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Up Regulation of Heat Shock Protein 70B (HSP70B) and *SSA1* in *Chlamydomonas Reinhardtii* via HSP70A-RBCS2 and PSAD Promoter

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UP REGULATION OF HEAT SHOCK PROTEIN 70B (HSP70B) AND *SSA1* IN *CHLAMYDOMONAS REINHARDTII* VIA HSP70A-RBCS2 AND PSAD PROMOTER

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
Biosystems and Agricultural Engineering
in the College of Engineering at the University of Kentucky

By

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Lexington, Kentucky

Director: Dr. C. L. Crofcheck,

Professor of Biosystems and Agricultural Engineering

Lexington, Kentucky

2015

ABSTRACT OF THESIS

UP REGULATION OF HEAT SHOCK PROTEIN 70B (HSP70B) AND *SSA1* IN
CHLAMYDOMONAS REINHARDTII VIA HSP70A-RBCS2 AND PSAD PROMOTER

Fabrication of effective algae cultivation systems adjacent to coal-fired power plants to fixate waste CO_2 would represent a sizable step towards achieving a carbon neutral energy cycle. However, emission gas would elevate the algal cultivation system temperature and decreases its pH without expensive preprocessing. Increased temperature and acidity constitutes a profound stress on the algae. Although stressed algae produce heat shock proteins (HSPs) that promote protein folding and protect against stress, the ordinary biological response is insufficient to protect against coal flue gas. Experimental upregulation of HSPs could make algae respond to the stress caused by high temperatures and low pH at an elevated level. However, no work has been done to determine whether HSPs can be experimentally upregulated in algae. Here, the *Chlamydomonas reinhardtii* algal strain was selected because it has a sequenced genome and singular cell structure ideal for genetic modifications. Two genetic modification methods: transformation with plasmids pCB720/pCB740, and cloned pchlamiRNA3/pchlamiRNA3int with yeast HSP gene *SSA1* were evaluated. pCB720/pCB740 up regulate algae production of native HSP, HSP70B. pCB720 transformation success was observed but statistically, data varied. pchlamiRNA3/pchlamiRNA3int were cloned with *SSA1*. Chlorophyll content measured growth indirectly. Quantitative HSP detection could be done using RT-PCR.

KEYWORDS: *Chlamydomonas reinhardtii,* heat shock proteins, temperature, pH, transformation, *SSA1*

B Kirtley Amos

December 9, 2015

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To friends and family, who believed in me and aided me in this process.

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CHAPTER 1:   INTRODUCTION

In 2008, the world's primary energy consumption was estimated at 11,295 million tons of oil equivalent with fossil fuels, oil, coal, and natural gas, accounting for approximately 88%. In 2003, the worlds biodiesel production was estimated at 1.8 billion liters (Huang, Chen et al., 2010). In the UK alone, an estimated 47 billion liters of transport fuels were consumed, 53% of which were diesel (Scott, Davey et al., 2010). In the United States it's estimated that slightly less than 20 million barrels of oil are consumed per day, with gasoline as the primary transport fuel being consumed at about 9 million barrels per day. Distillate fuel oil, a fraction of light petroleum that can be used as diesel or fuel oil, places second with a daily consumption of 4 million barrels (EIA, 2015). The use and consumption of these fuels will continue for years to come due to the vast supply of fuels within the globe, however, the industrial usage of these fuels can be linked to increased greenhouse gas emission.

In 2006, it was estimated that fossil fuels contributed 29 giga tons of CO_2 emissions to the biosphere with natural processes removing an estimated 12 giga tons, therefore, creating an annual increase in CO_2 concentration within the atmosphere (Brennan and Owende, 2009). Alternative energy methods involving chemical and biological CO_2 mitigation techniques have been the focus of research within the green energy sector since the creation of the Kyoto Protocol in 1997. This protocol calls for a 5.2% reduction in greenhouse gas emissions worldwide from the 1990 values (Brennan and Owende, 2009). Greenhouse gas emissions not only contribute to global warming but also encroach on other areas of natural function within environments. Oceans absorb approximately one third of the CO_2 emitted annually, and as the amount of CO_2 released into the atmosphere annually increases due to increased global economy, the ocean pH will gradually decrease, becoming more acidic, leading to loss of coral reefs and marine ecosystem biodiversity (Mata, Martins et al., 2009). To combat the increasing CO_2 annual emissions, alternative transportation fuel sources need to be discovered, refined, and exploited.

In order for biofuels to be considered, multiple parameters need to be met and sustained. Initially, from the eyes of industrial producers, biofuels must be economically competitive with petroleum fuels. Not only do biofuels need to be economically competitive, they must also be energetically competitive, meaning, almost equivalent energy output when ran through an engine. While simultaneously being energetically equivalent to petroleum based fuels, biofuels must support the improvement of air quality through cleaner emissions. Along with the requirement to be competitive with petroleum fuels on an economic and energetic basis, biofuels must also require very little to no additional land use. This technical requirement allows no infringement on lands currently used for agricultural commodities, enabling little to no competitive land usage. Along with these optimal parameters, biofuels should also require minimal water usage. This optimal condition allows drought susceptible areas to grow biofuel plants while also maintaining traditional local agricultural.

To replace all transportation fuel consumed within the United States, 0.53 billion m^3 annually would need to be converted (Chisti, 2007). Some biofuels can be used in existing engines and transport infrastructure with little to no modifications (Scott, Davey et al., 2010). Biofuels offer new opportunities to diversify income and transportation fuel supply sources, promote employment in rural areas, and develop a long term replacement for fossil fuels all while reducing greenhouse gas emissions. The most common types of biofuels are biodiesel and bio-ethanol which replace diesel and gasoline, respectively (Mata, Martins et al., 2009). Biodiesel usage could decrease sulfur and carbon monoxide release by 30% and 10%, respectively, while also decreasing 90% of air toxicity and 95% of cancers when compared to common diesel sources (Sharp, 1996, Huang, Chen et al., 2010).

Algae as a species have gained a large following due to their ability to produce various end products; methane through anaerobic digestion, biodiesel via lipid extraction and transesterification, and di-hydrogen production when deprived of sulfur (Chisti, 2007). Algal biomass can be used for multiple processes, varying from biofuels to the over expression of non-native proteins for pharmaceutical usage including but not limited to; nutritional supplements, antioxidants, cosmetics, natural dyes, and polyunsaturated

fatty acids (Rosenberg, Oyler et al., 2008). Carbohydrates and starches from algae can be used to make di-hydrogen and bio-ethanol while the lipids and triacylglycerols can be used to make biodiesel. Lipid composition within algae tends to be similar to those found within various vegetable oils, ranging from 20-50% (Chisti, 2007). Proteins and minerals associated with algal biomass can be used to make plant and animal foods (Dismukes, 2011). Nutritional and medicinal applications of algae show significant potential due the ability to produce biomolecules which are generally regarded as safe for human consumption. Along with the capability to produced biomolecules safe for human consumption, algae are extremely viable for genetic manipulation and high though put analysis due to their relatively small genome size. Algae also take advantage of light as a source of energy which could potentially provide an economical source of end products from biopharmaceuticals to bio energies on an industrial scale (Rosenberg, Oyler et al., 2008). Figure 1.1 displays a simplified diagram of algae inputs to produce outputs, such as hydrogen, ethanol, biodiesel, and nutraceuticals.
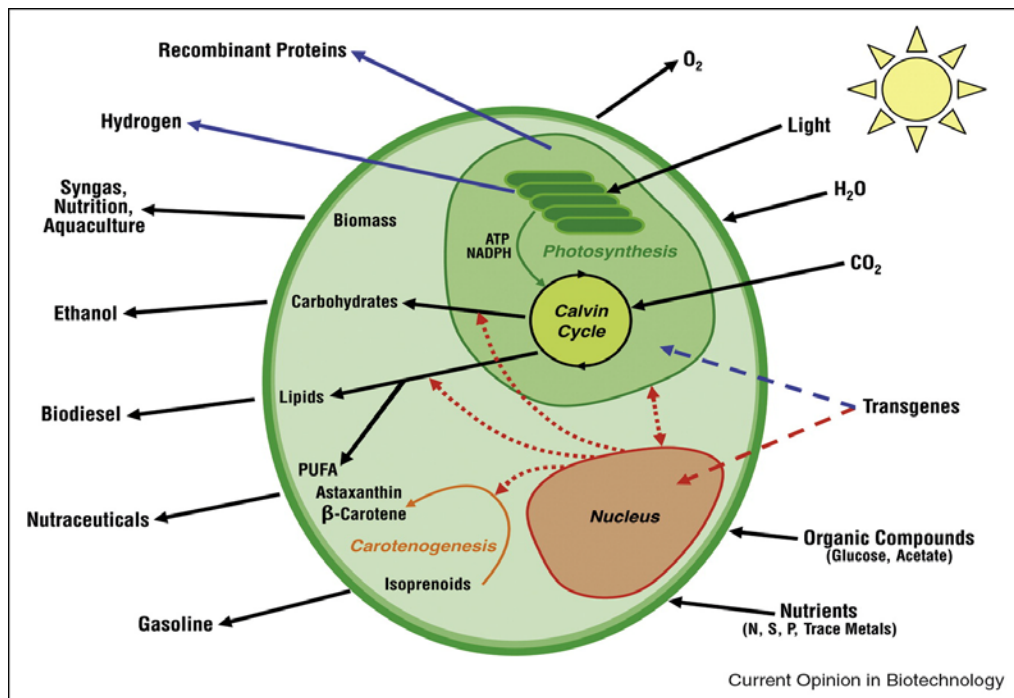
Figure 1.1 Diagram of green algae with inputs and potential outputs (Rosenberg, Oyler et al., 2008) .

The requirements and demands for clean and increased renewable fuels, ethanol, and biodiesels, allow using algae as a fuel source to be entertained. Benefits of algae as a

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fuel source when compared to other terrestrial biofuel sources are exactly that, they are terrestrial, and tend to compete with food commodities for adequate growing plots. In addition to non-competition of algal fuels with terrestrial crops for ideal land space, they produce more lipids/weight when compared to other biofuel producers, some in ranges of 30 to 50% on dry weight basis (Tornabene, Holzer et al., 1983, Miao and Wu, 2006, Xu, Miao et al., 2006).

The use of algae as an alternative biofuel feedstock, classified as a third generation biofuel, source has benefits that prior biofuel sources lacked. First generation biofuels, categorized as those which are derived from terrestrial crops, such as sugar cane, sugar beet, rapeseed oil, vegetable oil, animal fats, and maize place strain on the world due to increased water demand, crop displacement, and food market competition (Brennan and Owende, 2009). The use of these fuels will continue to grow in production and consumption but they have come under scrutiny due to the nature of food vs. fuel, requiring large acres of land for production, high levels of water and fertilizers, regionally constrained market structures, and lack of well managed agricultural practices in emerging economies (Brennan and Owende, 2009, Singh, Nigam et al., 2011). The savings in energy and greenhouse gas emissions over the life cycle of the first generation biofuel could be less than expected, averaging out to about 50% of the input energy is the energy contained in the fuel (Scott, Davey et al., 2010). The second generation biofuels derived from lignocellulosic plant materials, dedicated energy crops, provided an answer to the question of first generation biofuels; however, they also provided another issue of their own, competition for land.

The use of third generation biofuels in the form of algae provide answers to the problems related to first and second generation biofuels (Brennan and Owende, 2009). According to the United States Department of Energy, algae have the potential to produce up to 100 times more oil per acre land than many other terrestrial plants (Singh, Nigam et al., 2011). Not only do algae have the potential to increase biofuel production compared to other terrestrial plants, algae also consume large amounts of CO_2 during photosynthesis, transforming it into organic biomass, providing a greenhouse gas emission reduction, and some don't accumulate recalcitrant lignocellulosic biomass

(Dismukes, Carrieri et al., 2008, Singh, Nigam et al., 2011). Approximately 100 tons of algal biomass has the potential to fix roughly 183 tons of carbon dioxide (Chisti, 2007).

However, the prospects of algae as a third generation biofuel source also come with issues requiring hurdling. For one, maximizing lipid content and other precursor molecular compounds prior to processing. Nitrogen starvation allows algae increased lipid production while sulfur restriction allows di-hydrogen production through photosynthesis. One of the major hurdles is increasing the rate of algal cellular growth. If this could be modified, the uses of algae for carbon mitigation become immense, allowing more exhaust gas to pass though less volumes of algae while providing the same quality CO_2 sequestration. With these two hurdles to overcome, understanding the molecular pathways and chemicals which induce compound specific reactions must be understood and exploited. This would allow large scale production of compounds of particular interest. Once these questions have been addressed and answered, the implementation of multistage growth systems, providing quality controlled products, must be discovered and applied. This application would industrialize the processes of prior growth systems.

Algae range from small single celled to multi-cellular organisms, found in damp places or aquatic environments, therefore, naturally present in terrestrial and water body environments. Algae include seaweed, classified as macroalgae, and phytoplanktons, classified as microalgaes, surviving in both eukaryotic and prokaryotic cellular forms (Singh, Nigam et al., 2011). Algae thrive in both saline and fresh waters, depending on the species, and microalgae can be motile or non-motile based on the presence of flagella. An enormous range of microalgae exists including dinoflagellates, green algae, golden algae, and diatoms (Singh, Nigam et al., 2011). Ranges in algal lipid composition vary from species to species but can be increased when algae are deprived of nitrogen, however, there is a poor understanding of algal nitrogen retention in low nitrogen environments and lipid biosynthesis (Adams, Bugbee et al., 2014). They contain polysaccharides, carbohydrates, in their cell walls which accounts for a large amount of the carbon stored and under certain conditions, it has been quoted, that algae can have up to 80% oil by wet weight (Singh, Smyth et al., 2010). Diatoms are of particular interest

to the biofuels industry because they accumulate high levels of lipids, storing triacylglycerol lipid molecules as energy, which can be transesterified to create biodiesel. However, diatoms also contain a large percentage of phospholipids which are not easily transesterified to biodiesel (Singh, Nigam et al., 2011).

Conversion of lipids to biodiesels requires the process of mono-alcoholic transesterification where triacylglycerides react with a mono-alcohol, typically ethanol or methanol, with the aid of a catalyst of aklai, acid, or enzyme (Li, Horsman et al., 2008). Biodiesels produced through this process have combustion properties very similar to that of diesel. The conversion of algae to ethanol requires the carbohydrates undergo a process of saccharification (Matsumoto, Yokouchi et al., 2003). A major attractor of using microalgae to produce biodiesel is the amount of oil they produce on a per hectare basis, 58,700 L. Table 1.1 compares biodiesel sources based on oil yield, land area needed for yield, and the required cropping area to make it happen. All microalgae numbers were determined experimentally within PBR's.

Table 1.1 Comparison of oil yield, land area needed, and percent of existing US cropping area for various crops (Chisti, 2007).

Crop	Oil yield (L/ha)	Land area needed (M ha)[a]	Percent of Existing US cropping area[a]
Corn	172	1540	846
Soybean	446	594	326
Canola	1190	223	122
Jatropha	1892	140	77
Coconut	2689	99	54
Oil palm	5950	45	24
Microalgae [b]	136,900	2	1.1
Microalgae [c]	58,700	4.5	2.5

[a] For meeting 50% of all transport fuel needs of the US

[b] 70% oil (by wt) in biomass

[c] 30% oil (by wt) in biomass

Advantages associated with microalgae as a biofuel source are numerous, many of which trump terrestrial crops. One benefit, which many terrestrial crops can't sustain in particular portions of the globe, is year round production. Accompanying year round production is a greater average percentage of oils for biofuels. With various algae species having an oil content range from 20-50% dry weight of biomass, there could be potential for increased oil yield by modified growth conditions. Rates of photosynthesis efficiency within algae are higher than those of terrestrial plants, allowing increased CO_2 capture and biomass growth. The water requirements, when compared to terrestrial crops, tend to be less due to lack of runoff associated with traditional agricultural practices. Algae are either grown in ponds or re-circulating closed systems, allowing little evaporation and low amounts of runoff, respectively. Brackish water on non-arable land also poses as a potential growth model for algae, preventing land competition with terrestrial crops. Waste water can provide a nutrient source for algae growth with the usage of pesticides and herbicides applications at a lower rate than terrestrial crops. Using algae as CO_2 sequestration facilities also provide an advantage to algae as a biofuel source, averaging about 1.83 kg of CO_2 absorbed per 1 kg of dry algal biomass. In tandem with CO_2 sequestration, the use of algae for valuable co-products has high potential use as feed (Fleurence, 1999).

However, there are hurdles associated with using microalgae as a biofuel source, many of which must be overcome before industrial application of biofuels from algae becomes a potential. Algal species selection must balance the requirements for biofuel production and the extraction of valuable co-products. Potential attainment of higher photosynthetic efficiencies within algae would provide higher growth rates, allowing less volume of algae to sequester equal amounts of CO_2. Contamination is an enormous issue that algae cultivation must face; it's an extreme struggle to maintain single species cultivars especially in open ponds. This must be overcome with the development of sterilization techniques or equipment to prevent contamination. Along with the development of single species cultivation techniques, efforts need to be made to prevent evaporation from large scale algal production ponds. Life cycle analysis optimization of algae production will be required to ensure a positive energy balance is achieved. Flue gas from commercial plants being pumped into photobioreactors needs to have a

manageable NO_3 and SO_4 content which does not overcrowd the algae cultivar. This being said, the amount of CO_2 sequestered also depends on how much NO_3 and SO_4 the cultivar can handle. Once these goals have been reached, the operation and production of commercial plants capable of processing algae into biofuels must become widespread to reduce shipping costs and enable economic feasibility (Brennan and Owende, 2009).

The use of microalgae as a biological CO_2 mitigation factory provides an alternative to chemical CO_2 mitigation (scrubbing), both being two distinct techniques (Gupta and Fan, 2002). Chemical reaction mitigation is often referred to as scrubbing where the released CO_2 reacts with a solid metal oxide yielding a metal carbonate (Gupta and Fan, 2002). Advantages related to scrubbing include the process being performed under the temperature and pressure conditions typically associated with CO_2 associated flue gas streams. However, that being said, the process is energetically costly, expensive, and requires proper disposal of both captured CO_2 and absorbent material (Mata, Martins et al., 2009). Biological mitigation of CO_2 provides an alternative with potential for a bright future based off turning one waste, CO_2 from flue gas, into a nutrient source for microalgae, sequentially producing bioproducts, and congruently lowering CO_2 emissions (Mata, Martins et al., 2009).

1.1 Project Goal

The use of industrial coal-fired power plant flue gas as a nutrient source for algae attempts to mitigate greenhouse gas emissions while simultaneously producing an alternative product comes with challenges that must be overcome in order to provide algae within photobioreactors the ability to survive and function optimally in often sub optimal conditions. Most aquatic species require a moderate pH in order to function optimally, living in environments ranging from 6.5 to 8.3 for average fresh and salt bodies of water. Along with required pH ranges, the correct temperature range is also imperative in order to ensure adequate functioning. Challenges arise from incorporating coal plant flue gas into photobioreactors within adequate and optimal standards (Kumar, Ergas et al., 2010). The products of the coal plant flue gas, CO_2 and SO_4, drive the pH of the algae suspensions towards the non-inhabitable acidic range. Along with the coal plant flue gas decreasing the pH of the cultivar, algae respiration, the uptake of O_2, also

decreases the pH, due to release of gaseous CO_2. To compound the challenge, the temperature of the flue gas can be extremely high, making the photobioreactor supra optimal, limiting growth, and potentially causing algal death. Percentages of flue gas can be allowed in attempts to prevent culture overheating and these will require tinkering based on seasonal weather conditions. Hurdles associated with flue gas pretreatment also exist, requiring more money for the system to work.

Figure 1.2 shows the growth rate of *Scenedesmus acutus* in response to temperature (Cassidy, 2011). There is a drastic decrease in specific growth rate after 27°C is surpassed. This decrease in growth rate at 27°C provides potential for economic infeasibility. If the amount of flue gas passed through the photobioreactors could be increased along with allowing continual optimal growth at elevated temperatures and decreased pH, then the potential for an economically viable biofuel from microalgae in closed photobioreactors becomes more viable.

The ability of the algae to grow at higher temperatures and lower pH values, even if it is a minute change in either direction, allows for a potential large change to the system. Figure 1.3 depicts photobioreactor temperatures based on the percentage of flue gas allowed into the system along with the temperature at which the flue gas enters the system. Not only does it allow a change in the cultivar system, it allows the potential for much more economical feasibility in a larger area of the globe, affording less cost to cooling and pH altering substances.
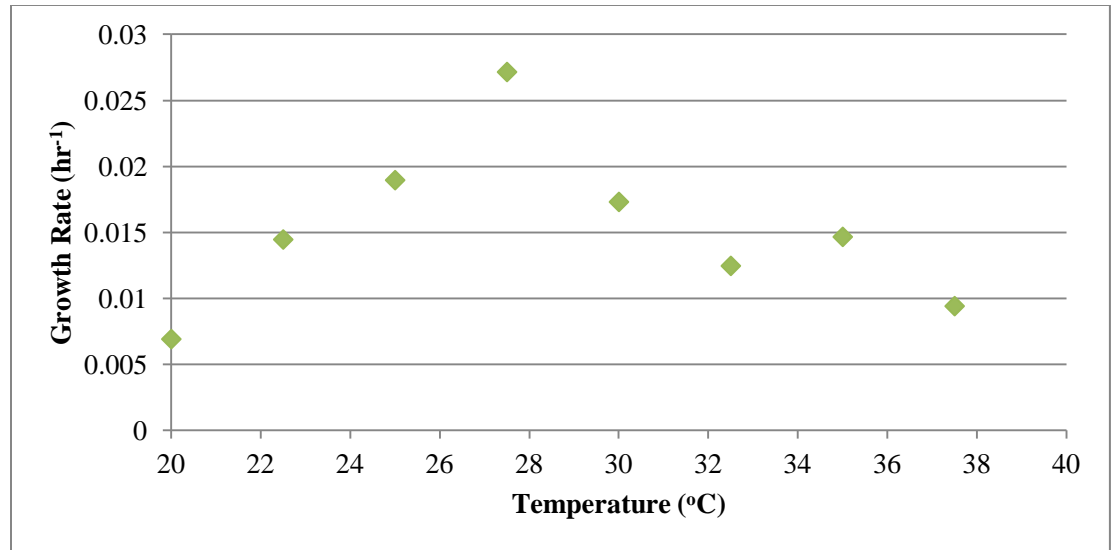
Figure 1.2. Growth rate of *S. acutus* in response to temperature (Cassidy, 2011).
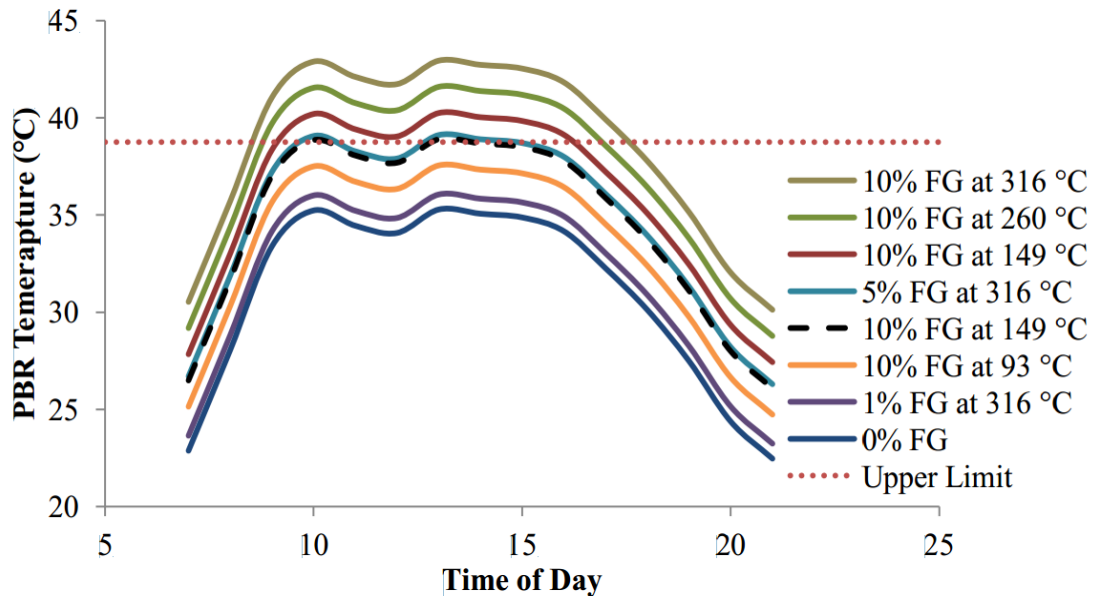
Figure 1.3. Estimation of photobioreactor temperature depending on flue gas temperature and percentage of flue gas circulating within an algae culture (Cassidy, 2011).

Therefore, the focus of this thesis is to modify the model algal species, *Chlamydomonas reinhardtii,* enabling the survival and optimal growth at higher temperatures, lower pH, and more intense light. It was thought that through the transformation of plasmids pCB720 and pCB740, actively promoting increased HSP70B

production within *Chlamydomonas reinhardtii,* a native chloroplast protein, that this could be achieved. Another approach includes the cloning of yeast heat shock gene *SSA1* into pchlamiRNA3 and pchalmiRNA3int and then transformation into *Chlamydomonas reinhardtii*. Therefore, the specific objectives of this thesis are:

1. Determine if over expression of *HSP70B* within *Chlamydomonas reinhardtii* elevates tolerable temperature ranges and broadens tolerable pH ranges.

2. Determine if over expression of *SSA1* within *Chlamydomonas reinhardtii* elevates tolerable temperature ranges and broadens tolerable pH ranges.

CHAPTER 2: BACKGROUND

2.1 Algal Culture

Algae of all species thrive within environments of adequate sunlight, carbon dioxide, and proper nutrients. Closed circulating ponds and photobioreactors have been the proposed methods of growth, containing both pros and cons. Associated negatively with closed system ponds are high chances of contamination from other microbes or native algae species (Rosenberg, Oyler et al., 2008). Photobioreactors cost more to maintain but enable strict culture containment and parameters, enabling control of some environmental variables. Under optimal conditions, microalgae populations are capable of doubling within hours, producing upwards of 60 g/L of heterotrophic biomass and 5 g/L of photoautotrophic biomass (Rosenberg, Oyler et al., 2008). Ideas related to biological CO_2 sequestration through microalgae stem from concepts associated with closed photobioreactors connected to coal plant flue gas which is circulated through at various percentages, having CO_2 converted to energy via microalgae during photosynthesis. Closed photobioreactors could be tubes, plates, or bags made of plastics, glass, or other transparent materials, where algae are supplied with light, nutrients, and carbon dioxide (Lehr and Posten, 2009). A major benefit to closed photobioreactors is the higher yield when compared to open systems, however, the downfall is the initial cost of installing and constant maintenance of closed systems when compared to open algal cultivation (Lehr and Posten, 2009). Table 2.1 outlines pros of photobioreactors. In addition, the application of lights, either natural or synthetic, add cost or reduced efficiency, meaning, natural light is seasonal in certain areas and during higher temperature months can cause algal death but requires no cost. Synthetic light is constant with a cost associated, increasing economic infeasibility (Chen, Yeh et al., 2011).

Raceway ponds, the open system model for algal growth, re-circulate algal biomass in a closed loop system on top of either compact earth or concrete at about 3 meters in depth. A paddlewheel provides constant circulation while also preventing sedimentation accompanied by baffles around each bend, also preventing sedimentation in corners (Chisti, 2007).

Table 2.1. Comparisons of photobioreactors to raceway ponds (Chisti, 2007).

Comparison of photobioreactor and raceway production methods		
Variable	Photobioreactor facility	Raceway ponds
Annual biomass production (kg)	100,000	100,000
Volumetric productivity $(kg\ m^{-3}d^{-1})$	1.535	0.117
Areal productivity $(kg\ m^{-2}\ d^{-1})$	0.048[a] 0.072[c]	0.035[b]
Biomass concentration in broth $(kg\ m^{-3})$	4	0.14
Dilution rate (d^{-1})	0.384	0.025
Area needed (m^2)	5681	7828
Oil yield $(m^3\ ha^{-1})$	136.9[d] 58.7[e]	99.4[d] 42.6[e]
Annual CO_2 consumption (kg)	183,333	183,333
System geometry	132 parallel tubes/unit; 80 m long tubes; 0.06 m tube diameter	978 m2/pond; 12 m wide, 82 m long, 0.30 m deep
Number of units	6	8

[a] Based on facility area.

[b] Based on actual pond area.

[c] Based on projected area of photobioreactor tubes.

[d] Based on 70% by wt oil in biomass.

[e] Based on 70% by wt oil in biomass.

2.1 *Chlamydomonas reinhardtii*

Chlamydomonas reinhardtii, the model algal species for genetics work, is a single celled green algae, classified as microalgae, and possesses two flagella allowing motility.

Chlamydomonas reinhardtii contain large chloroplasts and represent some of the simplest photosynthetic eukaryotes. *Chlamydomonas reinhardtii* can reproduce sexually or asexually while also possessing the ability to grow photoautotrophically, heterotrophically, or mixotrophically (both sunlight and a sugar source) (Neupert, Karcher et al., 2009). These algae live in multiple environments throughout the world, in both light and dark, having been used in areas ranging from pharmaceutical to biofuel experimentation and production. Large stocks of mutant collections have been established, enabling quick access to potential experimental strains. *Chlamydomonas reinhardtii* has all three of its genomes sequenced (plastid, nuclear, and mitochondrial), all genomes are responsive to transformation, allowing genetic engineering the benefit of reference, expediting any attempted synthetic biology (http://www.chlamy.org, and http://genome.jgi-psf.org/chlrez.infor.html).

   Chlamydomonas reinhardtii was chosen as a model algal species due to its mutant strains lack of cell wall and stable long term expression for multiple generations after transgenic experimentation. It has proven itself viable through sufficient studies in both up and down regulation of genetic expression (Schroda, Beck et al., 2002, Siripornadulsil, 2002, Shrager, Hauser et al., 2003, Rosenberg, Oyler et al., 2008). *Chlamydomonas reinhardtii* has also been used to determine organism multigenic response to varying abiotic stressors and is haploid, having half the number of chromosomes during vegetative growth, allowing for instant mutation detection (Hema, Senthil-Kumar et al., 2007). *Chlamydomonas reinhardtii* has also been used as a model organism for the study of many biological processes; photosynthesis, chloroplast development, and flagella motility and assembly (Grossman, 2005).

   There are downsides to *Chlamydomonas reinhardtii* but advances have been made over the years to combat these issues. One issue prevalent before strains/techniques were developed to combat the issue was *Chlamydomonas reinhardtii's* inability to express nuclear transgenes at high concentrations. This issue negates techniques used on higher plant species including all applications of in vivo gene expression promoters; promoter-YFP/GFP fusion for gene expression analyses, fluorescence resonance energy transfer,

and bimolecular fluorescence complementation for monitoring protein-protein interactions (Shao and Bock, 2008).

2.2 Synthetic Biology

Synthetic biology, a term of recent debate, encompasses fields related to biology; evolutionary biology, molecular biology, biotechnology, and systems biology. It is the design/redesign and fabrication of biological components and systems which do/do not already exist in the natural world (Schmidt, 2012). Synthetic biology is a study related very closely to genetic engineering, and, for example, of recent, *Escherichia coli* have been synthetically designed to require a specific novel manufactured protein and are therefore limited to life within the lab which they were created. Manipulated metabolic pathways are required to produce large quantities of desirable compounds allowing direct control over the cellular machinery function through the addition of transgenes. The advances accompanying algal transgenics include; efficient expression of transgenes (Neupert, Karcher et al., 2009), avant-garde mechanisms for genetic regulation in algae using riboswitches (Croft, Moulin et al., 2007), inducible nuclear promoters and luciferase reporter genes (Shao and Bock, 2008, Neupert, Karcher et al., 2009), and inducible chloroplast gene expression (Surzycki, Cournac et al., 2007).

The ability to efficiently express transgenes enables *Chlamydomonas reinhardtii* to be considered much more seriously as a piece of molecular farming machinery. A screen developed enabled identification of algal strains with transgenes expressed at high enough levels for potential biopharmaceutical and various compound extractions (Neupert, Karcher et al., 2009). This screen showed *Chlamydomonas reinhardtii* nuclear transgene levels as high as 0.2% of total soluble protein. The strains they developed through this technique enable overcoming of some of the largest hurdles associated with *Chlamydomonas reinhardtii* research. The understanding of metabolic pathways within species enables synthetic biologist the ability to more drastically control mechanisms of interest. For example, understanding that thiamine production within *Chlamydomonas reinhardtii* is regulated by riboswitches paints a clearer picture for synthetic biology (Croft, Moulin et al., 2007).

The identification of a highly sensitive luciferase reporter gene from *Gaussia princeps*, a marine copepod, serves as a reporter for constitutive and inducible algal promoters. This luciferase reporter gene discovery greatly surpasses the intensity of other promoters, enabling monitored response to environmental stresses in vivo and high throughput screenings for mutants of *Chlamydomans reinhardtii*. The luciferase gene from *G. princeps* generated more than a 7-fold increase in bioluminescence and about a 40-fold increase in luminescence imaging when compared to *R-Luc* gene from *R. reniformis* (Shao and Bock, 2008).

Chlamydomonas reinhardtii, Chlorella, Haematococcus, and *Dunaliella,* when discussing genetic engineering, are all highly probable to nuclear transformation required for successful metabolic control, apt to chloroplast transformation enabling large accumulation of protein expression, and are amenable to more spear pointed genetic transformation techniques when compared to other higher plants (Leon-Banares, Gonzalez-Ballester et al., 2004). The first stable chloroplast transformation was conducted using a biolistic approach in 1988 on *Chlamydomonas reinhardtii* (Purton, 2007). This enabled generational persistence after 65 generations of four various transformants within liquid medium, however, those grown on selective plate medium lost all chloroplast integrated plasmids. In another experiment, a nitrate reductase (provided ability to use nitrate as sole nitrogen source) and an unselected gene were co-transformed using glass beads, plasmids, and polyethyleneglycol. This experiment, performed in 1990, showed the high affinity for *Chlamydomonas reinhardtii* transformation, with the unselected gene present within nitrate reductase positive cells at percentages from 10-50% (Kindle, 1990).

2.3 *SSA1*, *SSA2*, and HSP70B Cellular Role

SSA1 and *SSA2* are found within *Saccharomyces cerevisiae*, also found within many other eukaryotes, encode for a 70 kilodalton protein, HSP70, responsible for transportation of polypeptides across mitochondrial membranes and into endoplasmic reticulum located in the nucleus, cytoplasm, and cell wall. The nucleic acid coding regions of *SSA1* and *SSA2* are nearly identical, having a minute three amino acid difference within the carboxy terminus of *SSA2* relative to *SSA1*, accounting for 3%

divergence (Slater and Criag, 1989). Under normal conditions, *SSA1* and *SSA2* are expressed constitutively with *SSA1* expressed at lower levels but when environmental temperatures are raised, expression of *SSA1* is increased drastically within *Saccharomyces cerevisiae* (Verghese, Abrams et al., 2012). Inactivation of either *SSA1* or *SSA2* does not have a visible phenotypic effect but inactivation of both causes slower growth at all temperatures and complete lack of growth at 37°C, a bit above *Saccharomyces cerevisiae* optimum growing temperature (Lopez-Ribot and Chaffin, 1996).

Major HSP families along with HSP70 include HSP40, HSP90, HSP100, HSP60, nucleotide exchange factors, and small HSP's (Vogel, Mayer et al., 2006). HSP70s are found in all known organisms, aside from Achaea, and are present in many compartments of the cell (Liu, Willmund et al., 2005). HSP70, a conserved protein within organisms, aids in refolding of mis-folded substrate proteins, partially unfolded substrate proteins, and newly synthesized proteins by binding hydrophobic regions, all of which is predominantly dictated by the bound co-chaperone (Tutar, Arslan et al., 2010). Along with correct substrate protein folding, HSP70s also aid in translocation across membranes, target proteins for degradation, and apoptosis (Vogel, Mayer et al., 2006, Woo, Jiang et al., 2009). Correct folding of proteins prevents aggregation, ensuring proper protein function, and HSP70 families of proteins have been shown to be expressed constitutively enabling stress protection (Witt, 2010). HSP70's can be viewed as having two domains, an ATPase and a substrate binding domain. Upon substrate binding, hydrophobic interactions force the misshapen protein to reach native states (Tutar, Arslan et al., 2010). The ATP bound state favors the release of the properly folded polypeptide while the ADP bound state facilitates polypeptide binding (Vogel, Mayer et al., 2006). Various chaperones, shuttle proteins, affect the activity of HSP70. HSP40 submits the polypeptide to the protein binding site of HSP70 in the correct spatial orientation allowing HSP70 to correctly fold and release the polypeptide (Vogel, Mayer et al., 2006, Witt, 2010). Within *Chlamydomonas reinhardtii*, the promoter HSP70A, when placed upstream of other promoters, significantly improves the transgenic expression within *Chlamydomonas reinhardtii* by decreasing the transgenes' chance of being acted on by transcriptional gene silencing (Schroda, Beck et al., 2002).

HSP70B, a chloroplast protein located in the stroma within *Chlamydomonas reinhardtii* and having similarity to prokaryotic HSP70's, has its gene, *HSP70B*, induced by heat stress and light intensity. It was shown to increase cellular ability to handle damage associated with high intensity light, either by protecting photosystem two from degradation or by stabilizing a damaged photosystem two, while cooperating with 5 different J-domain proteins termed chloroplast DnaJ homologs (CDJ) 1 to 5 (Liu, Willmund et al., 2005, Willmund, Dorn et al., 2008).

Figure 2.1, model A, with no client J domain protein binding interactions the client directly interacts with HSP70-ATP. Upon interaction, a J domain protein binds, dephosphorylating and releasing itself. A nucleotide exchange factor interacts with the dephosphorylated complex, facilitating client release. This results in the proper folding or movement of a particular client to its destined location for proper function. Model B, with the J domain protein tethered provides an example of how membrane transport can occur. Model C, client binding possible, offers the potential for both client binding and non-client binding depending upon the necessities of the client.

A) No Client Binding; J-protein un-tethered

B) No Client Binding; J-protein tethered

C) Client Binding Possible

ATP

Mis-folded protein

ADP

Properly folded protein

HSP70

J domain protein

Nucleotide exchange factor
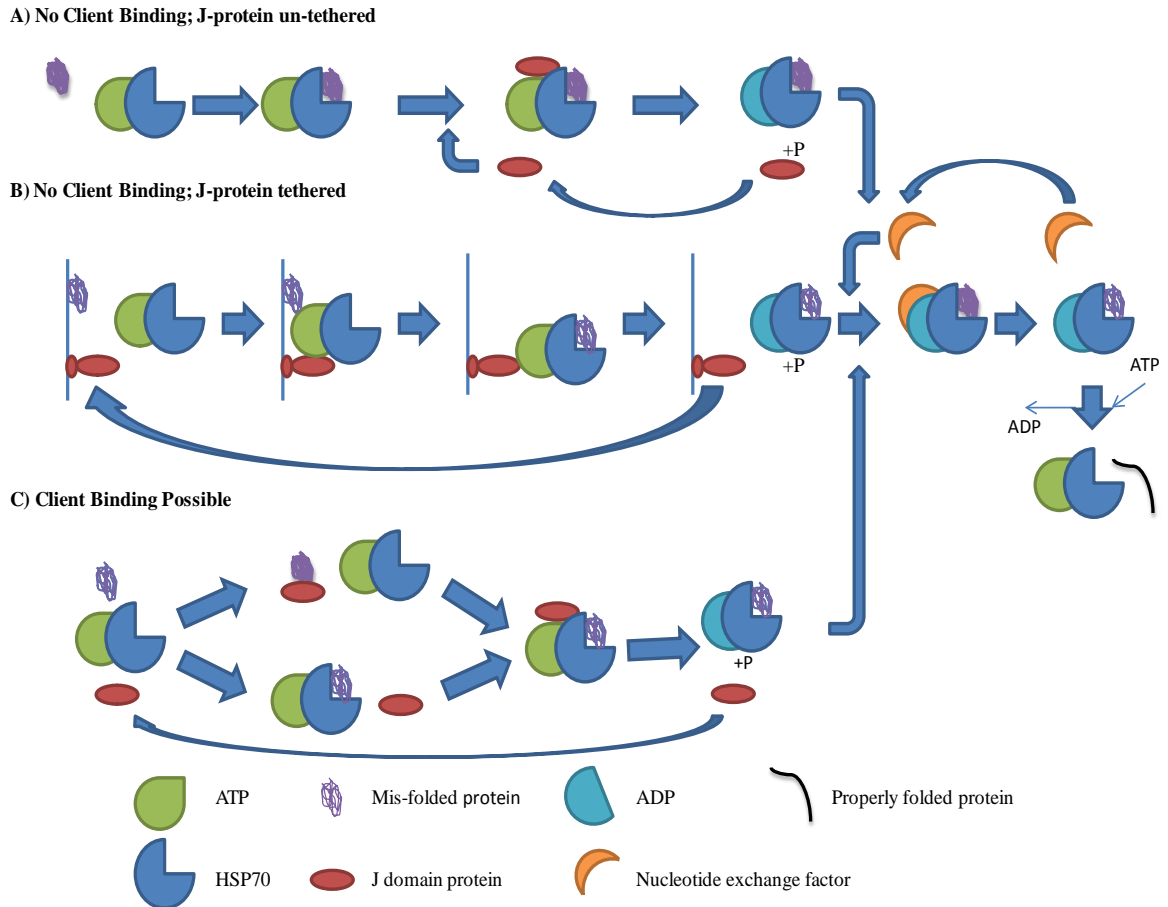
Figure 2.1 HSP70 and potential client interactions based off location of action within the cell; cytosol, nucleus, chloroplast, mitochondria.

2.5 *Chlamydomonas reinhardtii* Response to Abiotic Stress

The cycles of light throughout seasons and days can affect the rate of plant photosynthesis. For optimal photoautotrophic growth of *Chlamydomonas reinhardtii* about 30% of full sun light intensity is expected (Forster, Mathesius et al., 2006). During low light levels, light absorption is nearly equivalent to photosynthesis rates, reaching near maximum photosynthetic efficiency, while at high light levels the rate of photosynthesis becomes saturated but the excess light is still absorbed. *Chlamydomonas reinhardtii* can't handle the excess light, resulting in over excitation, and the production of biologically damaging chemical compounds which affect photosystem 1, photosystem 2, proteins, lipids, and nucleic acids, resulting in photoinhibition (Erickson, Wakao et al.,

2015). Depending on the duration of exposure to intensive light, *Chlamydomonas reinhardtii* has developed multiple coping methods.

Upon initial exposure to high light levels, *Chlamydomonas reinhardtii's* first response is to move away from high intensities using its dual motile flagella. When movement away from high intensity light isn't enough, on short time scales, rearrangements of light harvesting antenna, altering the electron transport chain, and thermal dissipation allows *Chlamydomonas reinhardtii* to handle high intensity light. When exposure to high intensity light is on a long term scale, changes in gene expression aid in coping while accumulation of antioxidants help eliminate reactive oxygen species within the chloroplast. It has been observed when *Chlamydomonas reinhardtii* is brought from low light levels to high light levels the amount of chlorophyll content can be halved within 6 hours (Shapira, Lers et al., 1997).

At a certain stage of abiotic stress, increased quantities of reactive oxygen species can be generated in the apoplast, mitochondria, peroxisomes, cytoplasm, chloroplasts, and endoplasmic reticulum inducing damage to DNA, proteins, and membranes (Chankova, Dimova et al., 2014). To combat oxidative stress, multiple genes, enzymes, and proteins are kicked on or off, depending upon cellular requirements, changing cell morphology, physiology, and cell metabolism to find homeostasis. Stress response within any plant comprises many genes, enzymes, and proteins acting in tandem, facilitating a functional stress response of valid proportion. About 100,000 expressed sequenced tags have been discovered within model plants in response to abiotic stressors (Hema, Senthil-Kumar et al., 2007).

2.6 Project Objectives

Molecular modification of *Chlamydomonas reinhardtii* has been a recent scientific ordeal in hopes of making the organism into a biopharmaceutical/compound producing factory. Modification techniques have been refined over the years and approaches to problems streamlined, making molecular modifications attainable. *Chlamydomonas reinhardtii* can undergo transformations from multiple plasmids; pCB720, pCB740, pchlamiRNA3, and pchlamiRNA3int. Plasmids pCB720 and pCB740

can up regulate the production of HSP70B due to the presence of promoter HSP70A and HSP70A-RBCS2, respectively. No plasmid modifications are required prior to transformation of pCB720 and pCB740.

Prior to transformation, plasmids pchlamiRNA3 and pchlamiRNA3int require cloning of yeast heat shock protein gene *SSA1* downstream of the PsaD promoter allowing efficient expression of *SSA1* within the nucleus of *Chlamydomonas reinhardtii*. Once accomplished, all of the new algae strains would be subject to temperature and pH tests. Temperature tests would be set at 20, 25, 30, and 35°C with a pH of 7. The pH tests would be conducted at 5.5, 6, 6.5, and 7. The hope is to show a statistical difference in algal mass between the wildtype, cc-4351, and modified *Chlamydomonas reinhardtii* strains at higher temperatures and lower pH. Biomass would be calculated indirectly via chlorophyll content. Once accomplished, it sets a platform for potential modifications of similar interest down the road. The molecular modification of algae for temperature and pH tolerance in order to be more industrially useful and economically viable as carbon mitigation systems adjacent coal-fired power plants is the overarching goal of the proposed genetic manipulation.

CHAPTER 3:   MATERIALS AND METHODS

3.1 Molecular Work

pCB720 and pCB740 were purchased from the Chlamydomonas Resource Center (St. Paul, MN, http://chlamycollection.org). pCB720 and pCB740 came in *E. coli* and required a mini-prep to isolate, the process of separating cellular debris from desired plasmid. Two protocols were followed named Plasmid DNA extraction from *E. coli* (Miniprep) (Appendix B, 55) and Thermo Scientific GeneJET Plasmid Miniprep Kit (Appendix B, 55). Both protocols yielded purified pCB720 and pCB740. Once isolated, pCB720 was transformed into algae following the protocol titled *Chlamydomonas reinhardtii* Transformations (Appendix B, 54). Transformation is the process of placing plasmids from solution into host organisms, affecting gene expression. There are multiple forms of transformation; heat shock, glass bead, electroporation, and biolistics to name a few. Positive confirmation of plasmid transformation was examined in the form of growing *Chlamydomonas reinhardtii*, strain cc-4351, on a medium lacking arginine. This would be a positive conformation because the plasmids chosen code for arginine while *Chlamydomonas reinhardtii* strain cc-4351, prior to transformation is incapable of producing arginine and must be supplemented in media for sustained growth.

Prior to pchalmiRNA3 and pchlamiRNA3int modification, multiples avenues were taken to amplify *SSA1* to a sufficient working concentration. One method, until a plasmid was obtained from Dr. Peter Nagy's lab, was isolating nuclear DNA from *Saccharomyces cerevisiae* using the protocol titled Isolation of Chromosomal DNA from yeast (Appendix B, 57). This repeatedly did not work but should have isolated large quantities of nuclear DNA, enabling PCR expression of *SSA1*. From there, the plasmid was obtained and forward oligos for *SSA1* were designed with both His-Tag and FLAG-tag at the 5' end with the restriction enzyme identifier for Nde1 also present. The purpose of the FLAG-tag and His-tag is identification of presence in transformed *Chlamydomonas reinhardtii* strains. The reverse oligo had the restriction enzyme Xba1 present, only. Design for oligonucleotides can be seen in Appendix A, 49. Design is based on the first 18 to 24 base pairs within the gene of interest. The restriction enzymes

and tags "hang off" the targeted gene when PCR is performed. Once oligos were designed, because there was a limited amount of *SSA1* plasmid present, a transformation protocol followed by a miniprep were required to bulk the gene of interest. The combination of the protocols places the plasmid of interest within *E. coli* for bulking (transformation) and then isolation of plasmids through miniprep procedures. The protocols followed; Plasmid Transformation into Competent Cells (Appendix B, 54) and Thermo Scientific GeneJET Plasmid Miniprep Kit, respectively (Appendix B, 55). Once *SSA1* had been bulked a PCR reaction had to be determined that worked consistently, 20 µL PCR Reaction (Appendix A, 48) displays the solution used and the protocol used titled Thermal Cycler Protocol to Amplify *SSA1* (Appendix B, 54). A gel was cast, enabling separation of DNA based on molecular weight, placing DNA with more base pairs towards the top and DNA with less base pairs towards the bottom, and protocol titled Gel Electrophoresis was followed (Appendix B, 57). With *SSA1* adequately amplified, *SSA1* had to be gel isolated via the protocol Axygen Bioscience PCR Clean-up Spin Protocol (Appendix B, 56), extracting *SSA1* from the gel, isolating the gene.

Regarding plasmids pchlamiRNA3 and pchlamiRNA3int, cloning work prior to transformation was required. These plasmids were purchased from the Chlamydomonas Resource Center. Prior to purchase, restriction enzyme analysis of the plasmid and *SSA1* gene was done using NEB cutter (http://nc2.neb.com/NEBcutter2). Analysis was done in order to determine which restriction enzymes will not cut *SSA1* while also cutting pchlamiRNA3 and pchlamiRNA3int in the proper locations. It was determined that restriction enzymes Nde1 and Xba1 were the best suited, both not found within *SSA1*, and both cutting plasmids pchlamiRNA3 and pchlamiRNA3int downstream of the PsaD promoter and upstream of the PsaD terminator allowing adequate and complete expression of *SSA1*.

Once adequate restriction enzymes were determined, and *SSA1* concentration high enough, plasmids pchlamiRNA3 and pchlamiRNA3int were digested with Nde1 and Xba1 according to Restriction Enzyme Digestion of Miniprep Plasmid DNA (Appendix A, 48) by following the protocol named Restriction Enzyme Digestion of Miniprep Plasmid DNA (Appendix B, 56). Digestion with appropriate restriction enzymes allows

adequate ligation, cutting both the plasmid and the insert in the proper location, creating "sticky ends". Once adequately digested, both pchalmiRNA3 and pchlamiRNA3int were added with *SSA1* to perform a ligation according to protocol titled Ligation with T4 DNA Ligase (Appendix B, 56) in quantities titled Ligation Reaction (Appendix A, 53). T4 DNA Ligase is an enzyme with the ability to stitch two strands of DNA together, in this case, stitching both plasmid and insert together, creating a fully circular plasmid.

With ligation complete, the newly created plasmids, pchlamiRNA3 and pchlamiRNA3int with *SSA1* inserted, must be transformed into *E. coli* for bulking. Protocol titled Plasmid Transformation into Competent Cells (Appendix B, 54) was used. Once transformed, *E. coli* containing modified plasmids were plated on LB Agar containing Ampicillin at a 100x concentration and allowed to grow overnight at 37°C. Colonies that grew either contained pchlamiRNA3 or pchlamiRNA3int with insert *SSA1* successfully ligated or pchlamiRNA3 and pchlamiRNA3int ligated on themselves, providing a false positive. This can occur easily with T4 DNA Ligase stitching the plasmid to itself. In order to differentiate between the two ligation potentials, colony PCR was performed. Colony PCR allows a quick check of individual *E. coli* colonies by identifying the insert, *SSA1*, by oligos used to originally amplify *SSA1*. Colony PCR Protocol (Appendix B, 57) with the Thermal Cycler Protocol to Amplify *SSA1* (Appendix B, 54) were followed sequentially to amplify potential *SSA1*. Once identified, liquid cultures made from positive identification were processed according to protocol titled Plasmid DNA Extraction from *E. coli* (Appendix B, 55).

Upon successful completion of Plasmid DNA Extraction from *E. coli*, resulting in solution containing pchlamiRNA3 or pchlamiRNA3int with insert *SSA1*. The last molecular step is to transform the newly created plasmid into cc-4315. This is potentially accomplished by the protocol titled *Chlamydomonas reinhardtii* Transformation (Appendix B, 54). Once successful transformations have been obtained, temperature and pH experiments would be run.

3.2 Temperature and pH Experiments

Once strains were modified, S1 and S2 with plasmid pCB720 in both, temperature and pH experiments were conducted. The control was the unmodified strain, cc-4351, at environmentally identical conditions. Three flasks of each strain, for a total of nine flasks, were cultivated simultaneously. In attempts to culture with identical quantities of algae, cell counts were performed prior to culture. Fifteen ml cultures were taken from well shaken flasks and examined under the Olympus MVX10 microscope at 6.3x under GFP filter. Three cell counts from each parent culture were taken by moving the slide three times and an average made to assume cell count. Ratio inoculation based on cell count followed.

Figure 3.1 Microscopic image (Olympus MVX10) of algae strain S2 used for cell counting.

Prior to chlorophyll content quantification, pH measurements from each sample were obtained. This was a measurement of growth; with increasing growth pH became more basic. Every 24 hours, three 10 ml samples were taken from each flask and the protocol Chlorophyll Content Extraction was followed (Appendix B, 58). Evolution 60S UV-Visible Spectrophotometer by Thermo Scientific was programmed to calculate the absorbance at 649 and 665 nm. The equation used to calculate chlorophyll a and chlorophyll b were found in (Ritchie, 2006) and are displayed in Equation 3.1. Three

separate experiments were conducted, the first and second in February and March, respectively, at room temperature (22 °C) with three replications each. The third was done in April at room temperature 30 °C with three replicates for each temperature. For each experiment, the cultivation was run for 96 hrs, having a total of 384 hours of experimental time and 36 flasks.

Chl a (ug/ml) = -5.2007 A_{649} + 13.5275 A_{665} ($\pm$.03125) (Ritchie, 2006)

Chl b (ug/ml) = 22.4327 A_{649} + -7.0741 A_{665} ($\pm$.02623) (Ritchie, 2006)

Equation 3.1 Chlorophyll a and b Calculations.

CHAPTER 4:   RESULTS AND DISCUSSION

4.1 Molecular Work

The presence of individual colonies on the plates in Figure 4.1 indicates a successful transformation. This is a positive identification due to the auxotrophic marker in place, arginine production. cc-4351 is incapable of producing arginine on its own, therefore, when a successful transformation takes place, cc-4351 is plated on TAP media without arginine supplementation and those colonies that have grown should have transformed plasmid DNA present.
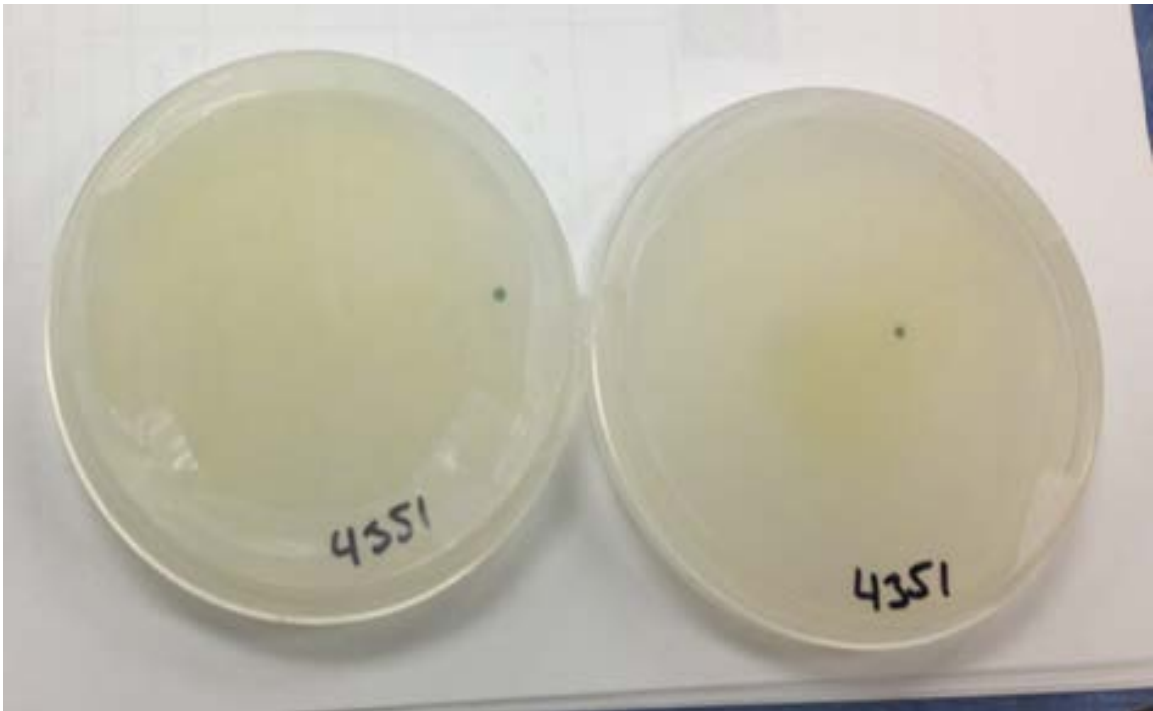
Figure 4.1. Glass bead transformation of pcb720 into algae strain cc-4351 creating strains S1 and S2 as displayed on a TAP plate lacking arginine.

Figure 4.2 depicts a successful expression of *SSA1* through PCR and then gel electrophoresis. This displays the higher affinity rate of Phusion as a DNA polymerase when compared to PFU due to the increased band intensity. Polymerases are the enzymes which elongate DNA both in vivo and in vitro. Also, this is the appropriate molecular weight to be confirmed as *SSA1*, being very close to 2000bp. 1 and 1P are

SSA1 without a tag present with the corresponding polymerases while 1F and 1FP are *SSA1* with a flag tag present and the corresponding Polymerase. 1H and 1HP are *SSA1* with histidine tags present amplified by the respective polymerase.
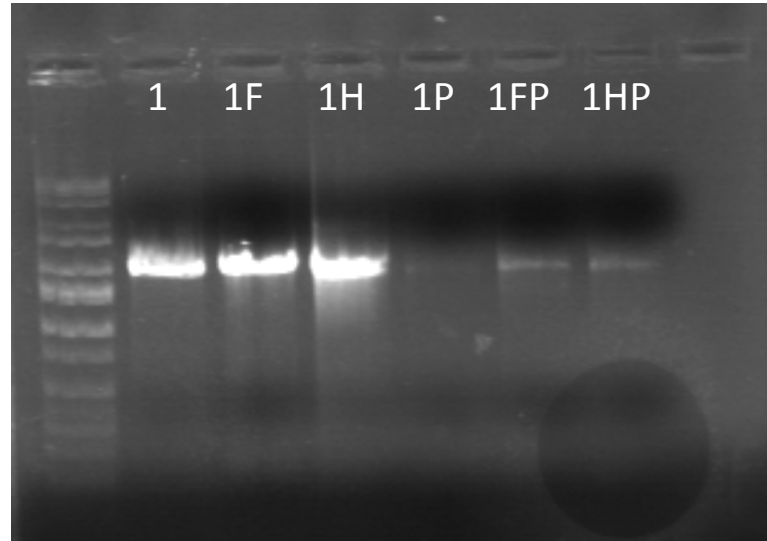
Figure 4.2. (Fotodyne/Analyst Express Systems) Successful expression of *SSA1* using Polymerase's Phusion and PFU; 1 = non tagged Phusion, 1F = FLAG tagged Phusion, 1H = His tagged Phusion, 1P = Non tagged PFU, 1FP = FLAG tagged PFU, 1HP = His tagged PFU.

Figure 4.3 depicts a successful digestion of pchlamiRNA3 and pchlamiRNA3int. Within lanes 3 and 3I, pchlamiRNA3 and pchlamiRNA3int respectively, are present in their undigested circular form. Within lanes 3D and 3ID, pchlamiRNA3 and pchlamiRNA3int respectively, are present in their linear digested form with the digested piece of a molecular weight around 200bp at the bottom of the lane, marking the successful excision. This is important because this allows progression to ligation of *SSA1* into either pchlamiRNA3 or pchlamiRNA3int.
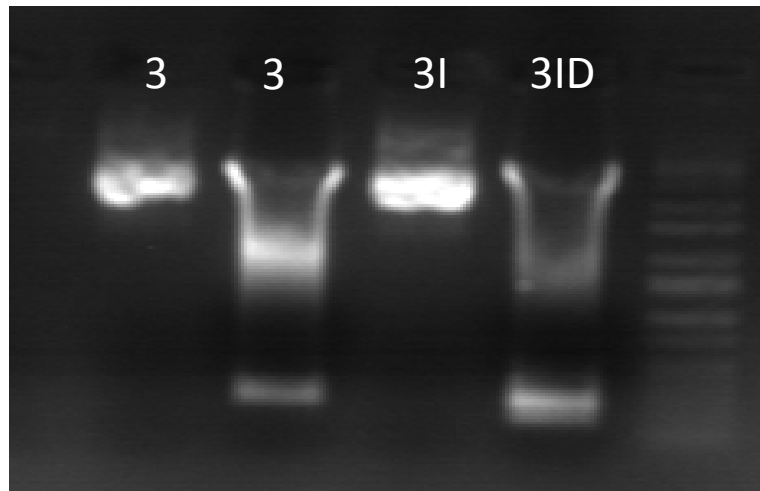
Figure 4.3. (Fotodyne/Analyst Express Systems) Successful digestion of plasmid pchlamiRNA3 and digestion of plasmid pchlamiRNA3int; 3 = non-digested pchlamiRNA3, 3D = digested pchlamiRNA3, 3I = non-digested pchlamiRNA3int, 3ID = digested pchlamiRNA3int.

Figure 4.4 shows successfully ligated plasmids, with the intensity corresponding to the quantity of *SSA1* present within the *E.coli* transformed. 4F and 6F represent flag tagged *SSA1* in pchlamiRNA3. 5F represents a flag tagged *SSA1* in pchlamiRNA3int. 4H and 6H represent a histidine tagged *SSA1* in plasmid pchlamiRNA3int while 5H represents flag tagged *SSA1* in plasmid pchlamiRNA3. All mixing ratios are described under Ligation Reaction (Appendix A, 49). The plasmid was transformed into *E. coli¸* and grown overnight and then colony PCR was performed. The bands are in the proper space, about 2000bp showing a successful expression of *SSA1*.
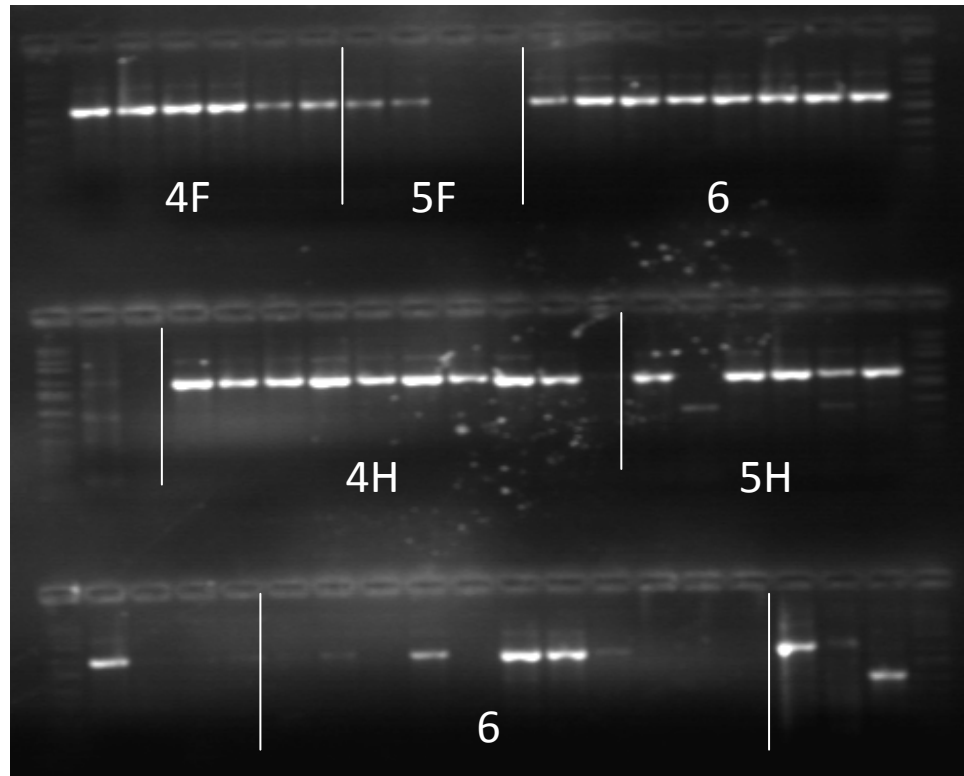
Figure 4.4 (Fotodyne/Analyst Express Systems) Successful expression of *SSA1* ligation in plasmids with molecular tag; 4F, 6F = Flag tagged *SSA1* in pchlamiRNA3, 5F = Flag tagged *SSA1* in pchlamiRNA3int, 4H, 6H = His tagged *SSA1* in pchlamiRNA3int, 5H = Flag tagged *SSA1* in pchlamiRNA3.

4.2 Temperature and pH

Figure 4.5 depicts a typical culture color at time 0, with approximately equivalent quantities of cells added to each flask based on cell count described within material and methods. Throughout the experiments, both S1 and S2 experienced possible contamination from diatoms, which were not removed from the algae prior to temperature experiments.

When comparing room temperature algae at time 0 hr and then at time 96 hrs, a visible color difference can be instantly notice, Figure 4.5 and Figure 4.6. A relatively linear growth rate based on chlorophyll a and b content can be observed from time 0hr to time 96hrs. Also, both figures show natural growth, free from noticeable contamination.
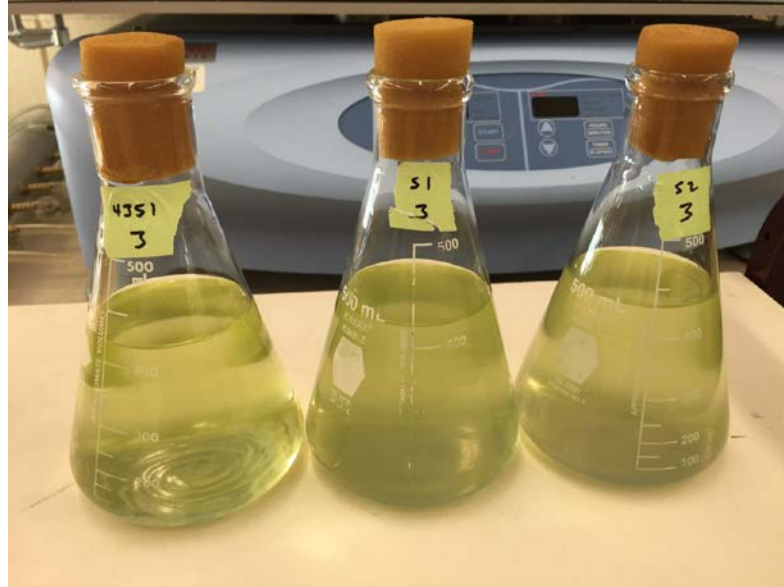
Figure 4.5 Room temperature comparison of algae strains cc-4351, S1, and S2 at the beginning of the experiment (t=0).
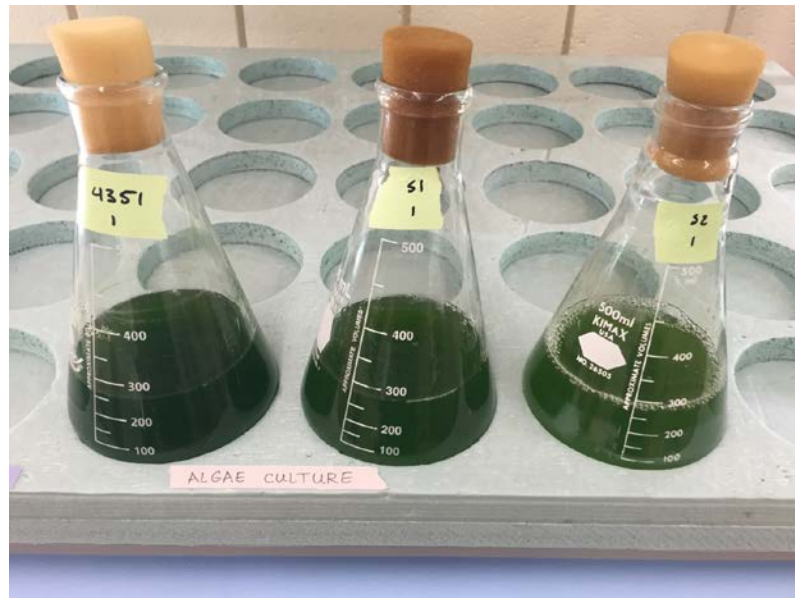
Figure 4.6 Room temperature comparison of algae strains cc-4351, S1, and S2 after 5 days of cultivation (t=96).

Contamination was an issue in multiple experiments, although steps were taken to prevent it, somehow it wrought havoc on the experiments. The circular clumps within flask 4351 b (Figure 4.7) show a form of contamination which was present during one of the experiments. When looking at growth from time 0 hr to time 96 hr (Figure 4.7, Figure 4.8, Figure 4.9) one can see how the circular clumping within cc-4351 inhibited

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optimal growth, skewing the results. When examining the algae flasks of S1 and S2 (Figure 4.9), one can notice a mild white film towards the top edge of media that rotates around the inner brim of the flask. This was the presence of a cohabitating microorganism of unknown specifics. We determined it was a diatom through multiple disinfection attempts including kanamycin, ampicillin, and paramycin at various concentrations up to 300x. There was also a UV sterilization attempt, which completely killed the algae and because the other microorganism was still living, it was proposed that the contaminating microorganism was a diatom.
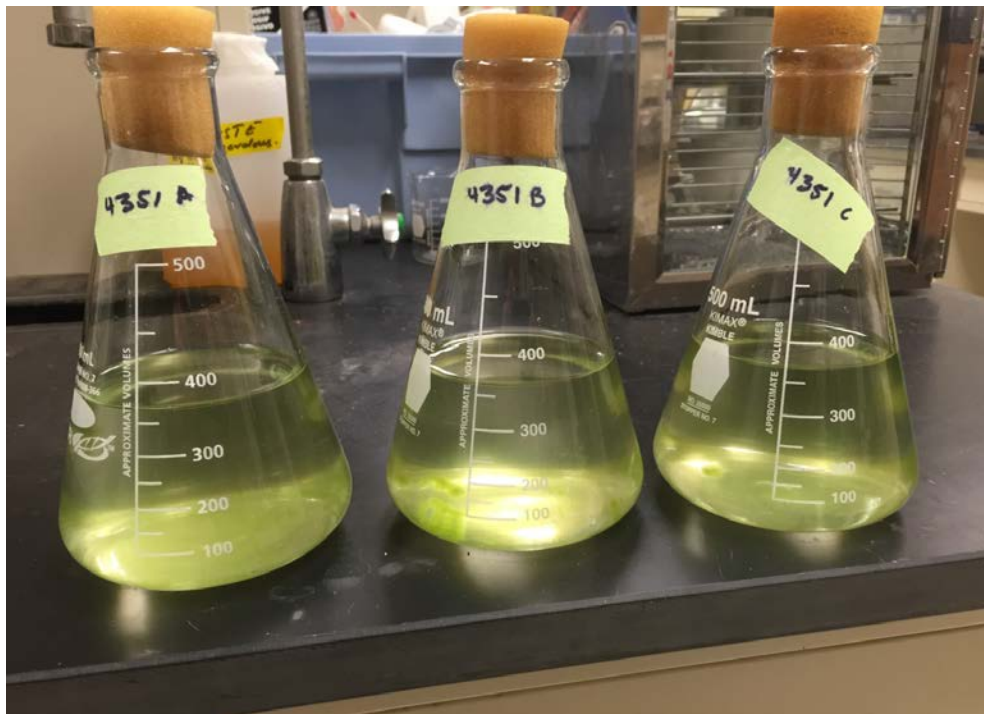
Figure 4.7 Room temperature cultures of cc-4351 on day 3 depicting odd circular algal cellular clumping (t=48).

Figure 4.8 Room temperature growth on day 3 of 5 comparing cc-4351, S1, and S2 during week 1 (t=48).

Figure 4.9 Room temperature growth on day 5 comparing cc-4351, S1, and S2 during week 1 (t=96).

When comparing modified strains to the wild type throughout the various experiments, the data are sporadic and inconclusive. This could potentially be due to the constant contamination issue with S1 and S2. Multiple separation techniques were attempted to remove the contaminant, various concentrations of multiple antibiotic and UV sterilization, to no avail. Ideally, S1 and S2 would be statistically similar to one another in all instances along with cc-4351 within room temperature settings, or at least display some sort of consistency from week to week. When placed in high temperature settings, the growth rate of cc-4351 would ideally be statistically different from S1 and S2, which should be higher; however, that was not the observed case. When comparing chlorophyll a content of cc-4351, S1, and S2 in repetition one, based off a pairwise t-test, all of the growth rates show that each growth rate is statistically different from the other. All growth rates calculated were based off the assumption of linear growth rates, however, that is not the case for some of the data trends.

Figure 4.10 Average chlorophyll a content at room temperature experiment 1. Error bars represent standard error (n=3).

Figure 4.11 Average chlorophyll b content at room temperature experiment 1. Error bars represent standard error (n=3).

Figure 4.12 Average growth rate based on chlorophyll content of strain cc-4351, S1, and S2 at room temperature replication 1. Error bars represent standard error (n=3).

When comparing cc-4351, S1, and S2 in experiment 2, based off a pairwise t-test, chlorophyll a based growth rate displayed each strain being statistically different from the other. When statistically comparing cc-4351 to S1 based on growth rate of chlorophyll b showed statistical similarity. When comparing cc-4351 to S2 based on chlorophyll b growth rate, they show statistical difference similar to S1 and S2. It seems odd that there would be statistical difference in growth rate based on chlorophyll a but not based on chlorophyll b. This could be based on an error within the spectrophotometer reading or the potential that S1, in that instance, produced more chlorophyll b than normal.

Figure 4.13 Average chlorophyll a content at room temperature experiment 2. Error bars represent standard error (n=3).

Figure 4.14 Average chlorophyll b content at room temperature experiment 2. Error bars represent standard error (n=3).

Figure 4.15 Average growth rate based on chlorophyll a content of strain cc-4351, S1, and S2 at room temperature experiment 2. Error bars represent standard error (n=3).

When comparing cc-4351, S1, and S2 in experiment 3 at room temperature, based off a pairwise t-test, cc-4351 showed a statistical difference when compared to S1 for chlorophyll b only. When comparing cc-4351 to S2 there is no statistical difference in growth rate between either chlorophyll a or b. When comparing S1 and S2 statistically, the pairwise t-test concluded S1 and S2 were statistically different. Examining these results leads to questions as to why there would be a statistical difference in growth rate based on two different chlorophyll contents from the same sample. Ideally, S1 and S2 would be statistically similar with each other while there is statistical similarity with cc-4351 across all temperature ranges.
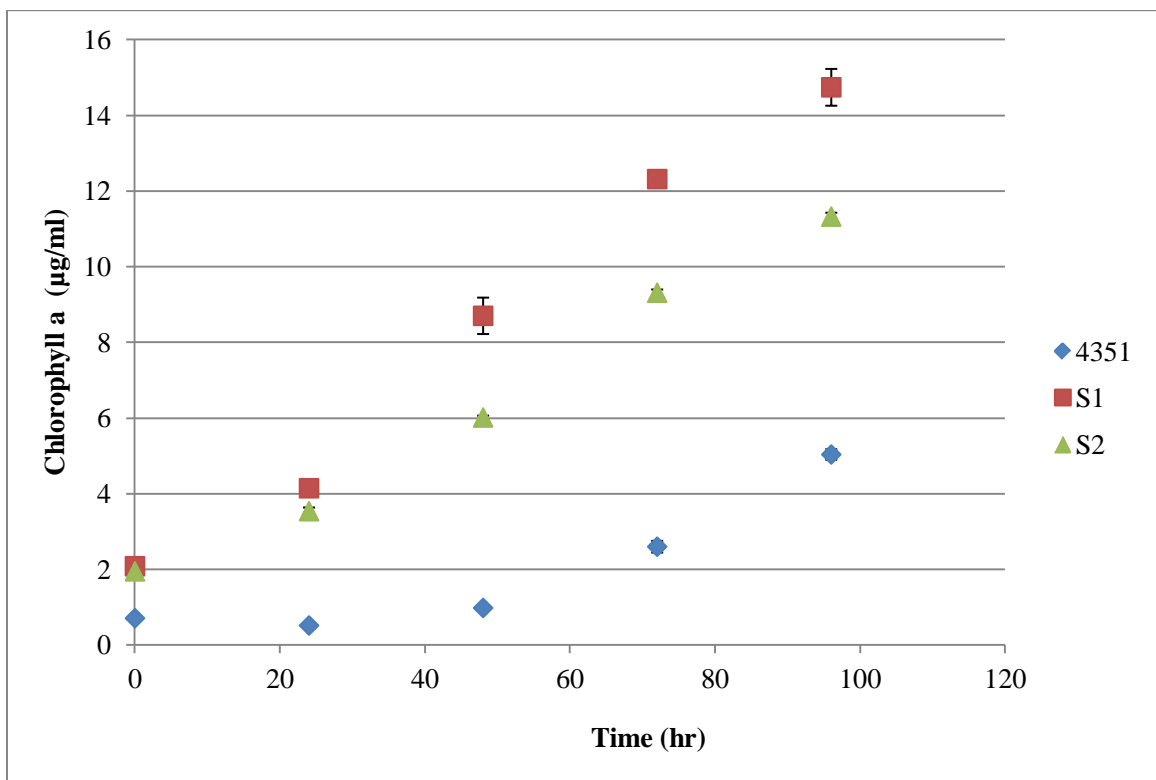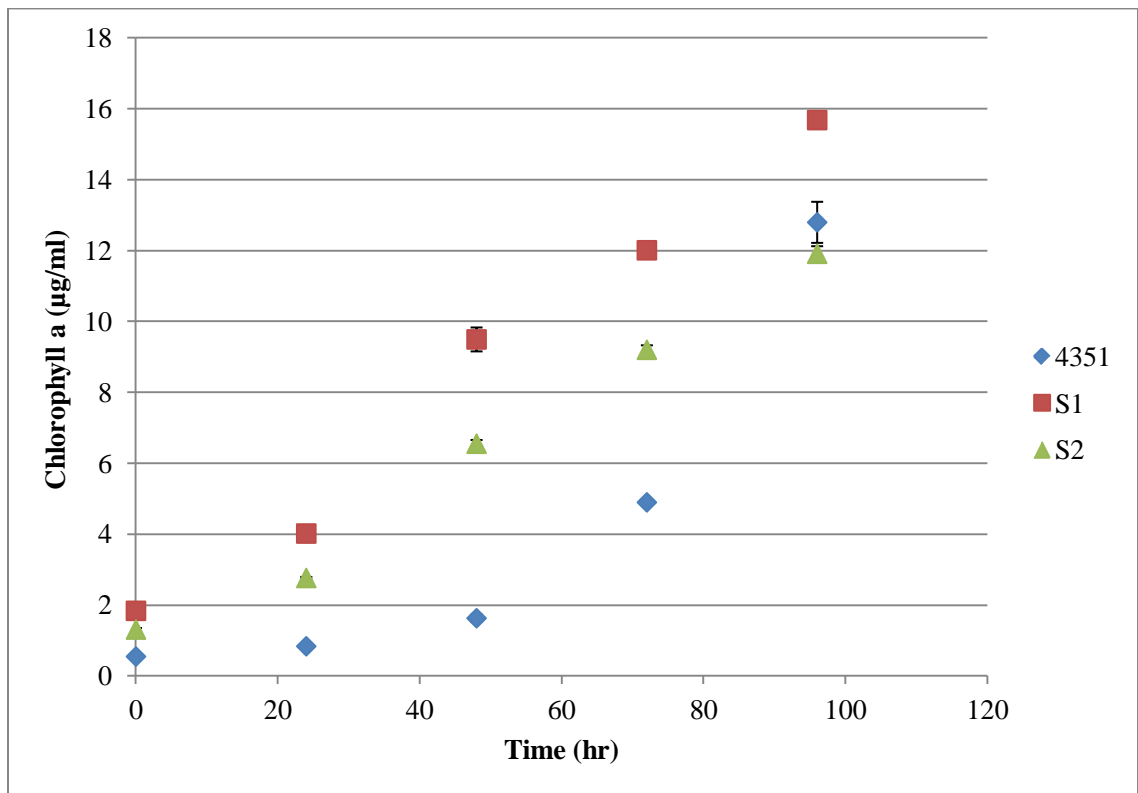

Figure 4.16 Average chlorophyll a content at room temperature experiment 3. Error bars represent standard error (n=3).
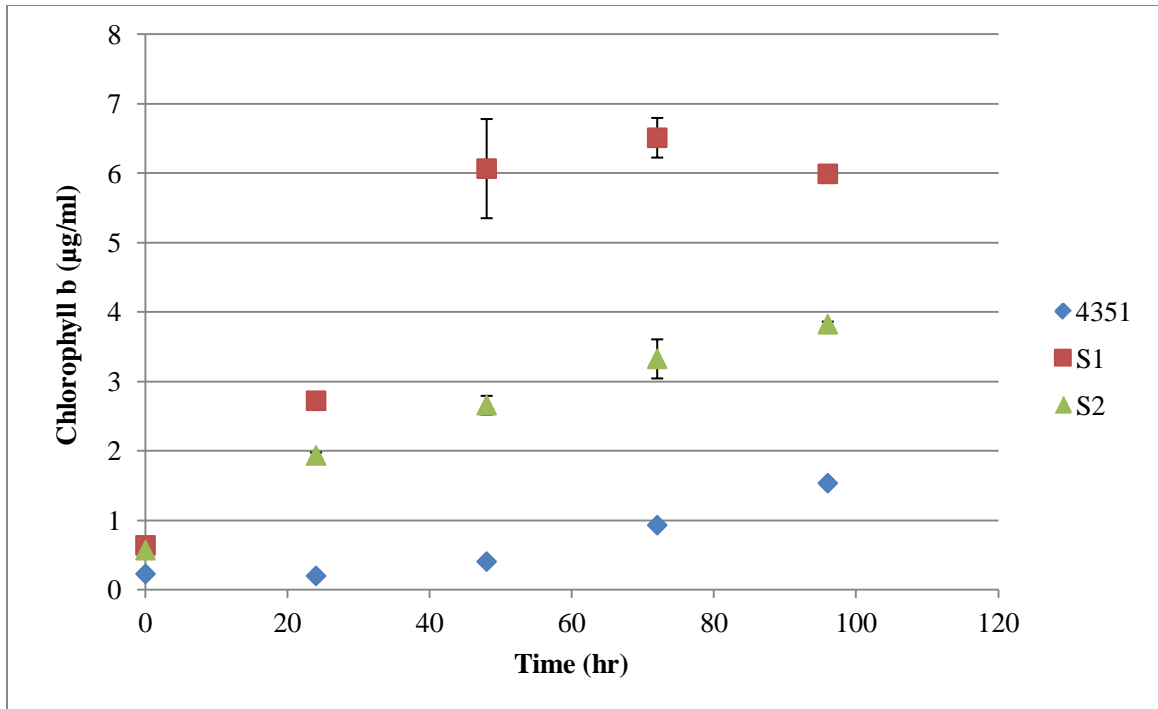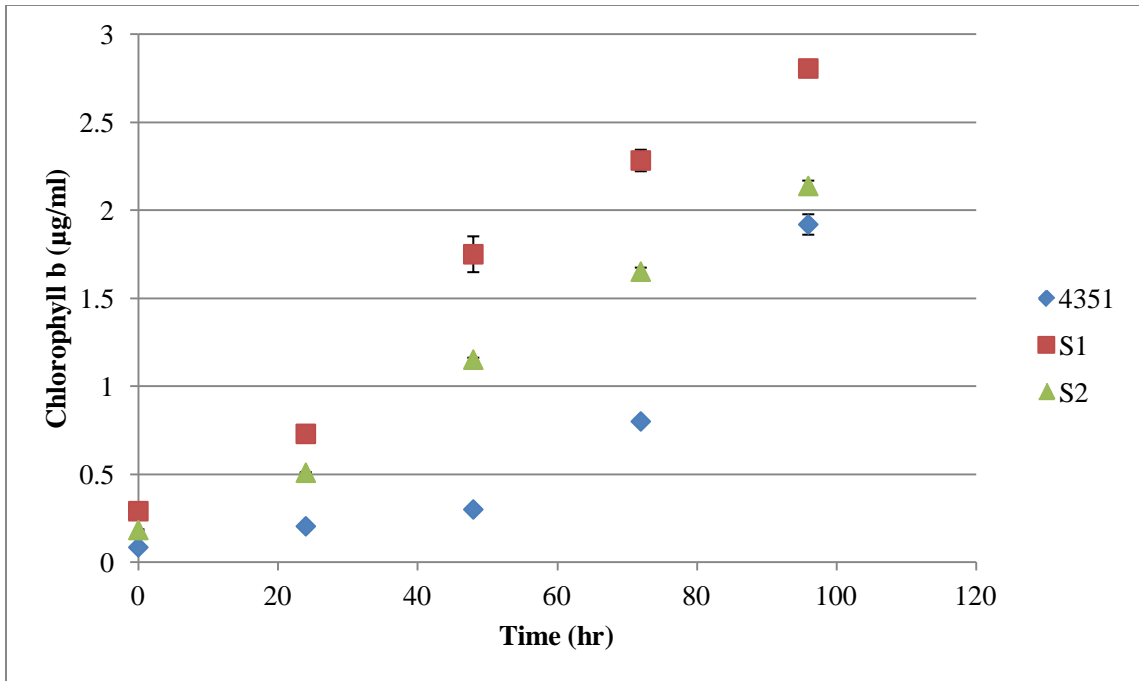
Figure 4.17 Average chlorophyll b content at room temperature experiment 3. Error bars represent standard error (n=3).
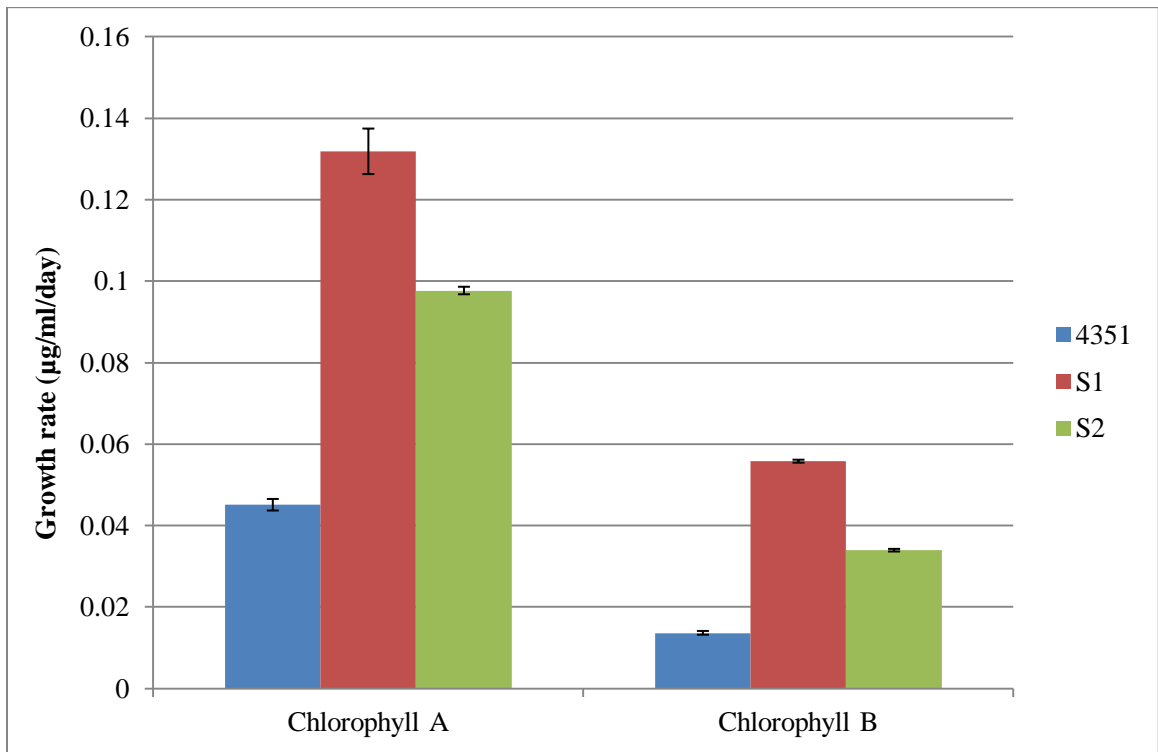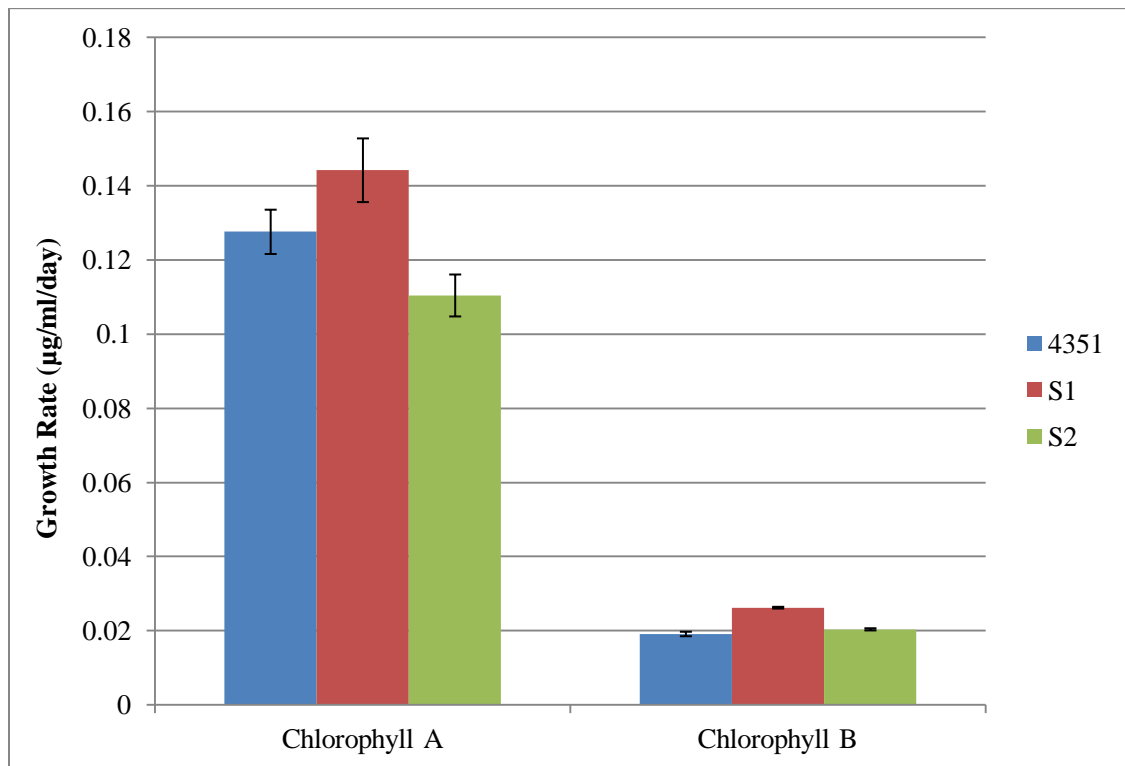

Figure 4.18 Average growth rate based on chlorophyll a content of strain cc-4351, S1, and S2 at room temperature in experiment 3. Error bars represent standard error (n=3).

When comparing cc-4351, S1, and S2 in replication 3 at 30°C, based on a pairwise t-test, neither of the three was different than any than the other, being statistically the same. Only the growth rate based on chlorophyll b showed statistical difference between S1 and S2, which is the exact opposite of what it should be. Ideally, at this temperature there should be a statistical difference between S1 and cc-4351 along with S2 and cc-4351. This could possibly be due to the contamination issues surrounding S1 and S2.
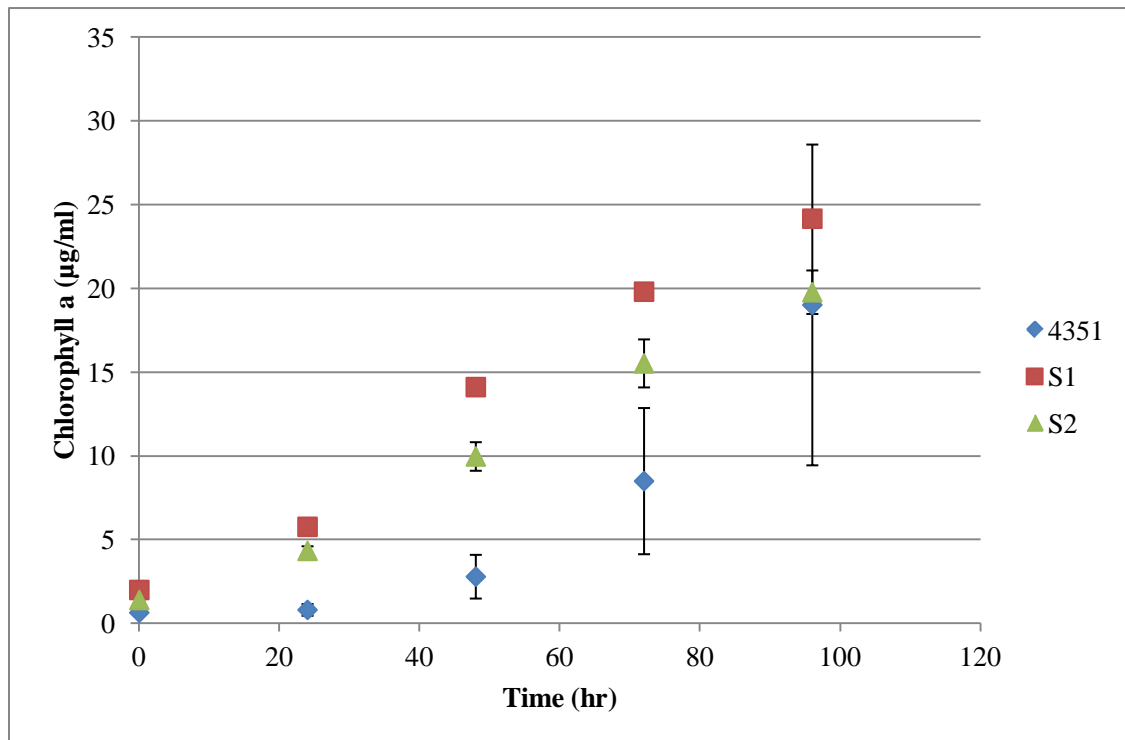
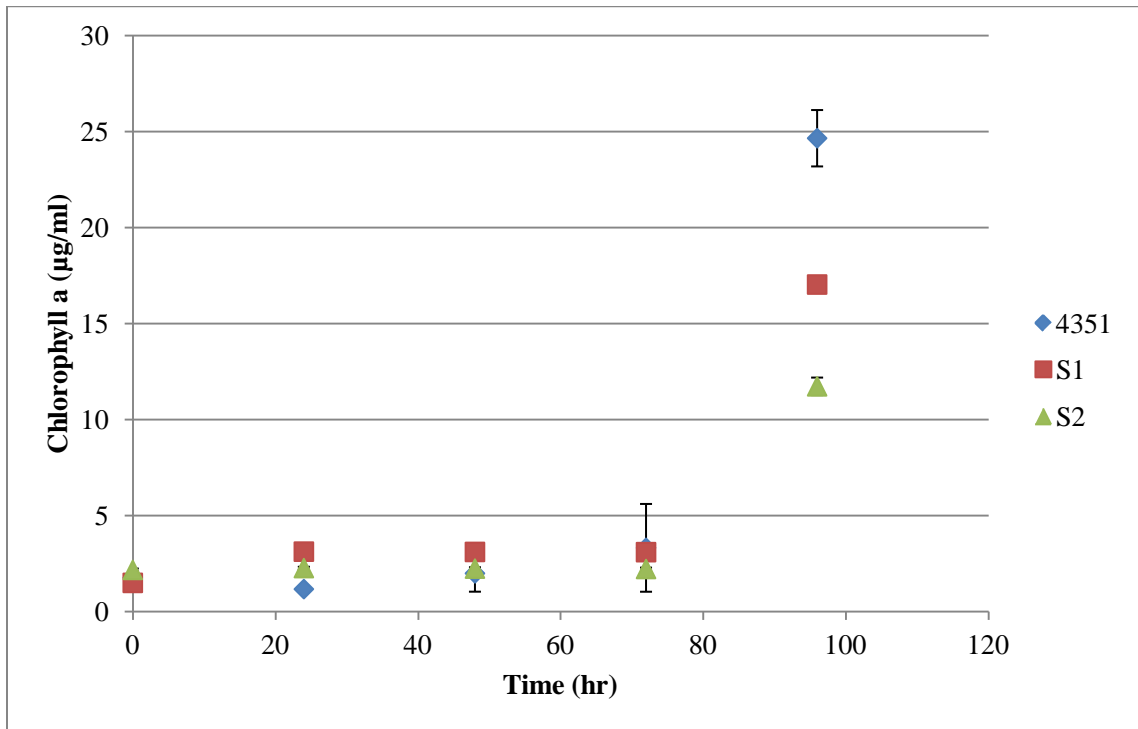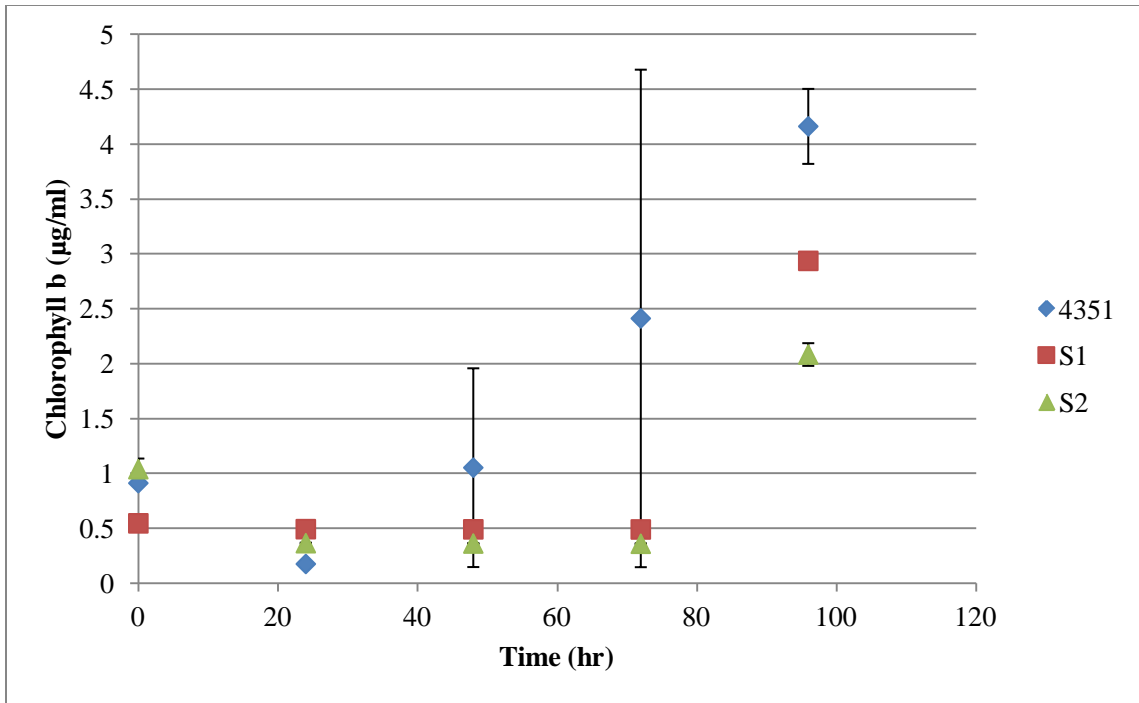**Figure 4.19 Average chlorophyll a content at 30°C experiment 3. Error bars represent standard error (n=3).**
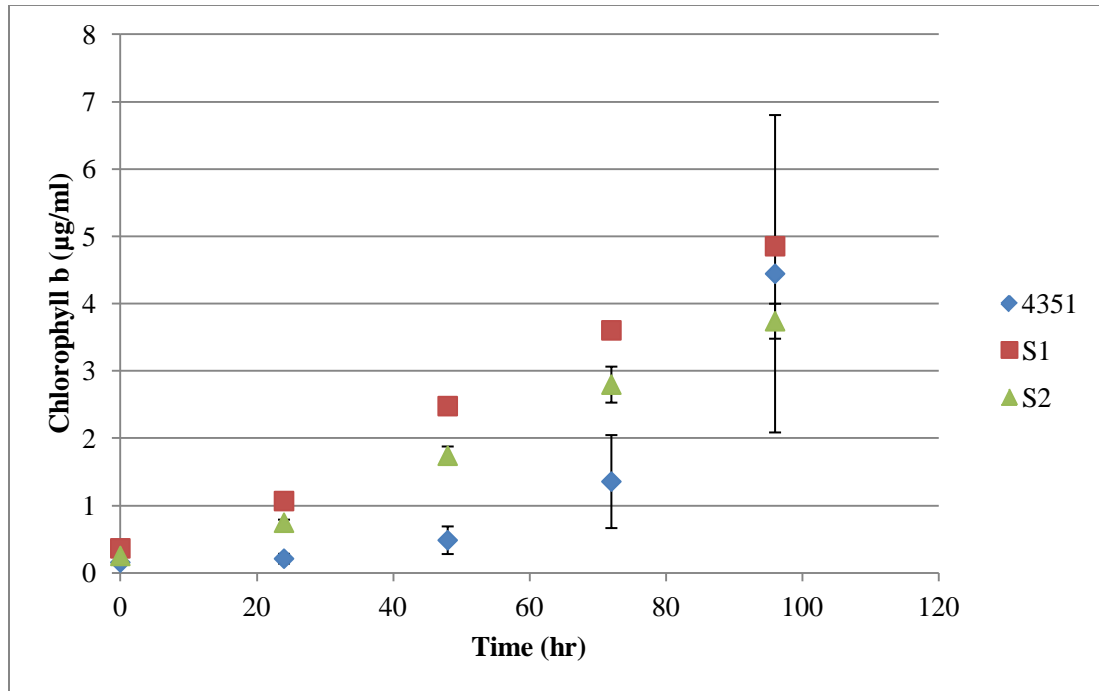
Figure 4.20 Average chlorophyll b content at 30°C in experiment 3. Error bars represent standard error (n=3).

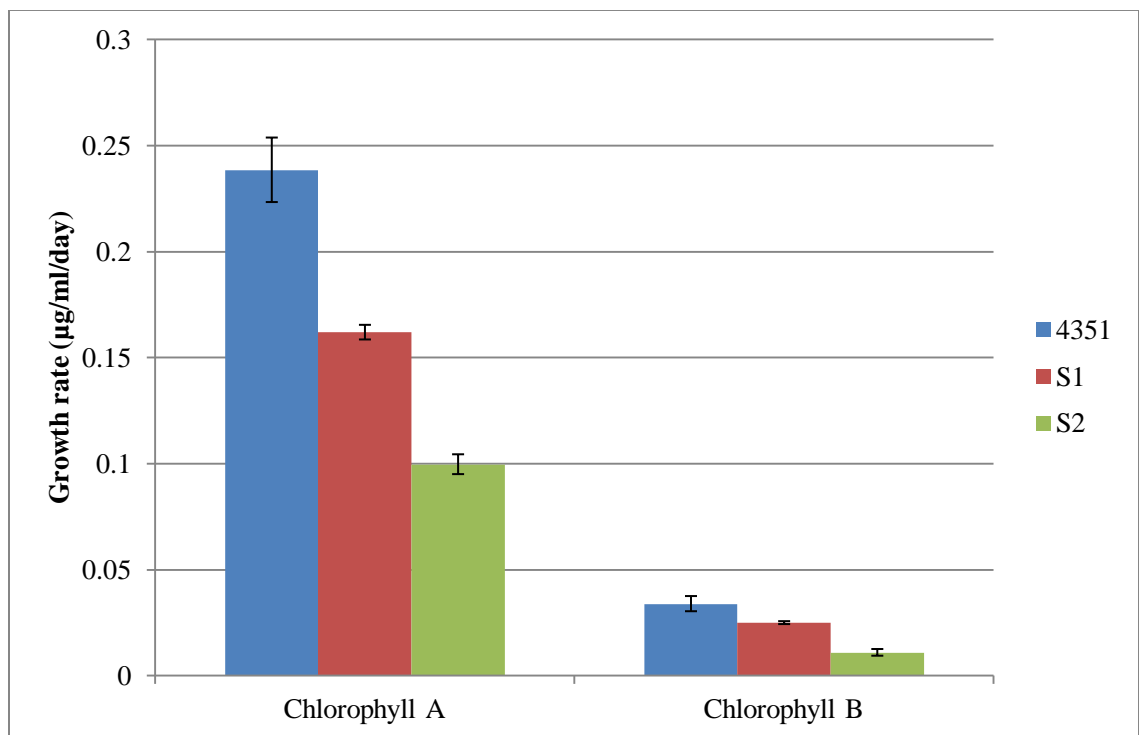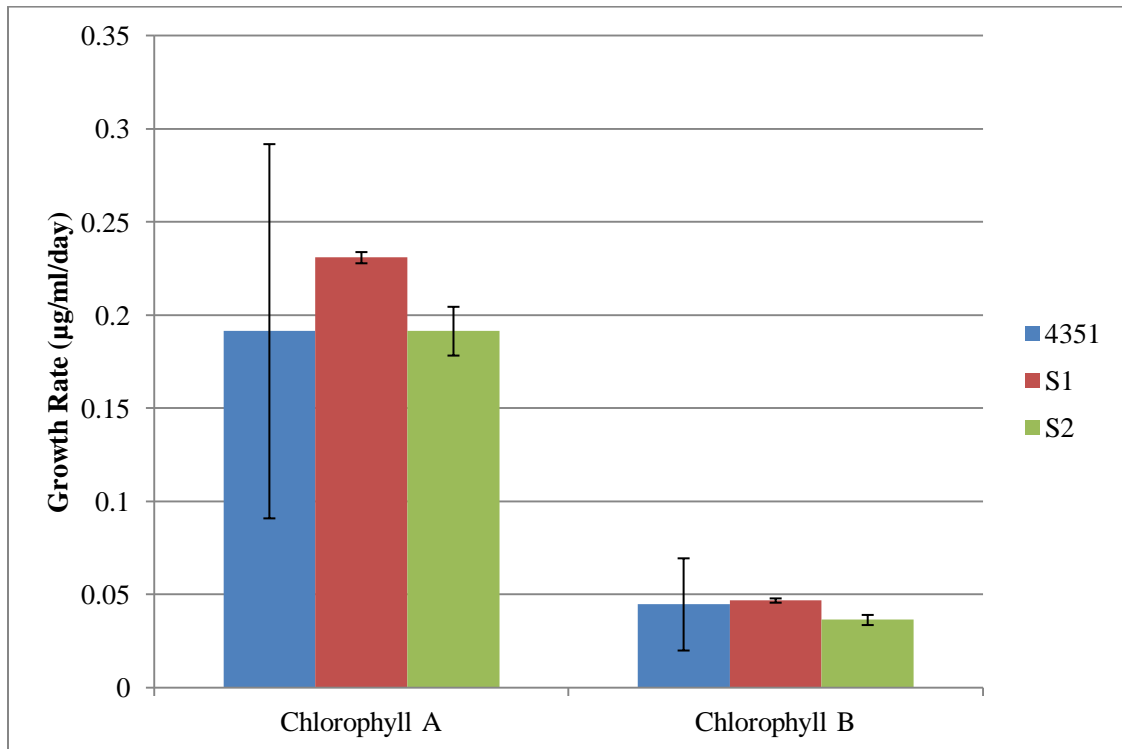**Figure 4.21 Average growth rate based on chlorophyll a content of strain cc-4351, S1, and S2 at 30°C experiment 3. Error bars represent standard error (n=3).**

Figure 4.22 and Figure 4.23 provide comparison between cc-4351, S1, and S2 at various temperatures as well as experiments. Figure 4.22 provides a comparison between chlorophyll a and chlorophyll b at both 22 and 30°C. It's easy to tell from this comparison that at higher temperatures, all strains grew at higher growth rates, but the standard error associated with 4351 chlorophyll a reading is staggering. This was due to one of the flasks having severe contamination issues, causing absolutely no algae growth. A repetition of this experiment could yield much better results. Figure 4.23 provides a comparison of cc-4351, S1, and S2 throughout all room temperature ranges, displaying the large level of variability from week to week. cc-4351 displays the most drastic changes in growth rate from week to week.
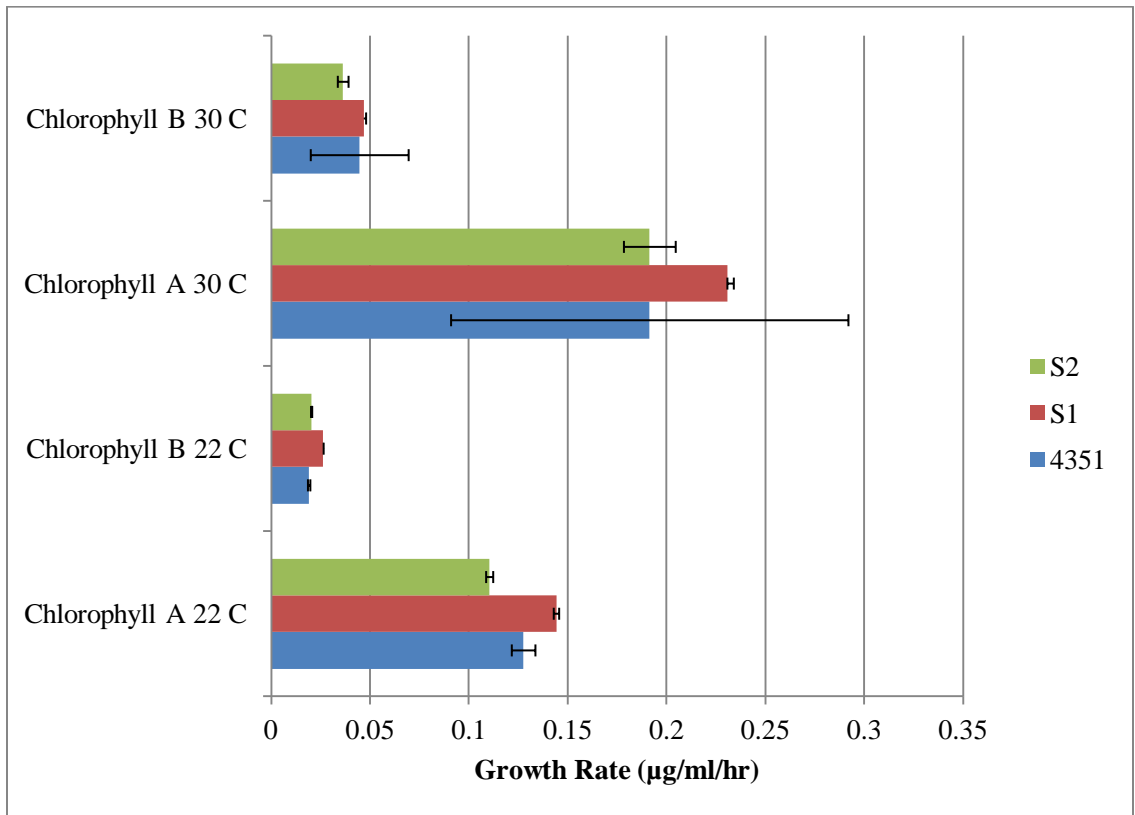
Figure 4.22 Average growth rate based on chlorophyll a of strain cc-4351, S1, and S2 at 25 and 30°C based on chlorophyll a and b. Error bars represent standard error (n=3).
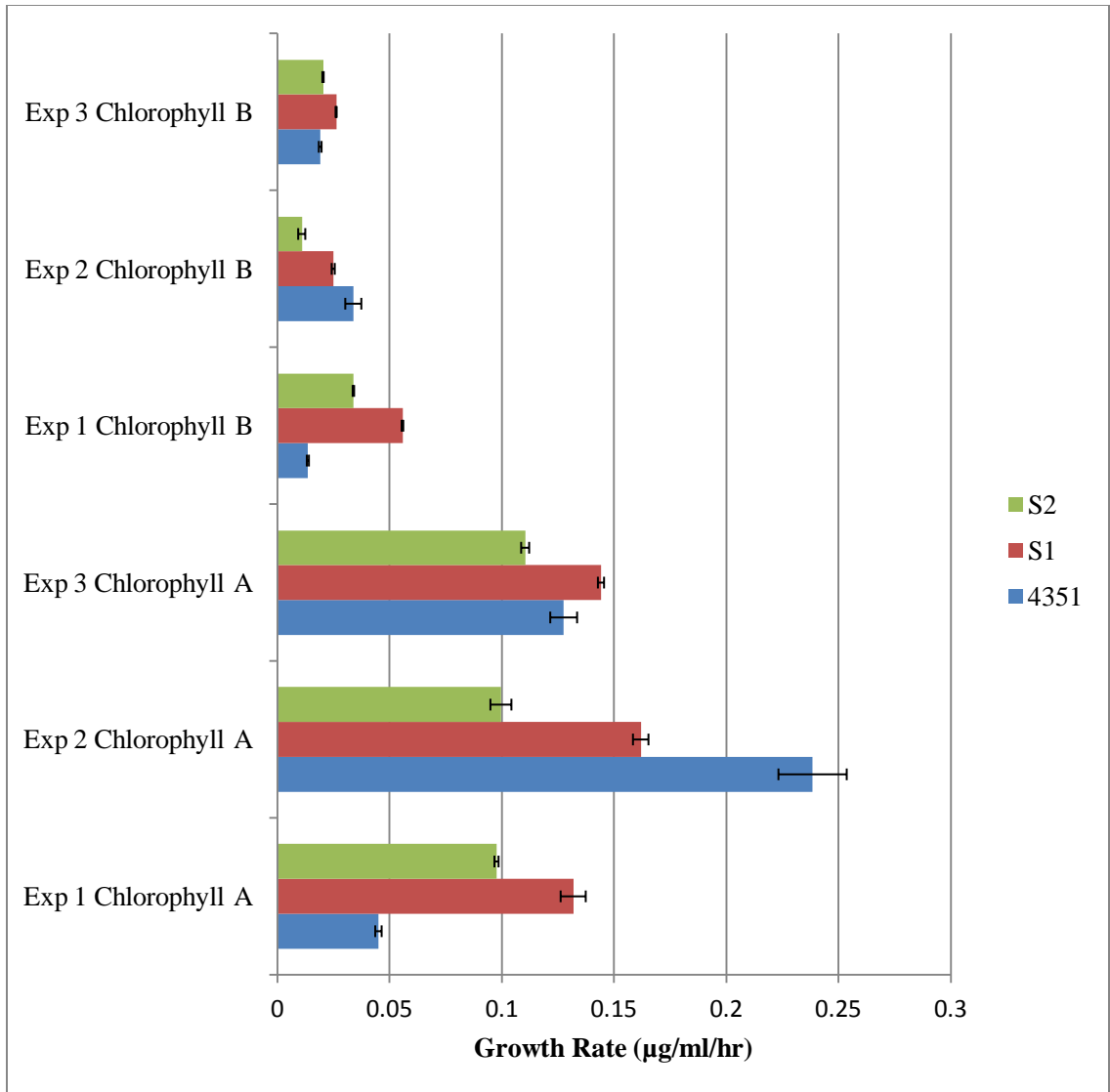
Figure 4.23 Room temperature growth rate comparison based on chlorophyll a content of strains cc-4351, S1, and S2 over three different experiments. Error bars represent standard error (n=3).

CHAPTER 5:   CONCLUSIONS

The potential use of algae as a carbon mitigation/storage system adjacent to coal-fired power plant presents a viable option if certain obstacles are hurdled. Optimal growth conditions are not presented by the flue gas produced via coal processing, being too hot and causing acidic pH changes in algae media. Initial approaches took the route of modifying preprocessing before adding the gas to the algal system, but, given current technological provisions, close to optimal conditions have been met. An alternative approach to this predicament is the modification of the carbon capturing organism, in this case, algae. The ability to adapt algae to specific environments or alternative environments present economic potential within an industrial setting, including but not limited to; textiles, building materials, cosmetics, biofuels, and biopharmaceutical compounds. The land usage comparison amongst other potential energy crops, or carbon mitigation crops, puts algae at another advantage. The liquid cultured species can be grown on a fraction of the land while producing alternative products not concerned with food commodity interference. In addition, particular species of algae have been documented to be 50% oil by weight, and if starved of nitrogen, even higher percentages. This is an extremely attractive feature for this organism as an alternative fuel producer. Within this experiment, in attempts to produce a more economical carbon capturing mechanism, algae heat shock protein production was the target mechanism of action.

HSP70B within pCB720 and pCB740 causes expression of a presently expressed protein within algae, but ideally at higher levels within transformed algae. This higher expression can be monitored by protein blotting, PCR, or RT-PCR. Large scale testing would be focused on temperature and pH testing. This would entail pH and temperature testing similar to those experiments found within this document along with photobioreactor experimental testing. Experiments within this thesis covered two separate transformations of plasmid pCB720 into algae strain cc-4351 resulting in S1 and S2. Both transformations became contaminated, and could not be isolated into monocultures again, even after numerous varied separation techniques. Multiple

transformation attempts occurred after the initial success of pCB720 and pCB740 with no success after.

Regarding temperature and pH testing, growth rates calculated based on slope were unreliable, and under the assumption of a linear growth pattern, very incorrect. Some experiments had strains with chlorophyll a being statistically different but chlorophyll b being statistically the same. Peak chlorophyll content after 96 hours, from experiment to experiment, displayed variability, potentially due to contamination or varying initial inoculation concentration, leading to inconclusive data. Large amounts of standard error occurred within some experiments due to circular algae formations, a form of contamination, or contamination which completely inhibited algae growth. Within the elevated temperature experiment, the unmodified strain performed much better, however the statistics disagree. If the highly contaminated flask of unmodified algae was removed from the data collection, the results could be very different.

With molecular work completed in the design and creation of plasmids pchlamiRNA3 and pchlamiRNA3int containing *SSA1*. The next step would be transformation of the modified pchlamiRNA3 and pchlamiRNA3int into algae of choice. These modified plasmids are ready for expression within *Chlamydomonas reinhardtii* and should be producing *SSA1* at a recognizable level, allowing better growth rate at higher temperatures and lower pH's. Detection of *SSA1* could be performed through PCR, RT-PCR (although not needed), and protein blotting. Once transformed and adequate results collected and concluded, pH and temperature comparisons could be started to determine large scale effects of yeast heat shock protein presence.

CHAPTER 6:   FUTURE WORK

Regarding this approach to increasing carbon capturing capabilities of microalgae adjacent to coal-fired power plants, further molecular work must be accomplished. Molecular work to be done would include the successful transformation of pCB720, pCB740, pchlamiRNA3 with *SSA1* insert, and pchlamiRNA3int with *SSA1* insert into *Chlamydomonas reinhardtii*. Once inserted, the back end work would begin, including but not limited to; temperature testing, pH testing, positive identification of plasmid insert within pchlamiRNA3 and pchlamiRNA3int, and quantification of HSP70B expression compared to a control in real time.

Temperature testing would require comparing the modified algal strains to a control strain, measuring either dry weight of algae or chlorophyll content, depending on the size of the algae. Adequate replicates would be required to ensure data statistics. Once temperature comparisons and thresholds were established, pH test would need to be performed comparing the modified strain to the unmodified strain. Ideally, the modified strain would perform better at lower pH that the unmodified strain. This would be determined by either chlorophyll content or dry weight measurement, depending on algae size. These would be the large scale lab tests of the modified algae, somewhat comparable to photo bioreactor scale rather than examining molecular expression.

Identification of insert upon successful transformation into algae would be made possible through molecular tagging of the inserted gene. This allows a positive confirmation of the genes presence by multiple methods, depending on the tag. RT-PCR would provide a real time analysis of gene expression comparison, allowing quantitative conclusions to be drawn. The molecular work required to ensure the inserted gene is present, active, and quantifying that activity will be demanding but doable. Once these analyses have been completed, the required remaining work involves large cultivation. The foreseeable future includes large scale monoculture becoming an issue, the ability to isolate organisms is incredibly tough in a large scale environment.

With the plasmid design completed, transformation of designed plasmids into algae would be the next step. This, once completed, would require a tremendous amount

of back end work to verify and support insertion, expression, and any potential hypothesis to be drawn. If the transformation of plasmids pchlamiRNA3 and pchlamiRNA3int into *Chlamydomonas reinhardtii* continue to fail, a new direction could be the attempted transformation of the same plasmids into a native strain of algae. This way, if transformed successfully, it would be more readily useable once testing was completed. Also, there are alternative methods to algae transformation other than glass bead that could and should be explored if this is to work. In addition to the back end molecular work, quantifying J-protein expression along with ATP usage upon over expression of HSP70 would be interesting. The over expression of HSP70 could result in the pulling of ATP from other energy dependent reactions or there could be a bottle neck affect due to the overabundance of HSP70 but lack of J-protein production. Essentially, a systems biology approach to this potential new expression could be analyzed, providing insight into the pathway flow rates. It could answer questions of expression rates and potential genetic modification avenues for the future.

APPENDICES

Appendix A. Media/Solution Recipes

Solution 1
1. 93 mL sterile distilled water
2. 2.5 mL glucose (dextrose) sterile
3. 2.5 mL 2M TRIS pH 8.0
4. 2.0 mL 0.5 M EDTA pH 8.0

Solution 2
1. 8.6 mL dH_2O
2. 0.4 mL 5M NaOH (add last)
3. 1 mL 10% SDS

Solution 3
1. 115.62 g ammonium acetate
2. Fill with distilled water to 200 mL

Restriction Enzyme Digestion of Miniprep Plasmid DNA
1. 1 µL 10x restriction enzyme buffer
2. 0.1 µL Rnase A (1mg/mL)
3. 0.1 µL BSA if needed (stock is 100x)
4. 2 µL miniprep plasmid DNA
5. Y µL 2U restriction enzyme (0.1 µL from 20,000U enzyme) for each restriction enzyme
6. X µL sterile distilled water to make total reaction mixture 10 µL

100 µL PCR Reaction
1. 10 µL 10x Buffer
2. 3 µL dNTP's
3. 1 µL Forward Primer
4. 1 µL Reverse Primer
5. 1 µL Template DNA
6. 3 µL DNA Polymerase of choosing
7. 82 µL DI Autoclaved Water

20 µL PCR Reaction
1. 1 µL Template DNA
2. 0.5 µL Forward Primer
3. 0.5 µL Reverse Primer
4. 2 µL dNTP's
5. 2 µL Buffer

6. 0.2 µL DNA Polymerase
7. 13.8 µL DI Autoclaved Water

Ligation Reaction
1. 2 µL 10X T4 DNA Ligase Buffer
2. 50 ng (0.020 pmol) Vector DNA (4 kb)
3. 37.5 ng (0.060 pmol) Insert DNA (1kb)
4. 1 µL T4 DNA Ligase (added last)
5. Nuclease-free water (add enough to make solution 20 uL)

One Liter 50X stock of TAE
1. 242 g Tris-base
2. 57.1 mL Acetate
3. 100 mL 0.5M sodium EDTA
4. Add distilled water up to one L

Spheroplasting Buffer (10 mL)
1. 1.9 mL sterile distilled Water
2. 6 mL 2M sorbitol
3. 1 mL 0.5 M EDTA
4. 1 mL beta-mercaptoethanol
5. 10 mg zymolyase/lysozyme (prepare only as much as needed for experiment due to instability)

Proteinase K Buffer (10 mL)
1. 8.9 mL sterile distilled water
2. 1 mL 0.5 M EDTA
3. 0.3 mL 10% SDS
4. 50 µL 20 mg/mL proteinase K

Oligonucleotide Design
----- *SSA1/SSA2* ----- FLAG tag ----- HIS tag ----- Restriction Enzyme Site

1. *SSA1*
   a. Forward Oligo 5' to 3' (Nde1)
      i. GGCC CATATG CATCATCATCATCATCAT ATGTCAAAAG CTGTCGG
      ii. GGCC CATATG gattacaaggatgacgacgataag ATGTCAAAAG CTGTCGG
   b. Reverse Oligo 5' to 3' (Xba1)
      i. GGCC TCTAGA TTAATCAAC TTCTTCAACG
2. New *SSA1* Primer Design without restriction enzymes or tags
   a. Forward Oligo 5' to 3'
      i. ATGTCAAAAG CTGTCGGTAT
   b. Reverse Oligo 5' to 3'

     i. <mark>TTAATCAAC TTCTTCAACG</mark>

3. SSA2
  a. Forward Oligo 5' to 3' (Nde1)
    i. Ggcc CATATG CATCATCATCATCATCAT <mark>ATGTCTAAAG CTGTC</mark>
    ii. Ggcc CATATG gattacaaggatgacgacgataag <mark>ATGTCTAAAG CTGTC</mark>
  b. Reverse Oligo 5' to 3' (Xba1)
    i. Ggcc TCTAGA <mark>TTAATCAACT TCTTCGACAG</mark>

4. New Primer Design
  a. Forward Oligo 5' to 3'
    i. <mark>ATGTCTAAAG CTGTCGGTAT</mark>
  b. Reverse Oligo 5' to 3'
    i. <mark>TTAATCAACT TCTTCGACAG</mark>

Bolds Basal Medium

This medium is highly enriched and is used for many of the green algae.
Reference: Stein, J (ED.) Handbook of phycological methods. Culture methods
and growth measurements. Cambridge University Press. 448 pp.

Stock	Stock Solution	mL/L
KH_2PO_4	8.75 g/500 mL	10 mL
$CaCl_2\ 2H_2O$	1.25 g/500 mL	10 mL
$MgSO_4\ 7H_2O$	3.75 g/500 mL	10 mL
$NaNO_3$	12.5 g/500 mL	10 mL
K_2HPO_4	3.75 g/500 mL	10 mL
NaCl	1.25 g/500 mL	10 mL
$Na_2EDTA\ 2H2O$	10 g/L	1mL
KOH	3.6 g/L	
$FeSO_4\ 7H_2O$	4.98 g/L	1 mL
H_2SO_4 (Concentrated)	1mL/L	
Trace Metal Solution	See Below	1 mL
H_3BO_3	5.75 g/500 mL	0.7 mL

Trace Metal Solution	
Substance	g/L
H_3BO_3	2.86 g
$MnCl_2\ 4H_2O$	1.81 g
$ZnSO_4\ 7H_2O$	0.222 g
$Na_2MoO_4\ 2H_2O$	0.390 g
$CuSO_4\ 5H_2O$	0.079 g
$CO(NO_3)_2\ 6H_2O$	0.0494 g

Dissolve each of the above substances separately prior to adding the next on the list.
Add MES to bring pH down accordingly.

TAP Medium

From Gorman, D.S., and R.P. Levine (1965) *Proc. Natl. Acad. Sci. USA* **54,** 1665-1669

This is probably the most widely-used medium at present for experimental work.

Make the following stock solutions:

TAP Salts Compound	Mass (g)
NH_4Cl	15.0 g
$MgSO_4$ 7H2O	4.0 g
$CaCl_2$ 2H2O	2.0 g
Water to 1 liter	

Phosphate Solution Compound	Mass (g)
K_2HPO_4	28.8 g
KH_2PO_4	14.4 g
Water to 100 mL	

Hunter's Trace Elements (ordered)

To make final medium, mix the following:

2.42 g Tris Buffer
25 mL Tap Salts Solution
0.375 mL Phosphat Solution
1.0 mL Hunters Trace Elements Solution
1.0 mL Glacial Acetic Acid
Water to 1 L

For solid medium, add 15g agar per liter

Autoclave

Autoclave Liquid Cycle Parameters	
One Container Liquid Volume (mL)	Minimum Recommended Sterilize Time at 121°C (minutes)
75	25
250	30
500	40
1000	45
1500	50
2000	55
>2000	55+10min/L

For Tris-minimal medium omit the acetic acid and titrate the final solution to pH 7.0 with HCl.

Ligation Reaction

Name	Buffer (µl)	Ligase (µl)	Insert (µl)	Plasmid (µl)	Tag	Plasmid
6F	2	1	6	1	Flag	3
5F	2	1	5	2	Flag	3I
4F	2	1	4	3	Flag	3
6H	2	1	6	1	His	3I
5H	2	1	5	2	His	3
4H	2	1	4	3	His	3I

Appendix B. Experimental Methods

Thermal Cycler Protocol to Amplify *SSA1*

Denaturing	Denaturing	Annealing	Elongation	Stabilization	Stopping Temp
1 Cycle	35 Cycles			1 Cycle	
98°C	98°C	37°C	72°C	72°C	4°C
3 min	30 sec	30 sec	1:30 min	7 min	∞

Chlamydomonas reinhardtii Transformation

1. Grow a 200 mL culture of an arginine-requiring strain in TAP media supplemented with 100µg/mL arginine under continuous light to a density of about 5×10^6 cells/mL or higher.
2. Transfer the culture to a sterilized GSA tube (70% EtOH, dry well is fine) and centrifuge for 5 minutes at 4000 rpm and 20°C.
3. Resuspend the cells in TAP to a density of 3×10^8 cells/mL (with 5×10^6 cells/ml in original culture, this is 3.3 mL TAP medium, i.e. sufficient for 10 transformations).
4. Place about 300 ng of pCB740 DNA into a 2-mL Eppendorf tube containing 0.3 g of glass beads (Sigma G-9268; beads need to be washed for 2-3 days with chromo-sulfuric acid and then many times with distilled water until pH is neutral again, then dry them at 80°C, fill in 0.3 g in each tube and autoclave them with lids slightly open).
5. Add 330 µL of the concentrated cell suspension into the glass beads-containing Eppendorf tube and vortex for exactly 15 seconds at highest speed.
6. Add another 300 µL TAP into the tube, mix and plate 200 µL onto a TAP plate, and the remaining onto another plate (spread the cells gently, they are cell wall-less, let the plates become dry, then seal with parafilm).
7. Transformations should be visible after one week.

Plasmid Transformation into Competent Cells

1. Place 50 µL of distilled water into an Eppendorf tube and add plasmid.
2. Add 1 µL of plasmid DNA into the dh5α E. coli competent cells and let set on ice for 20 minutes.
3. After 20 minutes on ice, place the plasmid DNA and competent cells in 42°C for 30 seconds, and no more, as it will potentially denature the competent cells to much.
4. Remove the competent cells from the 42°C water bath and immediately add 50 mL of LB broth.
5. Then let the solution set for 1 hour at 37°C.
6. Plate the solutions, letting extra solution evaporate, leaving a dry plate.

Plasmid DNA Extraction from *E. Coli* (Miniprep)

1. Pick a single bacterial colony from a plate with a sterile pipette tip and place it into a cell culture tube containing about 2 mL 2YT broth + ampicillin (appropriate antibiotic) (1 µL ampicillin from the 100 mg/mL ampicillin stock for 1 mL 2YT broth)
2. Shake cell culture tubes at 37°C at approximately 250 rpm
3. Pour about 1.5 mL of the bacterial culture into an Eppendorf tube and centrifuge at 6000rpm for 30 seconds to a minute, creating a cell pellet.
4. Discard supernatant. It's critical to remove 2YT broth because it contains a lot of NaCl and it can interfere with restriction digestion. Turn tubes upside down on a paper towel for a few seconds, then gently tap them upside down on a paper towel
5. Add 100 µL Solution 1 to pellet.
6. Resuspend pelleted *E. Coli* in 100 µL Solution 1 by bump vortexing.
7. Add 200 µL solution 2 to lyse cells and invert tube 6 times.
8. Incubate the solution at room temperature for 5 minutes, and no more.
9. Add 150 µL solution 3 to bind cellular proteins and invert tube 6 times.
10. Incubate on ice for 5 minutes where white flakey stuff should appear.
11. Centrifuge the solution at max rpm for 10 minutes and extract (approximately 350 µL) the supernatant (clear liquid at top) which contains the plasmid DNA and place in a new Eppendorf tube.
12. Add 700 µL absolute ethanol, invert tubes 6 times, centrifuge at max rpm for 10 minutes, and then discard all ethanol completely. Removal of ethanol is extremely important, turn the tube upside down and remove all excess ethanol through tapping of the tube.
13. Add 800 µL 70% ethanol, invert tubes 6 times, centrifuge at max rpm for 10 minutes, and then discard. It is once again very important to completely remove all ethanol within this step. Keep tubes upside down on a paper towel and then tap tubes on paper towel to get rid of the ethanol wash completely.
14. Resuspend the DNA in a storage solution of either distilled water or TE buffer depending on how long you wish to preserve the DNA.

Thermo Scientific GeneJET Plasmid Miniprep Kit

1. Resuspend the pelleted cells in 250 µL of the resuspension solution then transfer the ell suspension to a micocentrifuge tube. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.
2. Add 250 µL of the lysis solution and mix thoroughly by inverting the tube 4-6 times until the solution become viscous and slightly clear.
3. Add 350 µL of the neutralization solution and mix immediately and thoroughly by inverting the tube 4 to 6 times.
4. Centrifuge for 5 minutes to pellet cell debris and chromosomal DNA.

5. Transfer the supernatant to the supplied GeneJET spin column by decanting or pipetting. Avoid disturbing or transferring the white precipitate.
6. Centrifuge for 1 minute, Discard the flow-through and place the column back into the same collection tube.
7. Add 500 µL of the wash solution (diluted with ethanol prior to first use) to the GeneJET spin column. Centrifuge for 30-60 seconds and discard the flow-through. Place the column back into the same collection tube.
8. Repeat the wash procedure using 500 µL of the wash solution.
9. Discard the flow-through and centrifuge for an additional 1minute to remove residual wash solution. This step is essential to avoid residual ethanol in plasmid preps.
10. Transfer the GeneJET Spin column into a fresh 1.5 mL microcentrifuge tube. Add 50 µL of the elution buffer to the center of GeneJET spin column membrane to elute the plasmid DNA. Take care not to contact the membrane with the pipette tip. Incubate for 2 minutes at room temperature and centrifuge for 2 minutes.
11. Discard the column and store the purified plasmid DNA at -20°C.

Axygen Biosciences PCR Clean-up Spin Protocol
1. Add a 3x reaction volume of buffer PCR-A to the sample. If the required volume of Buffer PCR-A is less than 100 µL, add 100 µL of Buffer PCR-A.
2. Place a PCR column into a 2 mL Microfuge tube. Pipette the reaction from Step 1 into the PCR column. Centrifuge at 12,000xg for 1 minute.
3. Discard the filtrate from the 2 mL Microfuge tube. Return the PCR column to the 2 mL Microfuge tube. Pipette 700 µL of Buffer W2 into the column and centrifuge at 12,000xg for 1 minute.
4. Discard the filtrate and return the PCR column to the 2 mL Microfuge tube. Pipette 400 µL of Buffer W2 into the column and centrifuge at 12,000xg for 1 minute.
5. Transfer the PCR column into a clean 1.5 mL Microfuge tube. To elute the DNA, add 25-30 µL of Eluent (pre-warmed at 65°C) to the center of the membrane. Let it stand for 1 minute at room temperature. Centrifuge at 12,000xg for 1 minute.

Restriction Enzyme Digestion of Miniprep Plasmid DNA
1. Select restriction enzymes for characterization of the construct
2. Select the appropriate buffer and incubation temperature based on the supplier's recommendations. Often room temperature and BSA.
3. Mix gently by pipetting.
4. Let solution digest for 1 to 4 hours, typically 2 to 4 is better.

Ligation with T4 DNA Ligase
1. Set up the reaction titled Ligation Reaction on ice in a microcentrifuge tube.
2. Gently mix the reaction by pipetting up and down and microfuge briefly.

3. For cohesive (sticky) ends, incubate at 16°C overnight or room temperature for 10 minutes.
4. For bunt ends or single base overhangs, incubate at 16°C overnight or room temperature for 2 hours.
5. Heat inactivate at 65°C for 10 minutes.
6. Chill on ice and transform 1-5 µL of the reaction into 50 µL competent cells.

Colony PCR Protocol
1. Mix 20µL PCR reaction, minus template DNA (comes from insert in transformed *E. coli*).
2. Using a 10µL pipette tip, pick a colony from plate, dip the pipette tip into premade PCR reaction and then place into liquid LB media containing selection.
3. Run Thermal Cycler Protocol To Amplify *SSA1*.

Gel Electrophoresis (Standard 1% Agarose Gel)
1. Measure out 1 g of agarose
2. Pour Agarose powder into microwavable flask along with 100 mL of 1xTAE
3. Microwave for 1 - 3 min (until the agarose is completely dissolved and there is a nice rolling boil)
4. Let agarose solution cool down for 5 minutes
5. Add ethidium bromide (EtBr) to the final concentration of approximately 0.2 – 0.5 µg/mL (usually about 2 - 3 µL of lab stock solution per 100 mL gel). EtBr binds to the DNA and allows you to visualize the DNA under ultraviolet light.
6. Pour the agarose into a gel tray with the well comb in place.
7. Let newly poured gel sit at room temperature for 20-30 minutes, until it's completely solidified.
8. Add loading buffer to each of your digest samples.
9. Once solidified, place the agarose gel into the gel box.
10. Fill gel box with 1xTAE until the gel is covered.
11. Carefully load a molecular weight ladder into the first lane of the gel.
12. Carefully load your samples into the additional wells of the gel.
13. Run the gel at 80-150 V until the dye line is approximately 75-80% of the way down the gel.
14. Run OFF power, disconnect the electrodes from the power source, and then carefully remove the gel from the gel box.
15. Using a device that has UV light, visualize your DNA fragments.

Isolation of Chromosomal DNA from Yeast
1. Grow the yeast strain of interest in 2mL of YPD media at 30°C for 24hrs at 180 rpm.
2. Centrifuge cells at 5000 rpm, discard the YPD media, and then wash the yeast cell pellet with approximately 20 mL of distilled water.

3. Centrifuge cells at 5000 rpm, discard the supernatant, dissolve the yeast cell pellet in 0.5 mL shperoplast buffer, and then move it to an Eppendorf tube.
4. Incubate the solution for 30 minutes at 37°C, if lysozyme used, incubate up to an hour.
5. Add 0.5 mL Protienase K buffer.
6. Incubate for 30 minutes at 65°C, if lysozyme is used incubate for up to an hour.
7. Add 0.2 mL 5 M potassium acetate solution.
8. Incubate on ice for 10 minutes.
9. Centrifuge at max rpm for 15 minutes.
10. Pipette supernatant into a fresh tube, discarding the pellet, and add 2.5 volume of ethanol. If you choose to stop here, store at -20°C until next step.
11. Incubate on ice for approximately 15 minutes.
12. Centrifuge at max rpm for 10 minutes.
13. Wash pellet with 70% ethanol by centrifuging at max rpm for 10 minutes.
14. Dry the pellet in a speedvac.
15. Dissolve the pellet in 0.5 mL TE buffer.
16. Add 5 µL of 1mg/mL Rnase A solution.
17. Incubate at 37°C for 15 to 30 minutes.
18. Add 0.5 mL phenol – chloroform and vortex for 30 seconds.
19. Centrifuge at max rpm for 10 minutes.
20. Pipette the aqueous (top) phase into a fresh tube and add 2.5 volume of ethanol.
21. Incubate at -20°C for about 30 minutes.
22. Centrifuge at max rpm for 10 minutes.
23. Wash the pellet with 70% ethanol.
24. Centrifuge at max rpm for 10 minutes.
25. Dry pellet in a speedvac.
26. Dissolve DNA in 0.4 mL TE buffer expecting a final DNA concentration of about 10 to 20 mg/mL.

Chlorophyll Content Extraction
1. Centrifuge samples at 3000 rpm for 3 minutes, seperatin algal biomass from media.
2. Remove media and suspend cells in 5 mL 100% ethanol.
3. Incubate cells in a 40°C water bath for 30 minutes and then centrifuge at 3000 rpm for 3 minutes.
4. From each sample, take three 1 mL samples and place in Fisherbrand semimicro polystyrene 1.5 mL cuvettes.
5. Take spectral absorption values at 665 and 650 nm.

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