance to these chemicals is essential to achieve commercially feasible productivities and yields. Strain development techniques, such as directed evolution, require exposure of the microorganism to the chemical to develop resistance by selection of tolerant mutants. Since many of these chemicals are highly volatile,

directed evolution trials must take place in a sealed environment to maintain stable chemical titers. In Chapter V, cyanobacterial growth conditions were optimized in a sealed test tube environment using BG11 as the basal medium. Since it was not possible to provide carbon dioxide by sparging, I determined that 0.5 g/L NaHCO₃ was optimal for cultivating cyanobacteria in a sealed environment. Adding NaHCO₃ in a fed-batch mode only marginally improved growth, but increased the risk of contamination and loss of volatile chemicals.

In Chapter VI, a directed evolution study was conducted to increase cyanobacteria tolerance to next-generation biofuels. Directed evolution is a process in which a microbe is grown under a selective pressure that forces rapid evolution in order to tolerate that pressure. This technique has been used to improve production of a range of microbial products (Labrou, 2010). The study herein led to three confirmed cyanobacterial mutants with increased tolerance to specific biofuels: An *Anabaena* 7120 strain with a 220% increase in tolerance to farnesene, as well as an *A. variabilis* ATCC 29413 strain and a *N. punctiforme* ATCC 29133 strain with increased tolerance to linalool (60% and 20% increase in tolerance, respectively). This work served as proof-of-concept that directed evolution is a valid methodology to increase the tolerance of filamentous cyanobacteria to biofuels. These strains could then be genetically engineered to produce the chemicals for which they are tolerant. One might anticipate that the increased tolerance could also lead to increased productivity.

For any large-scale process involving filamentous cyanobacteria to become economically feasible, the cost of the cultivation medium must be minimized. As there was not a clear consensus in the literature regarding the optimal nitrogen source for

cultivating filamentous cyanobacteria, I evaluated various fixed nitrogen sources compared to atmospheric N₂ (Chapter VII). Of the nitrogen sources evaluated, sodium nitrate and ammonium chloride yielded 65% more cyanobacterial growth compared to the other nitrogen sources evaluated. An environmental comparative study was then used on a theoretical large-scale production process to down select the best nitrogen source, which was determined to be ammonium chloride as it had a substantially more favorable environmental impact than sodium nitrate. For example, sodium nitrate had a ~3-fold greater negative impact in human health, ecosystem quality, and resources categories.

In Chapter VIII, the economic feasibility of a theoretical facility that uses genetically engineered *Anabaena* 7120 to produce the cyclic hydrocarbon limonene in 25,162 m² of PBRs was analyzed using the Farm-level Algae Risk Model (FARM). FARM is an integrated systems compilation of numerous technoeconomic models that has been used previously in several algal production scenarios. FARM simulated 10 years of operation for the theoretical production facility. The process consisted of PBRs, gas stripping and limonene recovery units, a solid fraction recovery unit, an anaerobic digestion unit, a wastewater treatment unit, and a gas cleaning unit. CO₂ was directly transferred into the PBRs via internal sparging. Activated carbon (AC) was used for limonene collection in the adsorbing systems. The analysis determined that the average probability of success of the limonene production facility at year 5 using the current productivity rate (0.018 mg/L/h) of a genetically engineered strain of filamentous cyanobacteria was 0%. The average net present value at year 5 of this scenario was -588 M\$. A second scenario was evaluating assuming productivity can be increased 100-fold by future genetic and metabolic modifications. At year 5, this scenario had an average

probability of success of 100% and an average net present value of 392 M\$. This study shows strong evidence that a next-generation biofuel production facility utilizing genetically engineered strains of filamentous cyanobacteria could become economically feasible in the future if productivity can be improved.

In Chapter IX, a life cycle analysis was conducted on the theoretical production facility described in Chapter VIII. Two scenarios for limonene production were evaluated in the LCA. In scenario 1, limonene productivity from the genetically engineered cyanobacteria was 1.8 mg/L/h and in scenario 2, the productivity was 55.5 mg/L/h. This study showed that the production of limonene by genetically engineered filamentous cyanobacteria is less energy intensive than both fossil fuel based diesel and 3rd generation biodiesel production. The major environmental burdens associated with this facility are from the nutrient supply and electricity consumed by the PBRs. Electricity produced from the gas turbine can be used to offset some of the burden. Higher limonene productivities do not improve the environmental profile of the process due to biomass production being more environmentally friendly than limonene production. Of the two scenarios evaluated for limonene production, the environmental load caused by the consumption of activated carbon (AC) and electricity for recovery unit per 1 L of limonene was identical for both scenarios, but the amount allocated to 1 L of limonene was increased ~3-fold in scenario 2. Thus, by increasing the ratio of limonene to biomass, the overall environmental profile of the system became worse. Even considering this, the theoretical limonene production facility described in this project holds great potential as a future sustainable solution for producing next-generation biofuels.

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