DISSERTATION

METABOLIC ENGINEERING OF CYANOBACTERIA: DEVELOPING MOLECULAR TOOLS AND CHARACTERIZING STRAIN PERFORMANCE IN LIGHT:DARK CYCLES

Submitted by

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ABSTRACT

METABOLIC ENGINEERING OF CYANOBACTERIA: DEVELOPING MOLECULAR TOOLS AND CHARACTERIZING STRAIN PERFORMANCE IN LIGHT:DARK CYCLES

The conversion of CO_2 and light energy to biofuels holds promise for a renewable and environmentally responsible source of energy that could meet the growing demand for transportation fuels. However, early efforts to commercialize biofuels from plants were hampered by social, economic, and technological difficulties. Photosynthetic microbes present an opportunity for a more efficient conversion of fixed carbon to biofuels by bypassing the need of harvesting sugars from plants to be fermented by heterotrophic bacteria. More recently, cyanobacterial technologies have received considerable interest due to their ease of genetic manipulation that enables them to produce a myriad of biofuels and biochemicals directly from CO_2. This relatively nascent technology needs to be developed in order to realize its commercial potential.

Metabolic engineering is the systematic improvement of strains through the use of a variety of theoretical and experimental techniques. To date, heterologous pathways expression has been the most successful in model heterotrophic organisms (e.g. *E. coli*) and advances from these systems have to be carefully transferred over to cyanobacteria. Though several studies have demonstrated the capability of engineering cyanobacteria to produce biofuels, there is yet to be any commercially feasible production platform of fuels from CO_2. Amongst the challenges is the need to improve yields and titers from recombinant strains.

However, the physiology of cyanobacteria is distinct from that of heterotrophic organisms and therefore requires careful design and study in order to optimize for higher yields. This thesis contributes several technologies to foster the scale-up of cyanobacteria systems from the bench to industrial scale.

We first developed a markerless chromosomal modification method in WT *Synechocystis* PCC6803 that could reduce the metabolic load and cultivation cost compared to plasmid-based expression methods. We established a counter-selection method that necessitates two rounds of modifications in order to screen for the desired mutant harboring the gene(s) of interest. In the first round, a synthetic circuit consisting of a nickel inducible toxin gene (*mazF*) and a kanamycin resistance marker is integrated into a specific locus in WT *Synechocystis*. In the second round, a construct harboring gene(s) of interest is transformed into the prerequisite strains and screen on Ni^{2+} to obtain the desired mutants.

Next we established a free fatty acid (FFA) producing platform in *Synechocystis* PCC6803 by pursuing three goals: 1) deletion of acyl-acyl carrier protein (acyl-ACP) synthetase (*aas*), 2) optimize the expression of thioesterase I (*TesA*) with a promoter library and 3) examine the effects of light:dark cycles on FFA production in *Synechocystis*. For the first goal, we were successful in engineering an *aas* deletion strain that had increased FFA production. In the second goal, we developed four *Synechocystis* variants with increasing *TesA* expression strengths from the *aas* deletion strain. No increase in FFA production was observed between the *TesA* expressing strains (with *aas* deleted) compared to the baseline *aas* deletion strain. On the protein level, we found no evidence of *TesA* enzyme activity even though TESA peptides were detected in our *Synechocystis* strains. In the third

goal, we learn that diel light:dark cycles causes a significant decrease in production of FFAs in FFA producing mutants of *Synechocystis* compared to continuous light. We did not observe any transcriptional changes in the fatty acid biosynthesis pathway between our WT and FFA producing strains to explain these changes.

In summary, this thesis is impactful in two ways: 1) it entails the development of a markerless genetic modification method for use in cyanobacteria and 2) it characterizes the production of FFAs from engineered cyanobacteria under diel light:dark cycles. Overall, this thesis helps address the difficulties in the development of cyanobacteria systems for eventual use in an industrial setting.

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DEMAND FOR BIOFUELS

Environmental consequence of fossil fuel use

We can hardly gather our material needs without getting in the car, starting the engine, and stepping on the gas pedal. Liquid fuel is flushed into the engine and combusted to yield locomotion, a privilege of this modern era that allows us to get to our favorite destinations. It is affordable and available. In the U.S, there as an average of 1.86 vehicles per household in 2009, and this number has been rising since 1969 (from 1.12) [1]. Although this trend indicates a healthy growth of the transportation sector, the increase in number of vehicles on the road has its environmental repercussions. While producing locomotive energy, the combustion of gasoline and diesel produces CO_2 as a by-product. According to EPA estimates between 1990 and 2013, CO_2 emissions from fuels combusted for transportation have been steadily rising. Of the 1718.4 million metric tons (MMT) of CO_2 equivalent emitted in 2013, ~97% came from the combustion of petroleum [2]. But what is the harm?

Radiative forcing is a term used to describe the effect of gas concentrations on the net radiative flux change at the tropopause (a boundary in the earth's atmosphere) [3]. A positive radiative forcing indicates an increase in the energy of Earth-atmosphere system, thereby leading to the warming of the system. Between 1750 and 2005, increased CO_2 concentration (most likely due to human activities)

caused the largest positive radiative forcing known to date [4]. Given this relationship, it is even more unsettling to know that the current CO_2 level is at an alarming 400 parts per million (ppm) [5] and rising. This is the highest level seen in the past 400,000 years (predicted from Vostok ice cores) [6]. But what constitutes a dangerous CO_2 level? A comprehensive study by Hansen *et al.* [7] discusses this subjective question. Using a coupled atmosphere-ocean model (GISS ModelE), they concluded that a CO_2 level exceeding 450 ppm would be considered "dangerous".

The dangers of global warming are universal. Climate models have correlated the increase in CO_2 levels to rises in global temperature and rising sea levels [8]. A 2007 study by the Intergovernmental Panel on Climate Change (IPCC) presented the likelihood of more frequent hot days and nights as "virtually certain", while increases in heat waves and heat precipitation events as "very likely" [9]. The dire impacts on agriculture, water supply, ecosystem, coastal infrastructure, and human health is further detailed in this report. Another imminent danger is ocean acidification. When the oceans absorbs CO_2 released from the burning of fossil fuels, its pH rises [10]. This has led to adverse consequences on marine phytoplankton [11] and coral reefs [12] which would indirectly effect the fisheries and local economies of many nations [13].

Advent of Biofuels

So what happens to the CO_2 in the environment? Several models have predicted a very low decay rate of CO_2. Most of the CO_2 will remain in the environment "forever" [7, 14]. As global energy consumption and population is expected to rise in the future [15], fossil fuels combustion will likely follow this trend.

Ideally, fossil fuels should be exploited only if its CO_2 emissions are ameliorated. Current technologies provide two possible routes for CO_2 capture and sequestration: 1) long term storage that involves CO_2 capture, compression, and underground storage, and 2) biological capture (via photosynthesis) and conversion to useful products. The biological route is ideal because the biomass accumulated can be turned into useful products such as fuels and chemicals.

The advent of fuels from biomass (i.e. biofuels) holds promise for a renewable fuel with zero or lowered net CO_2 emissions. A life cycle bioenergy assessment by Schmer *et al.* conducted for ethanol production from switchgrass fields illustrates this potential [16]. Their highest yielding farms produced approximately 3000 L/ha of ethanol while completely displacing greenhouse gas emissions (net zero). On the other hand, the potential of photosynthetic microorganisms (algae in particular) is also viable depending on the unit operations. In a life cycle assessment for a microalgae to biodiesel process with filter press dewatering, Sander *et al.* reported a net CO_2 emission of -20.9 kg/functional unit [17]. Although there is yet to be a life cycle assessment on biofuels from cyanobacteria, a favorable analysis is probable because cyanobacteria are similar to microalgae in terms of efficiency of harnessing light energy and CO_2 fixation [18] while also being more genetically amenable.

Another benefit for the development of biofuels is energy security. In current times, the world is facing political turmoil in many oil-producing countries. Hence, the U.S's increased reliance on foreign oil also incurs risk to its political and economic security [19]. The country's transportation sector is particularly vulnerable because the US currently imports more than 50% of its oil. Politically, the trading of oil has benefited dictatorial regimes that could feed into anti-US fundamentalism and

unintended aggression (terrorism) in the future. Fortunately, discussions on the US's energy independence through the development of renewables is on-going, and a growing body of experts are highlighting rural development and income to farmers as benefits of home grown energy [20].

The formation of the National Renewable Energy Laboratory (NREL) was in response to energy crises in the 1970s. The Aquatic Species Program was a small but integral part within their biofuels program with the emphasis of producing hydrogen and biodiesel from algae [21]. The continued rise in crude oil prices and environmental concerns during the 1990s spurred the development of 1st generation renewables. The abundance of corn and the available fermentation technologies gave birth to ethanol as a 1st generation biofuel. However, due to their unintended influence on food prices, 2nd generation biofuels were developed based on non-food crops conversion technologies. Even so, the use of arable land for fuel production triggered the food versus fuel debate. This lead to the birth of 3rd generation biofuels from algae and cyanobacteria. Progress in this field is paving the way for a promising alternative to plant-based biofuels.

REVIEW OF CURRENT BIOFUEL TECHNOLOGIES

Biofuels from plants

As of 2006, ethanol accounts for 99% of US biofuel production with corn being the most common feedstock due to its high starch content [22, 23]. Two processes commonly employed for the production of corn ethanol are 1) dry grinding and 2) wet milling. Dry grinding has a slightly higher ethanol yield compared to wet milling (2.8 gallons of ethanol per bushel of corn versus 2.5 for wet milling) [22]. However, the

wet milling process allows for co-production of a variety of valuable products. A typical dry grinding process involves 5 steps: corn grinding, cooking, liquefaction, saccharification (process that breaks starch into sugars), and fermentation. Similar to dry grinding, the wet milling process separates the corn into its constituents (starch, fiber, gluten, and germ) prior to liquefaction. After saccharification, the fermentation to biofuels employs heterotrophic microorganisms such as *E. coli* and yeast (reviewed in the next section).

Second generation biofuels focuses on non-food crops as feedstocks for cellulosic biofuel production. The economic feasibility of cellulosic biofuels depends on feedstock availability in the given geographic region [24]. Under low CO_2 environments (200-400ppm) and high daytime growing temperatures (>30^{o}C), C4 grasses are most commonly used for biomass generation due to their higher photosynthesis rates compared to C3 grasses [25]. Examples of C4 grasses include maize, sugarcane, sorghum, and switchgrass (corn is also a C4 crop but not considered a grass). These feedstocks are processed to yield lignocellulose, simple sugars, and starch. While sugars and starch serve as immediate substrates for fermentation (reviewed in the next section), lignocellulose (the most abundant of the raw materials) will need to undergo hydrolysis prior to fermentation [26].

Dry lignocellulosic biomass consists of 40-60% cellulose, 20-40% hemicellulose, and 10-25% lignin [27]. The presence of lignin in the biomass hinders the hydrolysis of hemicellulose and cellulose and are usually removed [27] or degraded [28]. There are several physical, chemical, and biological hydrolysis processes that will simplify cellulose and hemicellulose to sugars. Physical methods such as wet oxidation, liquid hot water, CO_2 explosion, and steam explosion yields

high amount of sugar while forming minimal by-products (in-depth review in [29]). Efficient chemical methods include treatment with dilute (1-1.5%) sulfuric acid at high temperatures [30]. Alkaline treatment processes with sodium hydroxide (NaOH) or ammonia are costly but effective at solubilizing lignin while leaving hemicellulose in an insoluble form [27]. Both acid/alkaline methods require neutralization prior to fermentation. Milder biological processes use cellulases and hemicellases synthesized primarily by the fungi *Trichoderma reesei* to yield sugars [31].

Conversion of plant sugars to biofuels

Heterotrophic microorganisms play a significant role in the conversion of plant sugars to biofuels. An important obstacle prior to fermentation is the formation of five carbon sugars (arabinose, galactose, and xylose) that constitute up to 25% of the hydrolyzed cellulosic biomass [32]. In response, extensive metabolic engineering efforts have enabled the utilization of these sugars in *Sacccharomyces* [33] and *E. coli* [34]. Beyond that, the conversion of starch and sugars from plant feedstocks to a variety of products is briefly summarized in the following paragraphs (well reviewed in [35] and [36]).

Plant sugars can be fermented to two main categories of fuels: alcohols and fatty acid-derived fuels. The latter includes a conversion step to biodiesel. Reported maximum theoretical yields (yield calculations summarized in [37]) of select fuel molecules from glucose are summarized in Table 1.

Alcohols: The yeast *Saccharomyces cerevisiae* has been traditionally employed to ferment plant sugars to ethanol with upwards of 90% maximum theoretical yield [38]. Extensive metabolic engineering efforts in *E. coli* have resulted

in a strain with a maximum ethanol titer of 45 g/L [39]. Recently reported ethanol titers in continuous cultures of *Saccharomyces cerevisiae* were as high as 71.3 g/L [40]. Besides ethanol, *E. coli* has been successfully engineered to produce high titers of 1-butanol (1.2 g/L in 60 hrs [41]), isobutanol (~20 g/L in 100 hrs [42]), 1-propanol (3.5 g/L in 72 hrs [43]), and isopentanol (1.5 g/L in ~50 hrs [44]) through the introduction of heterologous pathways. Though *Clostridium acetobutylicum* has been successfully engineered to produce butanol (17.6 g/L [45]) and n-butanol (10 g/L in 360 hrs [46]), plasmid stability in the host poses severe challenges to long term production.

Table 1. Comparison of reported maximum biofuel theoretical yields from glucose (in g fuel per g glucose) and Lower Heating Values (in MJ/kg) to gasoline and diesel.

Fuel molecule	Lower heating value (MJ/kg)[1]	Maximum theoretical yield (g fuel per g glucose)[source]
Ethanol	26.95	0.51 [47]
1-propanol	30.68	0.43 [37]
n-Butanol	34.37	0.41 [46]
Isobutanol	32.96	0.41 [48]
Isopentanol	37.79[2]	0.33 [49]
Palmitic acid (C16:0)	39.26[3]	0.34 [36]
Hydrogen	120.21[4]	0.13 [50]
Gasoline	43.48	-
Diesel	42.79	-

Free fatty acids: Presently, the heterologous production of fatty acid-derived biofuels in *E. coli* stems primarily from free fatty acid (FFA) precursors (see Fig 1 for an overview of the fatty acid biosynthesis pathway is). This approach is based upon the fact that the fatty acid biosynthesis pathway is the rate-limiting step to growth in *E. coli* [51, 52]. The very first experiments that yield FFAs from this pathway dates as

[1] All values obtained from Oak Ridge National Laboratory (ORNL) appendix unless otherwise noted Link

[2] Estimated based on data for pentanol from NIST WebBook. No combustion data available for isopentanol.

[3] Estimated based on data for solid palmitic acid from NIST WebBook.

[4] Theoretical equilibrium yield.

far back as the 90s [53]. Today, two “textbook” approaches are commonly employed to increase FFA production in *E. coli*: i) deletion of genes leading to β-oxidation (a pathway that breaks down fatty acyl-coAs to generate acetyl-coAs), and ii) expressing thioesterases that hydrolyze fatty acyl-ACPs to FFAs. In *E. coli*, the highest total FFA titer achieved to date is ~8.6 g/L in ~60 hrs [54]. So far, the length and composition of FFAs produced is dependent on thioesterase expressed (‘*TesA* [53], *BTE* [55], *CcTE* [56], *RcTE* [57]).

Upgrading FFAs to biofuel: FFAs are biodiesel precursors. They need to be derivitized or esterified (either chemically or biologically) to fatty acid methyl, ethyl, or propyl esters (FAME, FAEE, and FAPE respectively) in order to be used as diesel fuel. Chemical routes to convert FFAs to biodiesel typically uses methanol as the methyl donor and can be catalyzed through an acid or a base reaction. Acid-catalyzed transesterification uses concentrated sulphuric acid (H_2SO_4) and excess methanol (methanol:oil of 20:1 to 245:1) to achieve almost complete conversion of FFAs to FAME [58]. Based-catalyzed transesterification with either potassium hydroxide (KOH) [59] or sodium hydroxide (NaOH) [60] and methanol (methanol:oil of 9:1 or 7:1 respectively) can achieve up to 88% conversion. Biological routes occur at much slower rates but uses comparatively less methanol (~3:1) with immobilized lipases (e.g. *Candida anarctica* lipase) to achieve up to 90% conversion [61]. Besides biodiesel, FFAs can be catalytically decarboxylated/decarbonylated to alkanes through the use of Palladium on carbon (Pd/C) catalyst at high temperatures [62]. Recently Lennen *et al*. demonstrated a continuous process for the conversion of FFAs from *E. coli* to decane [63].

The deployment of 1st and 2nd generation biofuels is hampered by social and economic complications. One of the severe consequence of increased plant-based biofuels is it poses competition for arable land for agriculture that is intended to provide food and feed for a growing world population. A farmer's dilemma also spans from the need to feed a growing population versus attaining more profit per acre of land [64]. Other sources also indicate that biofuel production from food crops would lead to increases in food prices and reduction in availability of calories [65]. In terms of process economics, vigorous pretreatment methods (i.e. hydrolysis to sugars) narrow the margin of profitability. Even so, the volatile prices of sugar has been known to influence the process economics of biofuel production (ethanol [66]). Methods to improve feedstock yields are limited and require breeding or genetic engineering. The former is utilized more frequently while the latter is discouraged due to its low public acceptance [24]. Thermochemical process (such as pyrolysis and gasification) to convert lignin to useful chemicals may further improve process economics [67].

Biofuels from algae (microalgae)

So far, it is obvious that the conversion of CO_2 to fuels via plant feedstocks involves several intermittent steps. Despite the remarkable yields and titers of fuel from heterotrophic microorganisms, the production of biofuels in these systems is inefficient at several levels. The first being the use of substrates, namely the sugar sources (i.e. glucose and starch) which have to be extracted from plant feedstocks. The extraction process is costly and produces waste. Secondly, once the substrates have been fermented, only a fraction of the substrates are converted to the product

of interest (see Table 1.), thereby incurring more waste. The numerous steps in conversion are costly and incur substantial waste, a factor that hampers the large-scale production of biofuels [68]. The development of technologies that minimizes the number of conversion steps would hence improve its economic feasibility.

The advent of algae biofuels (i.e. 3rd generation) alleviates economic inefficiencies in biofuel production by removing the influence of sugar from the process economics. Algae's major advantage is their ability to accumulate lipids (up to 90% dry weight) in the form of triacylglycerol (TAG) [69]; a biodiesel precursor. In terms of unit operations, algae biofuels consolidates the CO_2 harvesting and fuel production step, thereby bypassing several intermittent steps when compared to crop-based biofuels (i.e. pre-treatment, lignocellulose hydrolysis, and fermentation). Other advantages of algae over crops include year round production on non-arable land, higher biomass yields [70], and the ability to yield valuable co-products (i.e. recombinant proteins and nutritional supplements) [71]. Even so, several challenges in large-scale conversion of algae to biofuels remains prevalent: 1) cultivation and recovery of biomass, 2) nitrogen removal and induction of TAG synthesis and 3) efficient conversion of biomass to desirable products.

Open ponds are the cost viable option for large-scale cultivation of algae biomass. However, they produce exceedingly low biomass yield [72] and is frequently plagued by virus, bacteria, and zooplankton contamination [73]. Cultivation in photobioreactors offers higher biomass yields and more controlled environment at increased cost. Despite their variable investments, the cost of open ponds and photobioreactors will decrease with scale-up due to the economy of scale [74]. Besides cultivation, the recovery of biomass constitutes a large portion of the total

production cost [75]. Flocculation and filtration methods are needed to concentrate the algae biomass to a slurry prior to drying and recovery of metabolites [76]. Centrifugation can also be employed for biomass with high-value products.

Secondly, the large-scale induction of lipid accumulation in algae is no trivial task. It is well known that algae accumulates TAGs during nitrogen stress/depletion [21]. In a chemostat, algae TAG production is induced by ammonia (NH_3) stripping/volatilization [77] based on the following chemical reaction: $NH_4^+ + OH^- \rightleftharpoons NH_3\ (g) + H_2O$. By adjusting for NH_4^+ utilization and maintaining the pH of the culture broth above 7, the equilibrium can be shifted to the right (thereby volatilizing NH_3) by bubbling air [78]. However, nitrogen stress/depletion also decreases growth rate [79], and therefore the lipid content of the culture has to be carefully monitored to determine the best interval for harvesting [77]. In scaling up, monitoring the lipid content may pose a challenge in both open ponds and photobioreactor systems; likely more so in open ponds.

The ideal biomass conversion process would separate the algae biomass into its constituents (lipid, protein, and sugar) and process them to valuable products. Abundant conversion options exist. The lipid portion (TAGs) would undergo transesterification to biodiesel (methods compared in [80]) while the protein portion can be converted to animal feed and biofertilizers [81]. The sugars and starch portions can be fermented to a variety of fuel molecules.To further maximize profitability, the residual algal biomass (consisting of complex carbohydrates and cell wall material) can be hydrolyzed into fermentable sugars [82]. Alternatively, thermochemical conversion options exist whereby the biomass would be gasified (via fast pyrolysis) to yield bio-oil [83].

Algal feedstock improvement is possible through metabolic engineering. Although efforts to increase TAG production in *Chlamydomonas reinhardtii* via genetic engineering has had mixed results, the metabolic engineering of algae to produce therapeutic proteins such as vaccines and immunotoxins has seen more success [84]. From a genetic engineering standpoint, organelle compartmentalization in algae allows for several ways of genetically modifying the organism i.e. chloroplast, nuclear, endoplasmic reticulum, or mitochondrial engineering [85]. Chloroplast engineering has been the preferred method (at least in *Chlamydomonas reinhardtii*) due to the relative ease of transformation and the ability to accumulate high amounts of recombinant protein [84]. Nuclear engineering has been difficult because it generally results in random integration of genes [86], rapid gene silencing [87], and low amounts of recombinant protein [84].

Cyanobacteria: the algae alternative

Due to their relative ease of genetic amenability, the development of cyanobacterial technologies has garnered tremendous progress. Between 2009 and 2012, the increase in publications related to cyanobacterial biofuel technologies has been rapid [88]. To date (2015), several cyanobacteria strains have been engineered for production of a variety of fuel molecules and chemicals (comprehensively listed in [89]). Part of the reason is that many strategies employed in the engineering heterotrophic organisms (i.e. *E. coli*) are highly transferable to cyanobacteria. For example, the production of three and four carbon alcohols (e.g. isopropanol, 1-butanol, 2,3-butanediol, isobutanol) have been engineered in *E. coli* the prior to 2010

[35], and by the present time (2015) the production of these molecules have been largely successful in cyanobacteria [90-93].

Additionally, cyanobacteria technologies have several improvements over algal systems. While the conversion technologies of algae biomass to products can be used in a similar manner for cyanobacterial biomass, cyanobacteria cultures do not need to be stressed or induced to produce the desired product (unless the desired product is polyhydroxybutyrate (PHB)). Secondly, a unique feature of cyanobacterial production systems is that most fuel molecules that they produce are readily excreted. This directly benefits product recovery on an industrial scale as methods to separate fuel from media (e.g. adsorption, liquid-liquid extraction and gas stripping [94]), and its unit operations [95] are well established. Thirdly, the genetic amenability of cyanobacteria provides the flexibility of producing a variety of fuel molecules (versus being limited to biodiesel in algae) to accommodate the demands of other fuel combustion systems such as aviation fuel.

So why aren't there commercial biofuels?

While the prospects of cyanobacterial production systems seem optimistic, the deployment of biofuels is still not cost competitive compared to crude oil. Historically, the price of crude oil has been volatile as its supply is mainly derived from politically unstable regions. From the past, unstable crude oil prices has favored the development of renewables [21]. In the present, energy security correlates to national security [96]. For the near future, stable prices of transportation fuels can be maintained either by continued US presence in the Middle East, or a leap forward in efficiency of producing renewable fuels. The latter has to be the better plan.

A recent study shows that the productivity of a current promising cyanobacterial system (specifically Atsumi *et al.*'s system [90]) exceeds current yields of 1st, 2nd, and 3rd generation biofuels on a per hectare basis [97]. However, the increased capital cost to house these systems (closed photobioreactors) still hampers its economic feasibility. To address this limitation, the development of cost-effective photobioreactors and even higher yielding cyanobacterial strains has to be pursued. In literature, some promising production strains from cyanobacteria include ethanol (5.5g/L in 25 days [98]), 2,3-butanediol (2.38 g/L in 18 days [91]), isobutyraldehyde (1.10 g/L in 6 days [90]), and FFAs (~50mg/L in 17 days [99]). To date, there is yet to be any published literature on the life cycle assessment of biofuels from cyanobacteria. However, the economic feasibility of cyanobacterial systems is very heavily dependent on the microbe fuel yields from CO_2 (personal communication with Jennifer Markham, process engineer at NREL, ACS 2015 [100]). For the scope of my thesis, I will focus on the optimization of cyanobacteria strains to improve yields through metabolic engineering.

METABOLIC ENGINEERING – OVERVIEW AND COMMERCIAL SUCCESS

"Metabolic engineering is the directed improvement of cellular properties through the modification of specific biochemical reactions or the introduction of new ones, with the use of recombinant DNA technology."

- Gregory Stephanopoulos [101]

In the present, metabolic engineering has grown to encompass cross-field associations. Common associations include (but not limited to) "feedstock upgrading", "synthetic biology", "systems engineering", "omics", "cell physiology", "high-throughput sequencing", "metabolic flux-analysis", "flux balance analysis",

"expression and deletion libraries", and "genetic engineering". Principally, metabolic engineering always involves 2 practices: 1) incur changes and 2) detect changes (personal communication with Dr. Reardon sometime in 2011). For this thesis, metabolic engineering is interpreted as a field that applies numerous technologies to study, develop, and ultimately repurpose living systems for novel applications.

There are several accounts of commercial successes of metabolic engineering. In recent times, DuPont and the British firm Tate and Lyle formed a venture to produce biologically derived 1,3-propanediol (Bio-PDO^{TM}), a molecule typically produced from petroleum sources. They developed a system that converts corn sugar into Bio-PDO^{TM} by using engineered *E. coli*. Bio-PDO^{TM} is now used to make Sorona®, a fiber and fabric mimic that can be used to make apparel and carpet [102]. Besides commodity chemicals, the development of the precursor to the anti-malarial drug – artemisinic acid, has been pioneered in a collaboration between Jay Keasling's lab at University of California, Berkeley and Amyris Biotechnologies. The pathway for the production in artemisinic acid in *A. annua* plant was successfully transferred to yeast, and hence enabled mass production from sugars [103].

Challenges in engineering cyanobacteria

Overview of FFA production in *E. coli* and cyanobacteria

While the metabolic engineering of heterotrophic systems has enjoyed commercial success, the metabolic engineering of cyanobacterial systems is nascent and has to be optimized in order to realize its commercial potential. In metabolic engineering research, *E. coli* has primarily been the organism of choice. This is true especially in engineering of the fatty acid biosynthesis pathway to produce biofuels and biochemicals. The knowledge gathered and strategies employed in *E. coli* can

be harnessed and expedited to other organisms including cyanobacteria. The ensuing sections provide an overview of the fatty acid biosynthesis pathway and current efforts to convey the lessons learned in *E. coli* to engineering cyanobacterial systems.

Background of FA biosynthesis pathway

The FA biosynthesis pathway is a crucial pathway in *Synechocystis* (and prokaryotes in general) because it functions as the supplier of FAs that are building blocks for phospholipids. FA biosynthesis is one of the major anabolic pathways that use acetyl-CoA (a 2-carbon molecule, 2C) as a substrate. The reactions in FA synthesis are summarized in Fig 1. The first committed step in FA synthesis occurs with an ATP-dependent reaction where acetyl-CoA is carboxylated to malonyl-CoA (3C). Malonyl-CoA is then acylated to malonyl-acyl carrier protein (malonyl-ACP)

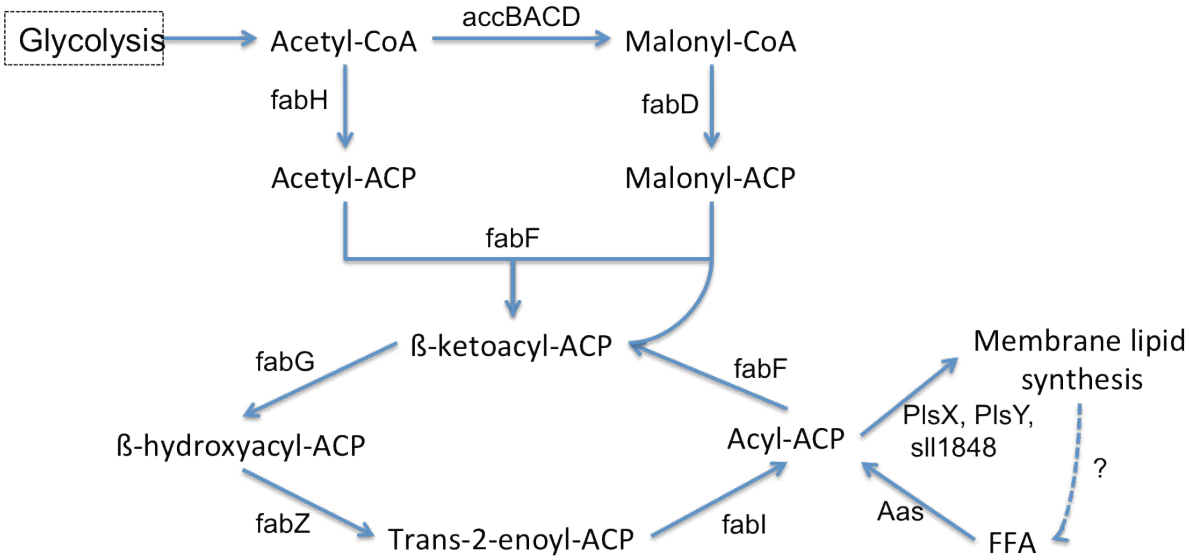

Fig 1. A simplified metabolic pathway of Fatty Acid Synthesis in *Synechocystis* PCC 6803. Blue solid arrows represent known reactions and dashed arrows represent unknown reactions. Text above or below the blue solid arrows represent known genes that code for enzymes that catalyze the reaction.

before entering the FA elongation cycle. Malonyl-ACP and a molecule of acetyl-ACP combine in a condensation reaction to form acetoacetyl-ACP (4C, represented by ß-ketoacyl-ACP in Fig 1).

Acetoacetyl-ACP then undergoes a reduction step to reduce its C3 carbonyl group to yield 3-hydroxybutanoyl-ACP (4C) followed by a dehydration on the now C3 hydroxy group to yield but-2-enoyl-ACP (4C). The C=C in but-2-enoyl-ACP is reduced to yield a saturated FA chain butyryl-ACP (4C). For further elongation, malonyl-ACP undergoes a condensation reaction with butyryl-ACP (instead of acetyl-ACP) to form 3-oxohexanoyl-ACP (6C) and goes through the same cycle of reactions to yield a saturated straight chain hexanoyl-ACP (6C). In *Synechocystis*, this elongation cycle repeats until the products consist primarily of palmitoyl-ACP (16C) and stearoyl-ACP (18C), which makes up the main constituents of their total FA composition. All varying FA chains bound to ACP are grouped into the term acyl-ACP.

FFA production in E. coli

The FA biosynthesis pathway is a promising route to produce FA derived fuels and chemicals such as fatty alcohols, alkanes/alkenes, and free fatty acids (FFA). FFA in particular, are precursors to the synthesis of fatty acid esters (FAME or FAEE), which could serve as biosynthetic replacement to diesel [104]. In the oleochemical industry, FFA can be used to produce antibacterial agents [105], polymer surface coatings [106], soaps [107], and surfactants [108]. Taken as a whole, the vast application of FFAs makes it attractive for industrial-scale production and a suitable precursor for the next generation of renewable fuels and chemicals.

There are several reactions that are known to use acyl-ACP as substrates. Thioesterases are enzymes that cleave the ACP moiety from acyl-ACP chains to yield FFAs. In *E. coli*, FFAs and exogenous fatty acids can be imported and activated to acyl-CoA (by *fadD*) to be used in β-oxidation (a pathway that breaks down acyl-coA to acetyl-coA) [109]. To produce excess FFAs in *E. coli*, two common strategies are employed [110]: 1) deleting genes that lead FFAs to β-oxidation, and 2) expressing thioesterases that hydrolyze acyl-ACPs to FFAs. Pertaining to 2), the length of the FFA chain depends on the thioesterase length preference for acyl-ACP.

The overexpression of thioesterase I (*TesA*) in *E. coli* has been most commonly pursued. *TesA* has a wide substrate preference for acyl-ACPs: C12:0, C14:0, C14:1, C16:0, C16:1, C18:0, and C18:1 [53, 110]. The N-terminal of *TesA* collocates the protein into the periplasmic space of cell membranes. By truncating the N-terminal (to yield *'TesA*), the protein is localized to the cytosol [53]. This is essential because the synthesis of acyl-ACP occurs in the cytosol and hence the presence of *TesA* in the cytosol would allow for the effective hydrolysis to FFA. In *E. coli*, the simultaneous deletion of *fabD* and overexpression of '*TesA* has been shown to increase the production of FFA [56, 110, 111]. The expression of plant thioesterase has also been successful in *E. coli*. When a plant C12:0 thioesterase (*BTE*) was expressed in a *fadD* knockout strain of *E. coli*, the resulting strain produced C12 and C14 saturated and unsaturated FFAs [55, 63]. Recently, the characterization of a library of plant acyl-ACP thioesterases in *E. coli* has been established [112]. In 2008, Liu *et al.* investigated the use of *E. coli* as a platform for production of FFAs [56]. The group's subsequent publication further improved the

production of FFA in a fed-batch fermentor to a titer of ~1g/L of total FFAs [111]. So far, the highest total FFA titer achieved in *E. coli* to date is ~8.6 g/L in ~60 hrs [54].

FFA production in cyanobacteria

In recent times, the advances of FFA production in *E. coli* have been adapted to cyanobacteria. In 2010, researchers at Arizona State University successfully engineered a glucose tolerant (GT) variant of *Synechocystis* PCC 6803 for the production of FFAs. Their work was subsequently retracted and republished in 2011 [113]. By expressing various plant thioesterases in GT *Synechocystis*, *Liu et al.* demonstrated that not all plant thioesterases worked as they would in *E. coli* [113]. Expression of a C8:0 and C10:0 thioesterase (*Ch FabtB2*) and a C12:0 thioesterase (*Uc FatB1*) lead to no significant improvement of FFA production in GT *Synechocystis*. On the other hand, expression of a C14:0 thioesterase (*Cc FatB1*) lead to a slight increase in secreted FFA. Surprisingly, the biggest contribution to FFA production and secretion came from the expression of '*TesA* from *E. coli*. This modification altered FA composition of the culture and resulted in the highest increase in total secreted FFA [113].

So far, Liu *et al.*'s work focuses on the effects of pathway perturbation around the key pathway intermediate acyl-ACP. In *E. coli*, the accumulation of acyl-ACP induces a negative feedback that down regulates the activity of several enzymes (*fabI*, *fabH*, and *ACC*) in the FA synthesis pathway [114-116]. Although the actual mechanism of regulation is unclear, acyl-ACP accumulation is known to have adverse effects on the FA synthesis pathway [114]. By hydrolyzing acyl-ACP to FFA, the size of the acyl-ACP pool would be reduced and therefore less of this inhibition would be expected. In *Synechocystis*, acyl-ACPs are synthesized in two ways: 1)

through the FA synthesis pathway and 2) through the recycling of FFAs released from membrane lipids (β-oxidation is not known in *Synechocystis*). The enzyme acyl-ACP synthetase (*Aas*) has been found to be responsible for recycling FAs released from membrane lipids. In 2010, *Kaczmarzyk et al.* tied the deletion of *Aas* to secretion of FFA and the inability of the cells to utilize exogenous FFA [117]. The simultaneous expression of '*TesA* and deletion of *Aas* would reduce acyl-ACP pool size and thus relieve the feedback inhibition of acyl-ACP on FA biosynthesis.

The role of genetic tools in improving yields

Liu *et al.*'s work illustrates one of the early developments of fatty acid-derived fuels and chemicals from cyanobacteria. Other work has since followed suit [99, 118]. Though promising, further development is essential in realizing the commercial potential of cyanobacterial systems. In general, recombinant systems always require optimization to fit their repurposed role. Generic metabolic engineering strategies including expressing novel pathways, deleting competing reactions, and overexpressing endogenous pathways, all of which occurs at the DNA level, has had success, though limited. In many cases, the more-is-better mentality (i.e. using the strongest promoters and highest copy number plasmids) struggles to achieve improvements in yield and titers. Major consequences of this strategy include 1) the buildup of toxic intermediates and 2) depravation of metabolites that are essential for the organism's growth [119].

To improve yields, pathways and metabolic balance of the system has to be carefully optimized. For pathway optimization, the interplay of several strategies is prevalent (reviewed in [120]): varying DNA copy number and promoters [63, 119],

optimizing ribosome binding sites [121], regulate enzyme turnover rate [122], regulate enzyme production ([123]), and post-translational balancing [124, 125]. Metabolic load is defined as the portion of the cell's resources (such as ATP, nucleotides, and cellular metabolites) directed toward to maintenance of newly introduced recombinant enzymes or pathways [126]. Plasmid maintenance is an example of metabolic load, where increasing plasmid size and copy number has been known to effect cellular growth and respiration rates in *E. coli* [127, 128]. In terms of metabolic balancing, reducing metabolic load (from plasmid replication and maintenance) and trimming cultivation cost (by having no antibiotic selection) can be attained by integration of DNA modifications into the chromosomal DNA [120]. The fine-tuning of expression of heterologous pathways (at the DNA, RNA, protein, and post-translational level) and reducing metabolic relies heavily on the use of genetic tools. Hence, the use of genetic tools is quintessential in optimizing recombinant systems. The following section will briefly summarize some of the genetic tools developed in cyanobacteria as well as several optimization strategies in *E. coli* that have been (and could be) conveyed to cyanobacteria systems.

DNA integration methods

It is unlikely that one strain of cyanobacteria strain can fulfill the function of producing diverse fuels across all geographic areas. Parameters such as the availability of PAR light, temperature fluctuations, types of water sources, and tolerance to toxicity will determine which strains are more suitable to produce a desired fuel or be employed at a desired location. Hence, the development of cyanobacterial tools plays a key role in enabling the metabolic engineering of various cyanobacterial strains. Fortunately, most cyanobacteria are easily transformable via

DNA integration into the chromosome or through the use of replicating plasmids (reviewed in [129]). Chromosomal DNA insertion is the more common method in cyanobacteria. The strategy involves tagging a gene of interest with an antibiotic resistance marker and introducing them into the chromosomal DNA via homologous recombination. As for plasmids, there are several broad host range plasmids that can be used in cyanobacteria: pDU1 [130], pPMQAK1 [131], pTCP2031V [132], RSF1010 [133], and pFC1 [134]. Both methods use antibiotics to screen for desired mutants. With this strategy, the number of available resistance markers limits subsequent modifications.

Markerless genetic modification

The ability to incur multiple genetic changes in cyanobacteria is essential in realizing the full potential of metabolic engineering. In GT *Synechocystis*, markerless genetic manipulation methods have been traditionally used for multiple rounds of genetic changes [135]. This system uses *sacB* which is an enzyme that converts sucrose to levans (a toxin which is lethal to the cell [136]) to screen for mutants that have taken up gene(s) of interest. However, this system is limited to cyanobacteria that are sucrose tolerant. In response, our lab pioneered the development of a novel counter selection method that does not rely on the ability to grow on sucrose [137] (manuscript in chapter 2). Other markerless methods have since been developed for *Synechococcus PCC 7002* [138], and *Synechococcus PCC 7942* [139].

Promoter characterization

Promoters can be used to fine-tune metabolism for optimal production of desired products or for developing novel tools for specialized and broad host range applications (e.g. markerless genetic modifications [137], enzyme turnover rate

control [122]). So far, most promoters have been built and tested for use in *E. coli* and has shown poor performances when used in cyanobacteria [131]. This could be attributed to differences in intracellular environment and regulation between the two systems. Cyanobacterial promoters are distinct from that of *E. coli* and are commonly found in three groups: 1) metal inducible, 2) light inducible and 3) constitutive (comprehensively list in [129]). Amongst them, the most responsive are metal inducible promoters P_{isiAB} (repressed 6000-fold GFP fluorescence by 30 μM EDTA [140]) and P_{nrsB} (induced 800-fold transcript abundance by 0.5 μM $NiCl_2$ [141]). The discovery of a super strong constitutive promoter (P_{cpc560}) in *Synechocystis* enables the production of up to 15% of total soluble protein from heterologous genes [142]. Alternatively, a weak constitutive promoter is *rnpB* [131]. A commonly used light inducible promoter is P_{psbAII} [143, 144]. Recently, a library of wide range inducible promoters (both light and dark inducible) was developed in *Synechocystis* [145].

Gene expression optimization

Optimizing the expression of known rate-limiting enzymes involved in the production of desired molecules has been shown to be effective. In *E. coli*, the production of polyphosphate and isopentyl diphosphate were increased by varying the copy number of plasmids harboring genes of rate limiting enzymes (*ppk* and *dxs* respectively) [146]. Using the same strategy, the optimized expression of thioesterases *BTE* [63] and *'TesA* [111] lead to increased FFA production in *E. coli*. The same strategy has been applied to cyanobacteria. Gao *et al.* optimized ethanol production in *Synechocystis* by expressing two copies of pyruvate decarboxylase

(*pdc*) [98]. Our lab has attempted to optimize *'TesA*^{CO} expression in *Synechocystis* via promoter libraries. Data will be discussed in chapter 3.

Module-based optimization

The culmination of the above mention genetic tools has allowed for Multivariable Modular Metabolic Engineering (MMME). In *E. coli*, this strategy involves grouping several interconnected pathways into "modules" and balancing each module to attain the maximum amount of target molecule of interest. In *Xu et al*., three modules consisting of glycolysis, acetyl-CoA activation, and fatty acid biosynthesis were modularly balanced (by mixing combinations of high, medium, and low copy number plasmid for each "module") to achieve maximum FFA production [54]. The same approach has been used to maximize taxol precursor [147] and isoprenoid production [119] in *E. coli*. The key successes of these approaches rely heavily on established expression technologies (i.e. promoters, plasmids, and codon optimization etc.) and the rapid generation of strains to cover a large solution spaces (32 strains generated and tested in [147], 24+ strains in [54]). Hence, the development of genetic tools and cloning methods in cyanobacteria will enable the use of MME to optimize production in cyanobacterial systems.

Two other genetic tools (i.e. RBS optimization and degradation tags) that show promise for optimization of cyanobacterial systems are discussed below:

Ribosome Binding Site (RBS) optimization

RBSs encoded in promoters, are sites of ribosome binding to mRNA. In prokaryotes, they are known as the shine dalgarno sequence. The effectiveness of ribosome binding to mRNA depends on the base-pairing potential with the anti-shine dalgarno sequence and the distance from the start codon [148]. Algorithms to

optimize RBS via thermodynamic models exist [121] which can generate several candidate RBS sequence that can be used to balance translation and indirectly, improve enzyme stability. Recently, RBS optimization of a 5-enzyme heterologous pathway in *E. coli* maximized NADPH regeneration rates and lead to increased terpenoid production [149]. For cyanobacteria, RBS optimization of a 3-enzyme heterologous pathway in *Synechococcus* PCC7942 lead to increased 2,3-butandiol production [150].

Degradation tags

Degradation tags can be used to control protein turnover rates and eliminate misfolded proteins. In addition, their potential can be extended to regulate the turnover rate of endogenous proteins to change the flux of metabolic reactions. *Synechocystis* has several endogenous degradation tags encoded by the peptide sequences ASV, AAV, and LVA. These tags have been shown to lead to the modular degradation of YFP (with LVA being the most efficient [131]). In *E. coli*, ssrA tags are peptide sequences (AANDENYALAA) added to the C-terminus of unfinished or misfolded proteins to be degraded by the proteases encoded by *clpX* [151]. Recently, a library of modified ssrA tags has been developed for *Synechocystis* [122]. The use of these degradation tags to improve production in engineered strains of *E. coli* or cyanobacteria has yet to be explored.

Understanding cyanobacteria growth physiology

Cyanobacteria are inhabitants of virtually any environment on the globe, even those that are strenuous. Freshwater cyanobacteria are commonly known to form blooms when nutrients conditions are suitable (low N to P ratios (<29) [152]) and

revert to their dormant state once the conditions change. In the Antarctic cold desert, a dry valley with no visible life forms, some cyanobacteria are known to survive in between the crystals of porous rocks [153]. Nonetheless, it is clear that their metabolism is highly flexible and adaptable to nutrient and light availability. Commercial use of cyanobacteria entails exposing them to forces out in the open (e.g. light:dark cycles). Understanding their physiology on the bench scale will help expedite their scale-up and reveal potentials that could be harnessed for metabolic engineering applications.

Light availability

Cyanobacteria exhibit circadian rhythms to allow for maximal productivity in daily sunlight fluctuations [154-156]. Diurnal changes in transcription, primarily studied via microarrays, suggest that mRNA expression patterns change drastically throughout the day and are highly affected by light:dark cycles [157-160]. In addition, several promoters in cyanobacteria are known to be light responsive (e.g. the high light induced *psb* [144] and the dark induced *lrtA* [161]) . Prominent changes on the metabolite level are also prevalent under diurnal cycles. For example, glycogen has been known to play a key role in energy storage: the glycogen stores are replete in the day and deplete in the night [162, 163].

Exposure to excess light can cause photoinhibition (defined as the decrease in the maximum efficiency and/or rate of photosynthesis [164]) and results in reduction in overall rates of carbon fixation and the ability to convert light energy into chemical energy [165]. On the contrary, the absence of light creates an anoxic environment (i.e. environment with low O_2 concentrations) that would be suitable for oxygen sensitive enzymes (an hence pathways). This would allow for production of

molecules during the dark periods (e.g. 1-butanol [166]). Therefore, light availability has to be considered in the design and characterization of recombinant cyanobacteria strains.

Nutrient deprivation

Cyanobacteria have evolved to survive under several stressed conditions such as nitrogen, iron, sulfur, and phosphorus depravation. In general, nutrient depravation causes several dramatic changes in cellular structure including the degradation of phycobilisomes to minimize absorption of excess excitation energy and the cessation of cell division [167]. Visually, cultures of cyanobacteria turns from green to pale white in a phenomena known as chlorosis [168]. In the case of nitrogen depravation, stress regulators (*ntcA* [169]) and response regulators (*sigE* [170] and *rre37* [171]) plays a significant role in up-regulating glycogen and sugar catabolism, leading to the production of PHB. The identification of these regulators in nutrient depravation studies can be beneficial for metabolic engineering. For instance, these regulators can be used to increase production of desired molecules in engineered strains (overexpression libraries discussed in chapter 4). Moreover, this potential can be expanded upon as similar responses are likely occurring during iron [172], sulfur [173], and phosphorus [174] depravation.

SIGNIFICANCE OF THESIS

Development of a markerless genetic modification method for cyanobacteria.

Our motivation for this work was to develop a markerless genetic modification method to be universally used in organisms that can undergo homologous recombination (particularly cyanobacteria and algae). To do this, we derived the idea

from a previous method that uses a toxin gene (*sacB*) to screen for mutants that have taken up the gene of interest [135]. Unfortunately, the use of *sacB* is limited to sucrose tolerant WT *Synechocystis*. In our work, we developed a system to effectively mimic *sacB* through the use of an *E. coli* toxin gene, *mazF*, under the control of a nickel-inducible promoter. Chapter 2 will discuss the development of this technology in greater detail. This study has been turned into a publication in 2012 [137]. We have since adopted this method as a "standard" protocol in our lab and use it to generate mutants of *Synechocystis*.

Optimizing gene expression of *'TesA* to increase production of FFA in *Synechocystis*.

TesA codes for thioesterase I from *E. coli*. This enzyme is localized in the periplasm and cleaves the ACP moiety of long chain acyl-ACP to yield FFAs. By truncating its leader sequence (yielding *'TesA*), the enzyme remains in the cytoplasm and has been shown to cause increased FFA production in *E. coli* [53]. In 2011, the expression of a codon-optimized version of this gene in *Synechocystis* (*'TesA*^{CO}) yielded the same effect [113]. Hence, we set out to see if we could optimize the expression of another *'TesA*^{CO} gene in *Synechocystis* through the use of the P_{psbAII} promoter suite developed in our lab. We learn that the promoter suite was successful at increasing mRNA abundance of *'TesA*^{CO}. Unfortunately, *'TesA*^{CO} did not translate to a functional protein as we saw no evidence of enzyme activity and little to no increase in FFA production compared to a baseline knockout strain (Δ*aas*). Though TESA peptides were detected, the abundance was too low to quantify. We

hypothesize that the protein is likely misfolding in *Synechocystis*. Further details of this work are discussed in chapter 3.

Understanding the effects of diel light:dark cycles on FFA production

While characterizing the effects of *'TesA*^{CO} in *Synechocystis*, we also worked on understanding the effects of 12 hr light:dark cycles on the growth and production of FFAs in our engineered strains. This work is intended to begin to replicate industrial conditions inside the lab in order to better understand the performance of cyanobacterial systems out in the open. This work was also motivated by the fact that light availability influences production; as cell density increases, light availability per cell decrease. Hence, we characterized their growth and production across 6 days to monitor their long-term performances. Our results indicate significant cessation of FFA production in our FFA producing strains under 12 hr light:dark cycles compared to continuous light. Transcriptionally, the fatty acid biosynthesis pathway showed no significant changes between the WT and a FFA producing mutant. We hypothesize that differences in cellular metabolism in the light phase versus the dark phase caused the decrease in FFA production.

Generation of an overexpression library in *Synechocystis*

Overexpressing upstream pathways can lead to improvements in production of desired downstream molecules [54]. In 2013, 4 gene targets (2 in FA synthesis and 2 in sugar catabolism) were proposed for overexpression in a FFA producing mutant of *Synechocystis*. The constructs designed to overexpress these targets was planned for the same loci in *Synechocystis* under the control of an endogenous Ni^{2+} inducible promoter. Since plasmid construction was methodical, Dr. Peebles

challenged me to come up with more potential targets. By the present day (2015), the library has been expanded to a total of 14 gene targets (comprehensively list and reviewed in Chapter 4). Of the 14 targets, 8 of them have been successfully transformed into a FFA producing strain of *Synechocystis* (strain GG1.3, Δ*Aas* with *'TesA*^{CO} under the control of $P_{psbAII\ \Delta hex}$). Upon discovery that our *'TesA*^{CO} expressing strains of *Synechocystis* had no enzyme activity, we halted the characterization of these strains and focused on creating a new metabolic sink. Chapter 4 discusses several candidates for a new metabolic sink in *Synechocystis*.

This work contributes to the field of metabolic engineering of cyanobacteria in two aspects: 1) development of novel tools and 2) the characterization of FFA production from cyanobacteria under light:dark cycles. The efforts in optimizing gene expression via promoter libraries to increase production in cyanobacteria are also described. The initiation of a gene overexpression library for *Synechocystis* that would be beneficial for increasing productivity of downstream products is highlighted.

SUMMARY

Since the energy crisis in the 1970s, the development of biofuel technologies has gained tremendous leaps in efficiencies [97]. Depending on geographic availability of substrates, light, land, and water, the efficient and sustainable production of biofuels in the near future would be derived from a mixture of 1st through 3rd generation biofuel technologies. Metabolic engineering has and will continue to play a substantial role in improving the efficiencies of all these systems. This thesis contributes to the metabolic engineering of cyanobacteria by developing genetic tools, optimizing strains, characterizing growth physiology, and generation of

overexpression libraries. The results of all these efforts will be detailed in the ensuing chapters.

- Ian Cheah (August 2015)

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