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EVALUATION OF HEAT SHOCK PROTEIN 70A (HSP70A) IN *CHLAMYDOMONAS REINHARDTII*

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THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in
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By

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ABSTRACT OF THESIS

EVALUATION OF HEAT SHOCK PROTEIN 70A (HSP70A) IN *CHLAMYDOMONAS REINHARDTII*

Algae are being considered as a possible tool for carbon dioxide mitigation because they uptake carbon dioxide during photosynthesis. Using flue gas from a coal-fired power plant as a carbon source would allow the algae to remove CO_2 from the flue gas before it is emitted into the atmosphere. Because algae do not grow well at the high temperature, low pH conditions presented by flue gas, the traditional approach has been to alter the flue gas to suit the needs of the algae; however, this work aimed to genetically modify the algae *Chlamydomonas reinhardtii* to grow better at less than optimal conditions. Heat shock proteins are important in the stress responses of many organisms; therefore, this work modified *C. reinhardtii* to overexpress HSP70A in order to increase the tolerance of *C. reinhardtii* to higher temperature and lower pH. Experiments yielded mixed results, but there were several instances in which the modified algae appeared to have gained an increased tolerance to decreased pH based on the chlorophyll concentration of the algae.

KEYWORDS: *Chlamydomonas reinhardtii*, heat shock proteins, carbon dioxide mitigation, temperature, pH

Sarah Short

April 23, 2012

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To my parents, who always believe in me and taught me to believe in myself.

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CHAPTER 1 : INTRODUCTION

As the world's population swells and developing countries increase their energy demands, the world's overall energy usage also continues to increase. During the UN conference in Copenhagen in December 2009, a non-binding objective was set to limit the average global temperature increase to 2°C above pre-industrial temperatures. In order to achieve this goal, the level of greenhouse gas (GHG) emissions would have to stabilize at 450 ppm CO_2-equivalent.[1] This stabilization would require far-reaching changes in the global energy market which has already begun to make small changes over recent years (IEA, 2010). The Kyoto Protocol, which was adopted in Kyoto, Japan on December 11, 1997, set binding agreements for 37 industrialized nations plus Europe in order to reduce global GHG emissions. This agreement went into effect on February 11, 2005 and required reductions during the period of 2008-2012. However, it was never enforced and, therefore, the reduction requirements were not met (Ghoniem, 2011; UNFCCC).

While the most well-known effect of GHG is the global warming phenomenon, there are other consequences for the environment. Using the oceans as a carbon sink has been considered a viable possibility in order to reduce the amount of CO_2 released into the atmosphere, but this would still have a negative impact on the environment. One-third of the CO_2 released from human activity is already absorbed by the oceans and the CO_2 absorption causes the oceans to develop a more acidic pH. Currently, the average ocean water has a pH of 8.2, but this is estimated to be at least 0.1 lower than pre-industrial levels. This decreased pH would have negative impacts for marine life, which would also affect terrestrial life. Because CO_2 solubility increases with lower temperatures and higher pressures, the acidity of ocean water would increase with decreased water depth and could more strongly affect marine life on the ocean floor, which may already be more sensitive to pH changes (Ghoniem, 2011; Ormerod, 2002).

[1] CO_2 equivalent denotes the amount of carbon dioxide that would have the same effects on global warming as other greenhouse gases and is used in order to compare the effects of different gases.

According to the EPA, in 2008 the United States emitted a total of 5,572.8 teragrams of CO_2 equivalent through the combustion of fossil fuels with 37.3% of those emissions resulting from the combustion of coal. Coal accounts for such a large percentage of these emissions in part because it has the highest amount of carbon per unit of energy, more than petroleum or natural gas (EPA, 2010). The Commonwealth of Kentucky is third in coal production with over 120 million tons produced in 2008 and is home to nearly one-third of all coal mines in the U.S. Beyond producing coal, Kentucky ranked seventh in total energy consumption per capita and sixth in coal consumption. Of the nearly 2,000 trillion BTU of energy consumed by Kentucky in 2008, 51% was supplied by coal (DEDI, 2010; EIA, 2010).

The reduction of greenhouse gas emissions is an absolute requirement in order to halt the trend of global climate change. Carbon dioxide emissions are the main target for these reductions and lower emissions can be achieved through decreased demands for fossil fuels and through carbon sequestration and mitigation. Many alternatives to fossil fuels are being developed and used, including solar energy (thermal and photovoltaic), wind energy, hydroelectric, geothermal, and biofuels, such as biodiesel and ethanol. There are advantages and disadvantages to each and usage is often dependent on global location and locally available resources. The production of biofuels continues to be more expensive than the traditional production of fuel from petroleum, but the increased environmental benefits have helped increase their demand and production (Mata et al., 2010).

Geologic carbon sequestration is the process of compressing and burying CO_2 generated from power plants in deep, underground reservoirs so that it is not released into the atmosphere. Biological sequestration can also take place naturally through plant growth in forest and grazing areas. It is estimated that roughly 3.6 billion hectares of grazing land exist globally and account for the removal of 20% of global CO_2 emissions due to deforestation and land-use changes (Follett and Reed, 2010; Froese et al., 2010; Ghoniem, 2011).

Carbon mitigation is an attractive option for situations in which CO_2 emissions continue to be generated because it prevents the entirety of the emissions from being released into the atmosphere. There are two general categories of CO_2 mitigation –

chemical reaction mitigation and biological mitigation (Wang et al., 2008). A common chemical reaction process used for CO_2 mitigation is the cycling of carbonation and decarbonation reactions in which gaseous CO_2 reacts with a solid metal oxide to yield a metal carbonate (Gupta and Fan, 2002). This technique is also referred to as "neutralization" because it is neutralizing the carbonic acid by producing carbonates, which are stable products that are already found in the natural environment (Lackner, 2003). This is also referred to as carbon dioxide "scrubbing" because it is removing the carbon dioxide after it is created but before it is released into the atmosphere. An advantage to this type of mitigation is that it can be performed under the temperature and pressure conditions associated with flue gas. This prevents the entire flue gas stream from having to be cooled or pressurized, which can be expensive considering flue gas is typically only 5-30% CO_2 with the remainder composed of N_2, O_2, and H_2O. Chemical reaction mitigation usually causes low concentrations of CO_2 to be left in the flue gas stream (Gupta and Fan, 2002). Despite these advantages, chemical reaction-based CO_2 mitigation is often an unattractive option because it is energy-consuming, costly, and has disposal problems (both the captured CO_2 and the absorbents must be disposed). Biological mitigation, on the other hand, has become a more attractive option because biomass is generated through photosynthetic reactions and the biomass contains energy that can be used later. This biomass energy can also help decrease the demand for fossil fuels, which would decrease the amount of CO_2 emissions (Mata et al., 2010).

Microalgae are being researched for possible biological CO_2 mitigation because many microalgae species can grow under harsh conditions and require few nutrients for growth. This allows microalgae to be cultivated in areas that are currently unsuitable for agricultural purposes which means that microalgae are not competing with current agricultural crops for arable land (Mata et al., 2010). Terrestrial plants are extremely inefficient at converting solar energy to biomass energy and are estimated to use less than 0.5% of the solar energy received. In comparison, microalgae are estimated to be 10-20% efficient at converting solar energy into biomass energy (Li et al., 2008). Microalgae are able to fix more CO_2 during photosynthetic growth because of their high efficiencies and growth rates, which makes them more effective at removing CO_2 from the atmosphere and an attractive option for biological CO_2 mitigation. A major

difference between sequestration and biological mitigation is that the CO_2 fixed during biological mitigation is not permanently removed from the atmosphere. Rather, the CO_2 fixed during photosynthetic growth is then recycled when the resulting biomass is burned as a biofuel. Therefore, no additional CO_2 is created and energy is created in a sustainable method through CO_2 recycling (Kumar et al., 2010). In a region where coal-fired power plants are ubiquitous, using microalgae for CO_2 mitigation from flue gas would help reduce GHG emissions without requiring the elimination of coal-fired power plants.

1.1 Project Goal

A possible strategy for carbon mitigation is to use microalgae to reduce the amount of carbon dioxide released into the atmosphere because algae uptake CO_2 during photosynthetic growth. By using flue gas as a source of CO_2 to grow microalgae, less CO_2 is released into the atmosphere, but the obstacle is that the flue gas must be altered in order to suit the needs of the algae. The CO_2 and SO_4 present in flue gas give it an acidic pH and the combustion processes from which flue gas is generated create high temperatures. Temperature and pH are two of the most influential environmental factors for microalgal growth and most microalgae species do not grow well or at all under the conditions presented by flue gas (Kumar et al., 2010). Figure 1.1 shows the growth rates of the reference algae *Scenedesmus acutus* as a response to temperature (Cassidy, 2011). There is a significant decrease in the growth rate once the temperature is raised above the optimal growth temperature of 27 °C. This work aimed to raise the growth rates at higher temperatures to equal or surpass the growth rate at the optimal temperature as well as to raise the growth rates at a lower pH to equal or surpass the growth rate at the optimal pH.

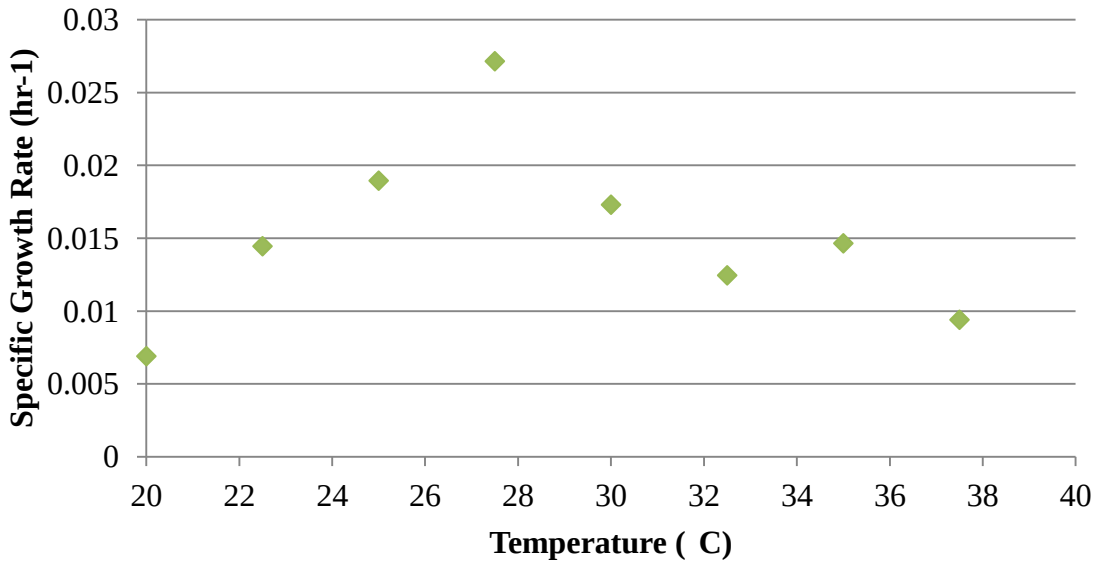

Figure 1.1 Growth rate of *S. acutus* in response to temperature (Cassidy, 2011).

In order to maximize algal growth and, consequently, CO_2 fixation, the current strategy consists of altering the environmental conditions associated with the flue gas so as to meet the needs of the algae. However, another possible approach is to genetically modify the algae to grow better at the conditions associated with flue gas, which are elevated temperature and low pH. This project aimed to genetically modify the algae using two different methods which are explained further in Chapter 3:

- Overexpress HSP70A in order to increase the thermotolerance and pH tolerance of the algae.
- Overexpress HSP70A and transform yeast SSA1 (Stress Seventy A) into the algae to increase the thermotolerance and pH tolerance of the algae.

While it may not seem significant to modify the algae so that it can grow normally at a temperature just a few degrees higher than normal or a pH a point lower than normal, small changes such as these actually allow for much greater system changes. Figure 1.2 shows the allowable temperature in a photobioreactor (PBR) where algae are being grown and the subsequently allowable amounts of flue gas. If the amount of flue gas being circulated is increased, the temperature of the flue gas must be decreased and if the temperature of the flue gas is increased, the amount circulated must be decreased. Therefore, if the allowable temperature in the PBR were raised by even a few degrees, either the amount or temperature of the flue gas could be increased dramatically.

Allowing a greater amount of flue gas to be circulated would increase the amount of carbon mitigation and allowing the temperature of the flue gas to be higher would mean less time, energy, and money would have to be spent on cooling the flue gas before circulation.

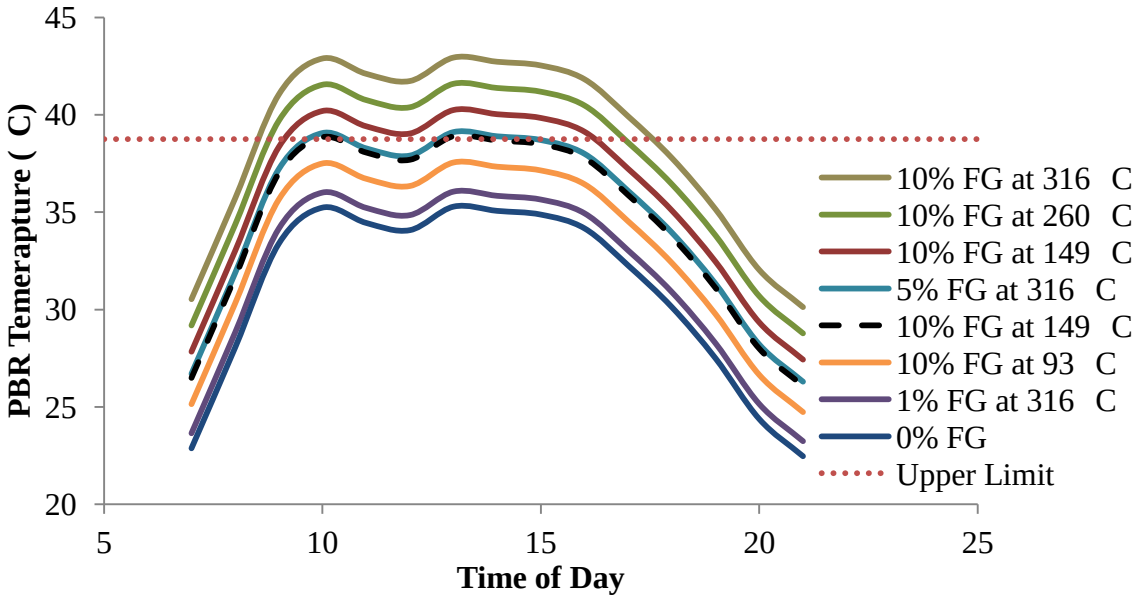

Figure 1.2 A model showing the temperature of a PBR depending on differing amounts and temperatures of flue gas circulated in a photobioreactor in which algae cultures are growing (Cassidy, 2011).

CHAPTER 2 : BACKGROUND

2.1 Microalgae

Microalgae can be either prokaryotic or eukaryotic organisms that are photosynthetic and have high growth rates. A variety of species are present in any existing ecosystem (terrestrial or aquatic) due to the wide range of conditions in which the species can survive. With the increasing demand for biofuels such as ethanol and biodiesel, many feedstocks have been considered, but second-generation feedstocks, such as algae, are advantageous because they are not already used as major agricultural crops. Some algal species are used as food supplements or for pharmaceutical activities, but this does not constitute a large market share. The unique advantage of microalgae as a biofuel feedstock is their higher growth and productivity rates as compared to traditional crops. The oil content in microalgae is much higher than in other feedstocks, such as soybean, which makes it more efficient as well (Mata et al., 2010). Many researchers are looking into methods to induce the algae to have an even higher oil content by increasing the lipid content. Metabolic engineering is one possible method to achieve this goal. Using either mutagenesis or transgenics, the algae's metabolic pathways can be manipulated to shift production to a desired product or to increase production of a product (Rosenberg et al., 2008).

One of the few green algae species that have exhibited stable, long-term expression after transgenic manipulation is *Chlamydomonas reinhardtii* (Rosenberg et al., 2008). *C. reinhardtii* is a photosynthetic, unicellular green algae that is often used and studied as a model organism. Historically, it has been used to study eukaryotic photosynthesis because it can grow either photoautotrophically or heterotrophically and to study the functions of eukaryotic flagella (Merchant et al., 2007). Researchers have also used *C. reinhardtii* to study the effects of different environmental stresses and the organism's responses to those stresses (Hema et al., 2007). Because of its use as a model organism, researchers began sequencing the *Chlamydomonas* genome so as to better understand the organism and facilitate future research. The completed sequence was finished and published in 2007 and is now available online for public use (Chlamydomonas Center, 2011; Merchant et al., 2007).

2.1.1Growth Model

A homogeneous batch culture of algae generally follows a growth model with several phases, which may not be distinct from one another and the length of which can vary based on growth conditions. The first phase is the lag phase, during which the cells must adapt to the new environment. The length of the lag phase depends on how different the new environment is from the previous environment and on the physiological age of the cells when the new medium is inoculated. If the cells are too young or too old when used to inoculate a new culture, it will have a longer lag phase. The next phase in the growth model is the accelerating growth phase, during which the specific growth rate of the cells increases because they are more adapted to the new environment. Next, the cells enter the log phase of exponential growth. During this phase, the cells are fully adapted to the environment and the algal culture is not so dense as to limit light penetration to all cells and the growing cells have not used enough nutrients to become nutrient limited for growth. The next phase is decreasing log growth where the specific growth rate is more linear. This occurs when the cells have grown dense enough to begin to have light limitations. At this point, the cells have also used up much of the nutrients and release an increasing amount of toxic wastes, which can both limit growth. Once the cells have reached a point where the light is limited, they have reached the stationary phase where a growth equilibrium is reached. Degradation of dying cells offsets biomass increase, which causes the growth curve to increase to a point representing the maximum attainable algae concentration. The next phase is the accelerated death phase where the algal cells begin to die in greater numbers, which releases growth-inhibiting materials into the culture. This phase can be caused by the cells being too old and running out of nutrients, but it can also be caused by unfavorable environmental conditions. Finally, the cells reach the log death phase where the death rate is exponential and the culture completely dies (Becker, 1994).

The human eye can detect some general differences in the phases of the growth model, but not all of them are distinctly discernible. The algal cultures tend to start out as a very pale green and then become a darker green as they grow denser. It is difficult to detect a difference in the phases at this point until the culture enters the death phase at

which point it begins to turn yellowish-brown. As more and more of the cells die, the culture will increasingly become less green and more brown.

As the algae grow, the pH increases because of hydroxide molecules (OH^-) released during photosynthesis. The water (H_2O) in the media combines with the carbon dioxide (CO_2) in the air to form carbonic acid (H_2CO_3), which can then break into hydrogen ions (H^+) and bicarbonate (HCO_3^-). The algae take up the carbon in the bicarbonate form and, through the photosynthesis process, release oxygen (O_2) and OH^- molecules, which raises the environmental pH.

2.2 Genetic Engineering

On its most basic level, genetics is the "study of heredity and hereditary variation" (Campbell and Reece, 2005). Researchers attempt to determine which gene or group of genes in an organism is responsible for a certain phenotypic trait, metabolic activity, or function. The manipulation of the genome is called genetic engineering. Even after genomes are sequenced, often there are sequenced genes with no known purpose or function.

Numerous studies involving the genetic engineering of *Chlamydomonas* have been conducted and this has led to improved techniques and targets for future manipulation (Beer et al., 2009; Léon-Bañares et al., 2004). One successful method that has been demonstrated is RNA silencing, which has successfully been used to reduce the expression of 30 different genes in the organism. Antisense or dsRNA can be used for RNA silencing although dsRNA has proven to be much more effective at reducing the expression of the targeted gene. One problem often encountered is the transient nature of the gene silencing; however, researchers have demonstrated stable and heritable, if still variable, effects in *Chlamydomonas* (Kim and Cerutti, 2009; Rohr et al., 2004; Schroda, 2006). Metabolic engineering through the use of transgenes has also been effective for *C. reinhardtii* with studies showing that it is possible to transform genes via a vector into the algae (Wang et al., 2010).

2.2.1 Reverse Genetic Engineering

Reverse genetic engineering aims to manipulate an organism's genes in order to determine their function for the organism. There have been several reverse genetics techniques developed for plants in order to relate gene to function such as virus-induced

gene silencing, RNA-mediated interference, and insertional mutagenesis (Gilchrist and Haughn, 2010). Metabolic engineering specifically denotes the purposeful manipulation of metabolism using recombinant DNA techniques. Therefore, it includes the creation of new metabolic pathways in organisms as well as the modification of existing pathways, both of which can be used to create new or improved products. While metabolic manipulation has been used to select the best strains of *S. cerevisiae* for beer fermentation through crossing strains as well as the most productive strains of *P. chrysogenum* for penicillin production by repeated mutations, metabolic engineering allows for a more targeted approach for choosing and/or creating an optimal strain (Nielsen, 1998).

Previous studies with *Chlamydomonas reinhardtii* provide possible reverse genetic engineering techniques for that species. Transformation techniques such as *Agrobacterium tumefaciens* (Kumar et al., 2004), electroporation (Ladygin, 2004; Shimogawara et al., 1998), and silicon carbide whiskers (Dunahay, 1993) have been used to introduce DNA into *C. reinhardtii* cells. However, the most common transformation method for *C. reinhardtii* is the glass beads method (Deng, Li, and Fei, 2011; Schmollinger, Strenkert, and Schroda, 2010; Shroda et al., 1999; Shroda, Blöcker, and Beck, 2000; Wang, 2010) popularized by Kindle (1990).

2.3 Environmental Stress Responses in *Chlamydomonas*

When algae are exposed to environmental stresses such as elevated temperatures, high-light conditions, or salinity changes, there are certain reactions triggered in the organism. Environmental stresses for plants and their responses have been studied extensively (Allakhverdiev et al., 2008; Wahid et al., 2007), but there have also been studies specifically involving algae (Schroda et al., 1999; Tanaka et al., 2000). Heat stress is generally defined as a transient temperature increase of 10-15 °C above ambient temperature (Wahid et al., 2007). The common theme among the studies is that the most heat-sensitive component of the plant or algae is the oxygen-evolving complex (OEC) in the photosystem II complex. Photosynthetic cells use a protein matrix to arrange chlorophyll and other pigment molecules to form light-harvesting matrices, which are called photosystems. There are two photosystems, photosystem I (PSI) and photosystem II (PSII) (named in order of discovery but functionally occur in reverse order). These photosystems are embedded in thylakoids, which are cellular membranes within the cell

(Bauman et al., 2007). Elevated temperatures have been shown to affect the photosystems in such ways as a reorganization of the thylakoid membranes and a reduction in the rate of electron transport during the photosynthetic processes. However, the OEC in PSII is still considered the most sensitive component of the photosynthetic process and its sensitivity to heat stress comes from the heat-induced release of two of four manganese atoms from the catalytic site in the OEC (Allakhverdiev et al., 2008; Tanaka et al., 2000).

2.4 Heat Shock Proteins

Environmental stresses cause both immediate, direct damage and continued, indirect damage. The direct damage from the stress causes problems in the photosynthetic process, which immediately affects the organism. The stress also causes the inhibition of protein synthesis, which prevents the repair of the damaged photosynthetic machinery. Thus, the stress causes both a problem for the organism and prevents the organism from fixing the problem, which prolongs the stress and exacerbates the damage (Allakhverdiev et al., 2008). Some of the proteins which are most important to the organism during environmental stress are heat shock proteins (HSPs). They were named because they were observed to be upregulated with a sharp temperature increase, but they were later observed to be upregulated by other environmental stresses, such as high light levels, salinity changes, and dehydration. Heat shock proteins are classified and grouped in reference by their molecular weight and function (e.g. HSP100, HSP90, and HSP70) (Gerloff-Elias et al., 2006). There are some HSPs that are present in the cell regardless of stress level and aid in basic functions, such as the correct folding and assembly of other proteins, which explains why these proteins are also referred to as chaperone proteins (Hartl, 1996). Certain HSPs are important during stress events because they help prevent harmful protein aggregation and interactions and act as shields for denatured proteins so that they do not interact with other proteins. They also help refold and reassemble proteins after the stress event and, therefore, aid in the repair of stressed cells (Parsell and Lindquist, 1993). While there are some differences in how the cell responds to different environmental stresses, there are also many commonalities and interactions between the responses. For instance, light is sometimes required for a cell to properly stimulate its repair mechanisms for heat stress, but high levels of light can

exacerbate the damage of heat stress. Termed "cross-tolerance," cells can gain increased thermotolerance from pre-exposure to strong light or increased tolerance to light from pre-exposure to elevated temperatures (Allakhverdiev et al., 2008). Both light and heat stress cause damage to the D1 protein in PSII, which causes several problems including the loss of manganese atoms. Once a D1 protein is irreversibly damaged, it is replaced with a newly synthesized D1 protein so as to repair the functionality of PSII, which is why it is important that HSPs maintain the protein synthesis capabilities of cells (Schroda et al., 1999).

While many different heat shock proteins play important roles in cell function, HSP70s have been identified as having a particularly significant role during cell stress. Members of the HSP70 family are present in almost all known organisms and exist in every component of a eukaryotic cell. In *Chlamydomonas reinhardtii*, there have been three HSP70s identified: HSP70A, located in the cytosol; HSP70B, located in the chloroplast; and HSP70C, located in the mitochondria. There are other genes which are thought to potentially code for other members of this family, but they are not well studied (Schroda, 2004). These proteins have been shown to be induced by heat shock as well as by light exposure after a period of dark incubation. HSP70B is integral in the resistance of *C. reinhardtii* to photoinhibition as evidenced by a reduction in photoinactivation of PSII with the overexpression of HSP70B and an increase in photoinactivation with the underexpression of HSP70B. Additionally, the overexpression and underexpression of HSP70B augment and retard, respectively, the recovery of the photoinactivated PSII. Therefore, HSP70B works by both preventing damage to PSII and by aiding in its repair if damage was not completely prevented (Schroda et al., 1999).

Along with heat or light stress, pH changes can be very stressful for microorganisms. Changes in the internal pH of a microorganism can cause damage to a cell through protein destabilization and denaturation. Studies in microorganisms have shown that during acid-shock, there is an increased level of HSPs, such as HSP60s and HSP70s, in the cell which leads to increased acid tolerance. Cross-tolerance provides the cell with increased thermotolerance as a result of a pretreatment in an acidic environment and exposure to a mild heat shock can provide increased acid tolerance. Most pH stress studies have been conducted using prokaryotic microorganisms, but there has been

research into the role of HSPs during temperature and acid stress for the algae species *Chlamydomonas acidophila*. The results of the research showed that this acidophilic algae has two growth maxima at pH 2.6-3 and pH 5-6, which is much lower than the neutral pH required for optimal growth for the mesophilic *C. reinhardtii*. A neutral pH of 7 was actually observed to cause a decreased growth rate as well as decreased PSII function in *C. acidophila*. As in *C. reinhardtii*, HSP70 was shown to have an increased level as a result of exposure to elevated temperatures, but it was also observed that *C. acidophila* had higher basal levels of HSPs such as HSP70 than did *C. reinhardtii*, which supports the theory that HSPs are at least partially responsible for the increased acid tolerance of *C. acidophila*. The results suggest that acid stress is just as, if not more, stressful than temperature stress for *C. acidophila* (Gerloff-Elias et al., 2006).

2.5 Yeast SSA (Stress Seventy A)

Heat shock proteins are ubiquitous to almost all living organisms and have important functions for normal protein activities such as folding, assembly, and transport. HSP70 has been identified as having a particularly important role for normal functions in the cell as well as in the event of stress events. In yeast, these heat shock proteins are referred to as SSAs, but they are homologous to HSP70s and have the same functions. In fact, HSP70s across different species have such similar structures that they can be interchanged across different species. They are not absolutely compatible, but multiple studies have shown that the HSP70s from one species can be transformed into another species and provide beneficial results (Sharma et al., 2009). As with *Chlamydomonas reinhardtii*, in yeast there are several members of the SSA family, but it is possible to induce increased thermotolerance with the overexpression of just one of the SSAs (Werner-Washburne, 1987). It has been shown that a pre-treatment of yeast cells with a mild heat shock can provide tolerance to a lethal heat shock because it stimulates the synthesis and expression of heat shock proteins (SSAs) (Sharma et al., 2009).

2.6 Project Objectives

Heat shock proteins are important in the response of algae to environmental stresses. Therefore, the goal was to increase the tolerance of the algae cultures to elevated temperature and low pH by overexpressing these proteins. The specific thesis objectives, followed by the resulting tasks, were:

- Determine if the overexpression of algal heat shock proteins (HSPs) causes increased tolerance of algae cultures to elevated temperature and/or low pH. The main target will be HSP70, which has been identified as particularly important to the algae's stress response.
    - Transform wild-type strains of *C. reinhardtii* (CC-400, CC-503, and CC-1690) with plasmid pSI103 harboring the HSP70A-RBCS2 double promoter, creating "overexpression" modified strains (400p, 503p, and 1690p).
    - Compare the growth rate of the modified strains to unmodified strains with different cultivation temperatures (22 and 35 °C) and different cultivation pH values (6 and 7).
- Determine if the addition of yeast SSA into algae along with overexpressed algal HSP70A causes increased tolerance of algae cultures to elevated temperature and/or low pH. The transformation was of SSA1 from *Saccharomyces cerevisiae*.
    - Transform wild-type strains of *C. reinhardtii* (400, 503, and 1690) with the modified plasmid pSI103-SSA, creating "overexpression" modified strains (400p-SSA, 503p-SSA, and 1690p-SSA).
    - Compare the growth rate of the genetically modified strains to unmodified strains with different cultivation temperatures (22 and 35 °C) and different cultivation pH values (6 and 7).
- Quantify any differences in tolerance gained from the overexpression of algal HSPs versus algal HSPs plus yeast SSAs.

CHAPTER 3 : MATERIALS AND METHODS

3.1 Microalgal Culture Conditions

The algal species chosen for this experiment was *Chlamydomonas reinhardtii*. There were three strains of *C. reinhardtii* selected for transformation: CC-400, a cell wall deficient strain with a purported greater tolerance for pH and CO_2 variation; CC-503, a cell wall deficient strain that was used as a DNA source for part of the genome sequencing project; and CC-1690, a wild-type strain that was used for part of the genome sequencing project (Chlamydomonas Center, 2011). The CC-400 strain was purchased from the Chlamydomonas Center. The CC-503 and CC-1690 strains were generously donated by Dr. Joseph Chappell in the Plant Physiology Department at the University of Kentucky.

The plasmid used to overexpress HSP70A was pSI103, which was also donated by Dr. Chappell. pSI103 contains the aphVIII gene conferring paromomycin resistance behind the HSP70A-RBCS2 promoter (Sizova, Fuhrmann, and Hegemann, 2001). pSI103 was then altered to insert the yeast SSA gene between the double promoter and hereafter will be referred to as pSI103-SSA.

Chlamydomonas reinhardtii was grown in TAP medium (see Media Recipes). Pre-cultures of *C. reinhardtii* were grown at 22°C with a 16 hour light/8 hour dark cycle using Sylvania cool white bulbs. The pre-cultures were grown on a shake table which provided continuous motion.

3.2 Genetic Modification

Each strain of *Chlamydomonas reinhardtii* was genetically modified to overexpress HSP70A. The expression of SSA1 was not able to be completed in time for testing, so only the algae cultures overexpressing HSP70A were tested in this study. Each modified strain was compared to its unmodified strain to determine whether or not the modifications made the algae more thermotolerant and/or acidophilic than the unmodified strain.

To create the modified strains that overexpress HSP70A, the pSI103 plasmid could simply be transformed into the algae cultures using a modified glass beads method (Kindle, 1990; Appendix B). Several days after the plating process, new colonies appeared, as seen in Figure 3.1. The original algal cultures died when plated on TAP

plates supplemented with paromomycin, which allowed for the certainty that any new colonies that formed in the presence of paromomycin contained the pSI103 plasmid and, therefore, were successfully modified and could be tested as such. The newly created modified algal colonies were named such that the strain number was following by the letter "p". Therefore, the three modified algae cultures were 400p, 503p, and 1690p.

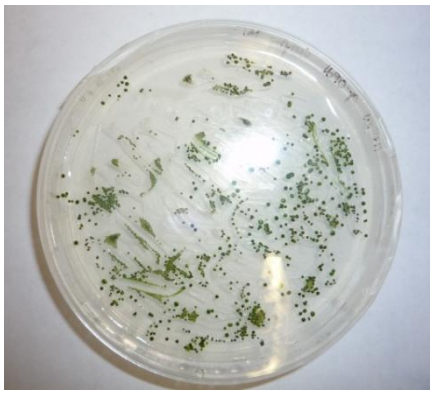

Figure 3.1 After 4-7 days, new colonies formed on plates supplemented with paromomycin from algal cultures transformed via the glass beads method.

Another successful method that was used to transform the algae was electroporation. See Transformation Methods for Chlamydomonas for more information about the electroporation protocol. Figure 3.2 shows two plates with new colonies that formed on paromomycin-supplemented plates, which were successfully transformed and modified algal cultures. Ultimately, the glass beads transformation method was chosen as the preferred method over electroporation because the former was easier, more familiar, and less costly.

Figure 3.2 After 7-10 days, new colonies formed on TAP plates supplemented with paromomycin from algal cultures transformed via electroporation.

To create the modified strains that overexpressed HSP70A and expressed SSA1, the plasmid pSI103 had to be altered to include the yeast SSA1 protein between the HSP70A-RBCS2 double promoter present in the plasmid. First, the yeast protein was amplified using PCR (forward: GCCGGCTAGCATGTCAAAAGCTGTCGGTATTG; reverse 1: GGTGATGGTGATGTCGACCTCCTCGATCTT; reverse 2: GCC GGC TAG CTT AGT GAT GGT GAT GGT GAT GAT CAA CTT CTT C) and then the PCR product was cloned into a TA vector containing ccDB resistance. The TA vector was used to make sure that the PCR product was digested by NheI because the TA vector was linear and would be broken into two linear pieces if the insert was digestible by NheI. Once the insert was confirmed to be digested by NheI, both the insert and the pSI103 plasmid (hereafter referred to as the vector) were digested with NheI. Once the vector was digested, it was then dephosphorylated so it could not easily close up by itself. Next, the insert and vector were gel-isolated using a Qiagen gel-isolation kit (Qiagen, Inc.) and ligated. After ligation, they were transformed into competent cells. The cells were then plated on ampicillin plates and new colonies grew after 12-16 hours. From these colonies, liquid cultures were started using LB media (Bacto-Tryptone 10 g/L; Bacto-yeast extract 5 g/L; NaCl 10 g/L) and were left for at least 8 hours to grow. Once the liquid cultures were matured, the plasmid was extracted using a Qiagen miniprep kit (Qiagen, Inc.). A small sample was then digested with NheI and run on a gel to check for the presence of the insert. Any mini-preps that had the insert confirmed would have then been taken and transformed into algae via the modified glass beads method (Kindle, 1990; Appendix B).

3.3 pH Acclimation

The chosen stress level for pH for this study was pH 6, which is not far from the optimal pH of 7, but the algae were not able to survive initially in media with a lower pH. Attempts were made to grow algae in media with a pH of 5 or a pH of 5.5, but the algae cultures died within 24 hours, as seen in Figure 3.3. The cultures were left for a few days to allow for a possible recovery after an extended lag phase, but they never recovered. A pH of 6 was chosen for the experiments so the algae would be able to survive the initial environmental shock of the pH, but later efforts were made to acclimate the algae to a lower pH so that a lower pH could be used in testing. The algae were first grown in

media with an initial pH of 6 and then were transferred to media with an initial pH of 5.8. Because the algae grown with an initial pH of 6 would have had a much higher pH by the time of the transfer, the cultures were first spun down so that all media could be removed from the algal biomass before it was resuspended in the new media and transferred to the new flasks. Cultures that grew at the initial pH of 5.8 were then transferred to media with a pH of 5.7 following the same procedure. Finally, cultures that grew successfully in media with an initial pH of 5.7 were transferred to media with an initial pH of 5.6 Figure 3.4 shows the acclimated, unmodified algae and Figure 3.5 shows the acclimated, modified algae. The figures show that the 503 and 503p cultures were not as well acclimated as the other strains and grew more slowly because their cultures were not as dense and dark given the same cultivation period. This difference in acclimation could be due to the fact that strain 503 is cell-wall deficient whereas strain 1690 is a wild-type strain. Strain 400 is also a cell-wall deficient strain and it acclimated well, however, so there must be a difference in the cell-wall deficiency of these two strains.

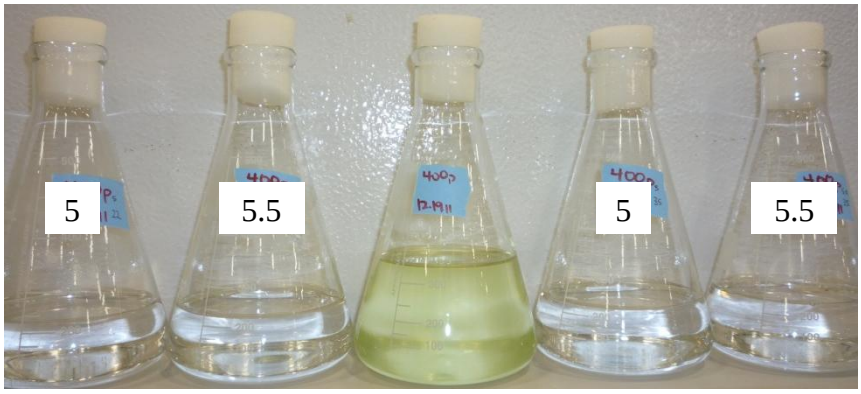

Figure 3.3 *C. reinhardtii* strain 400p algae cultures in lowered pH media after 24 hours. The two left flasks are *C. reinhardtii* 400p with a pH of 5 and 5.5 in 22°C. The middle flask is *C. reinhardtii* 400p with an initial pH of 7 in 22°C. The two flasks on the right are *C. reinhardtii* 400p with an initial pH of 5 and 5.5 in 35°C.

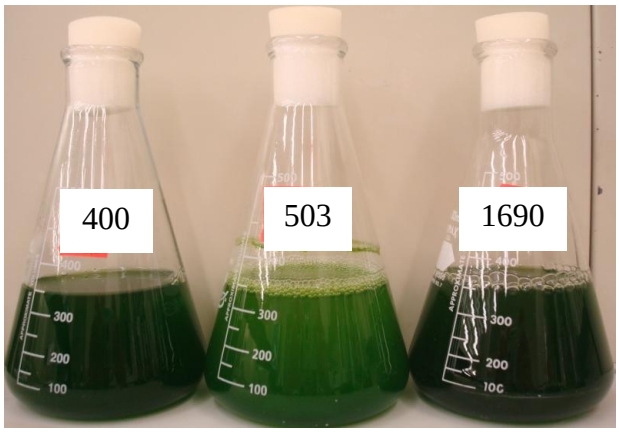

Figure 3.4 Unmodified *C. reinhardtii* cultures acclimated to pH 5.6.

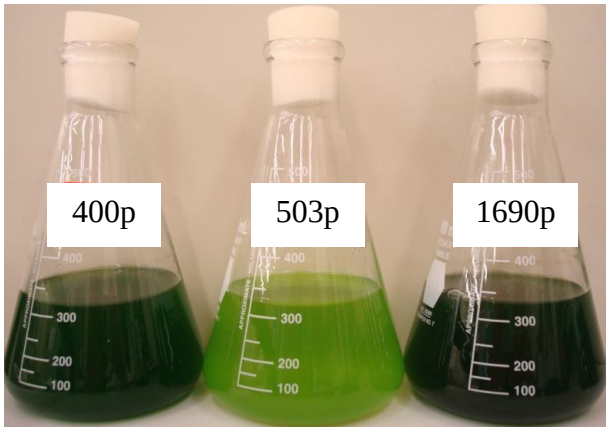

Figure 3.5 Modified *C. reinhardtii* cultures acclimated to pH 5.6.

3.4 Experimental Design

Once the proteins were confirmed in the strains, the algae were grown in liquid cultures. After the liquid pre-cultures reached the exponential growth phase, a 5 to 15 ml sample was removed and placed in a 500 mL Erlenmeyer flask with 400 mL TAP medium. The mixture of algae sample and medium was cultured for six days on a shake table in a temperature-controlled growth chamber. For the preliminary experiments, the treatments were randomly assigned to different flasks within the same strain using a 2x2 factorial for temperature and pH as shown in Table 3.1.

Table 3.1. Treatment descriptions for preliminary experiments.

Treatment	Temperature (°C)	pH	Abbreviation
1	22	7	22-7
2	22	6	22-6
3	35	7	35-7
4	35	6	35-6

For the main experiments, the treatments were randomly assigned to different flasks based on a 2x2x2 factorial for temperature, pH, and modification, as shown in Table 3.2. The temperature was controlled in each growth chamber and the medium had a controlled pH at the beginning of the test.

Table 3.2. Treatment descriptions for main experiments.

Treatment	Temperature (°C)	pH	Modification	Abbreviation
1	22	7	Unmodified	22-7-u
2	22	7	Modified	22-7-m
3	22	6	Unmodified	22-6-u
4	22	6	Modified	22-6-m
5	35	7	Unmodified	35-7-u
6	35	7	Modified	35-7-m
7	35	6	Unmodified	35-6-u
8	35	6	Modified	35-6-m

3.5 Analytical Methods

3.5.1 pH

Each treatment was applied to three flasks (n=3) of each unmodified and modified strain. Three 10 ml samples were taken daily in order to determine the growth rate via chlorophyll content. The pH of each sample was measured by Model AR15 pH meter (Fisher Scientific) and recorded for each sample throughout the experiments so it could be monitored throughout the algal growth.

3.5.2 Chlorophyll a content

After the pH was measured, the samples were processed so as to extract the chlorophyll from the algal cells. Dry weight was first used as the growth metric for this study, but it was discovered that other materials in the samples besides the algae cells (such as minerals and other nutrients from the media) were artificially increasing the dry weight. Chlorophyll content gives a more accurate representation of algal biomass because only the algae cells contain chlorophyll and, therefore, it does not matter if there are other substances in the sample.

The following protocol is a modified version of the chlorophyll extraction protocol found in Becker (1994). Ten algae samples were centrifuged at 3000 rpm for at least 3 minutes to separate the algal biomass from the liquid media. The media was removed and the algae were resuspended in 5 ml 100% ethanol. The samples were then placed in a water bath at 40°C. After 30 minutes, the samples were again centrifuged at 3000 rpm for 3 minutes. From each original sample tube, three 1 ml samples were pipetted into Fisherbrand semimicro polystyrene 1.5 ml cuvettes (Fisher Scientific) and spectral absorption (A) was measured at 665 nm and 650 nm using an Evolution 60 spectrophotometer (Thermo Scientific). The spectral absorptance values of the three cuvette samples from the same original sample were averaged to obtain the true absorptance values for that sample. From these absorptance values, the chlorophyll a content (mg/L) of the algae culture was calculated using the following equation:

(3.1)

The spectrophotometer had a spectral absorptance range of -0.1 to 3.0, which required changes to be made to the chlorophyll extraction protocol when the algae samples were too densely concentrated. Usually, starting on day 3 (72 hours) or day 4 (96 hours), the 10 ml samples were too dense and the spectrophotometer would no longer give accurate readings. Therefore, a 2:1 dilution was used with DI water.

CHAPTER 4 : RESULTS AND DISCUSSION

4.1 Preliminary Experiments

Before the modified strains were tested, the unmodified strains were tested using the preliminary treatment levels to determine the original growth rates of the algae at those specific growth conditions. Each treatment was applied to three flasks (n=3) and all three strains were tested at the same time.

The first experiment compared 22-7 and 22-6 for all three strains of *C. reinhardtii*. All 18 flasks were grown on a shake table in the 22°C growth chamber and were randomly placed on the table. The chlorophyll content over time of strains 400, 503, and 1690 are shown in Figure 4.1, Figure 4.2, and Figure 4.3, respectively. These figures show that strains 400 and 1690 were not affected much by the pH decrease for the first two days because the chlorophyll content is not significantly different (at the 0.05 level) between the cultures grown at the different pH levels at those times. Strain 400 (Figure 4.1) shows that the flasks with the initially lower pH actually finished the experiment with a higher chlorophyll content than those with the optimal pH. For strain 1690 (Figure 4.3) the flasks with the initially optimal pH finished the experiment with a higher chlorophyll content than the flasks with the decreased pH, as expected. Strain 503 (Figure 4.2) was different from the other two strains in that the flasks with the decreased pH grew very slowly for the first couple days and then had an equal chlorophyll content as the flasks with the optimal pH by the end of the experiment.

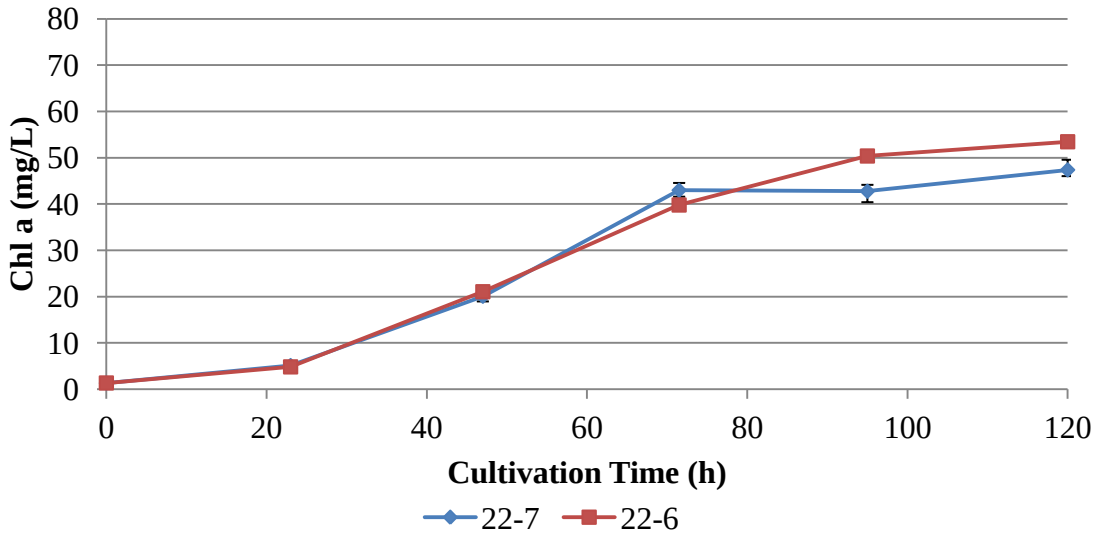

Figure 4.1 Chlorophyll content of *C. reinhardtii* strain 400 over time at 22°C and different pH (22-7 and 22-6). Error bars represent standard error (n=3).

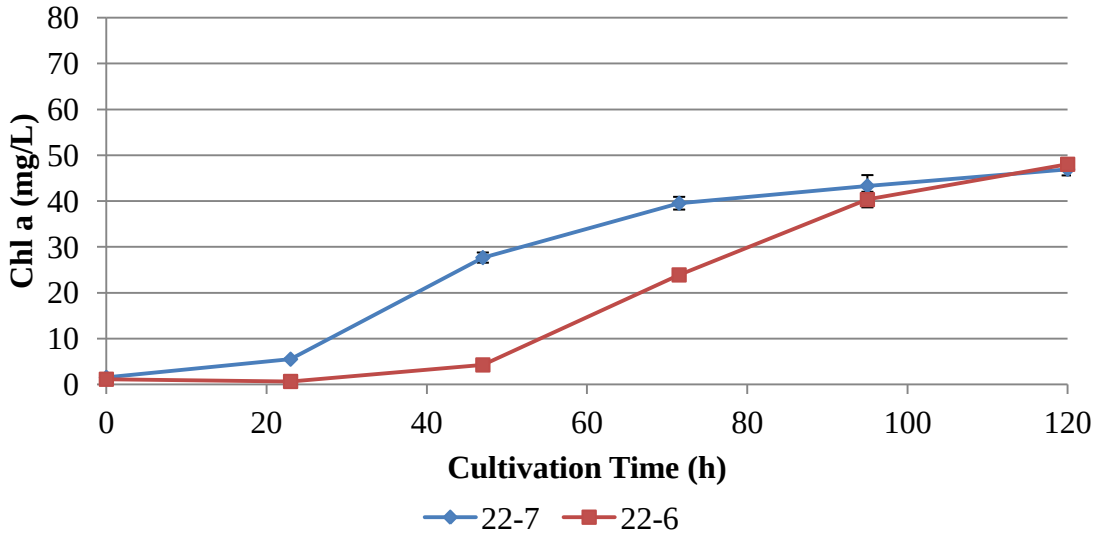

Figure 4.2 Chlorophyll content of *C. reinhardtii* strain 503 over time at 22°C and different initial pH (22-7 and 22-6). Error bars represent standard error (n=3).

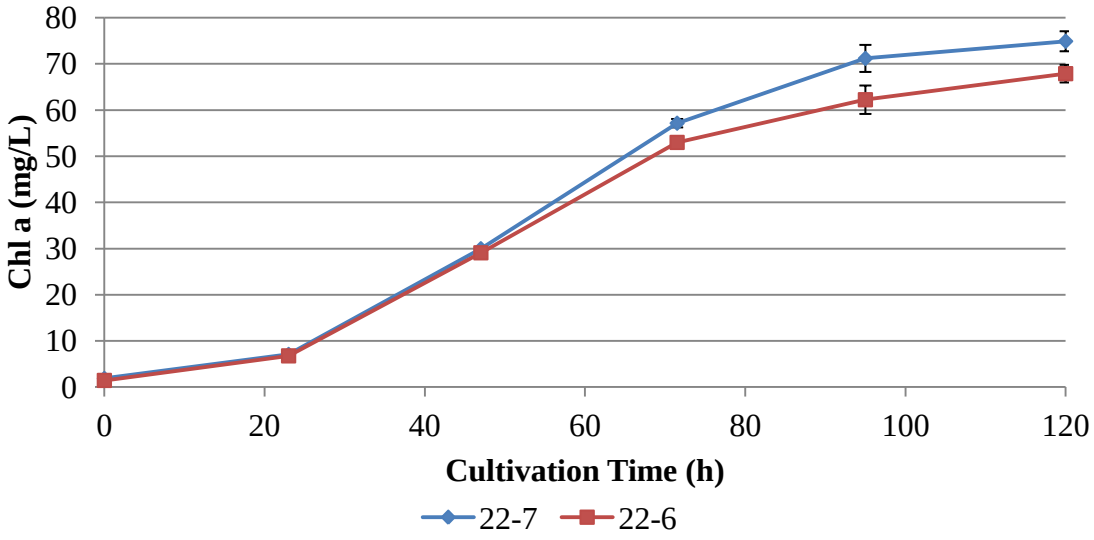

Figure 4.3 Chlorophyll content of *C. reinhardtii* strain 1690 over time at 22°C and different initial pH (22-7 and 22-6). Error bars represent standard error (n=3).

The second experiment compared 22-7 and 35-7. Nine flasks (three for each strain) were grown on a shake table in the 22°C growth chamber and nine flasks (three for each strain) were grown on shake tables in the 35°C growth chamber. All 18 flasks contained medium with the optimal pH. The chlorophyll content over time for strains 400, 503, and 1690 is shown in Figure 4.4, Figure 4.5, and Figure 4.6, respectively. By the end of the experiment, there was a significant difference in the chlorophyll content between the flasks at the two temperatures for all three strains. Looking at each strain individually, the cultures in the elevated temperature chamber finished the experiment with roughly half the chlorophyll content of the cultures in the optimal temperature chamber. At both the optimal and stress temperature, strain 1690 (Figure 4.6) had the highest growth rate of the three strains. Initially, the strain 1690 cultures in the elevated temperature chamber had a higher growth rate than those in the optimal temperature chamber, but after the first two days, the cultures in the elevated temperature chamber reached a growth plateau whereas the cultures in the optimal temperature chamber continued to grow.

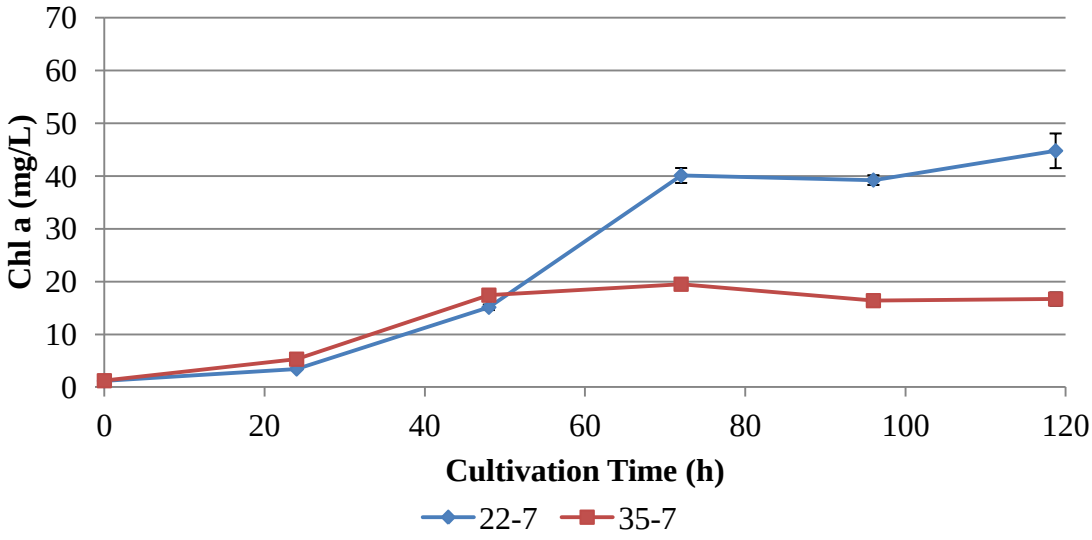

Figure 4.4 Chlorophyll content of *C. reinhardtii* strain 400 over time with pH 7 at different temperatures (22-7 and 35-7). Error bars represent standard error (n=3).

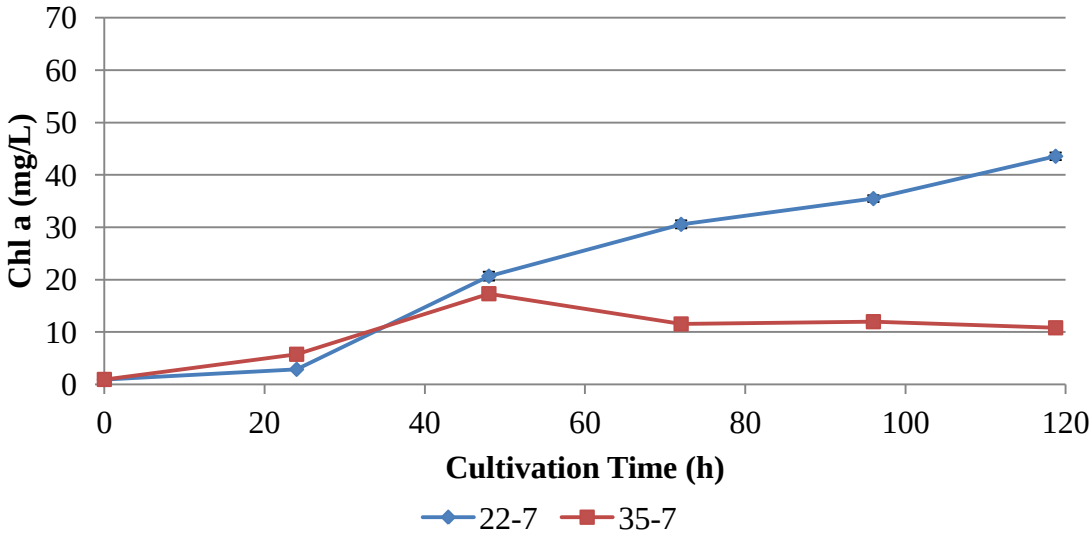

Figure 4.5 Chlorophyll content of *C. reinhardtii* strain 503 over time with pH 7 at different temperatures (22-7 and 35-7). Error bars represent standard error (n=3).

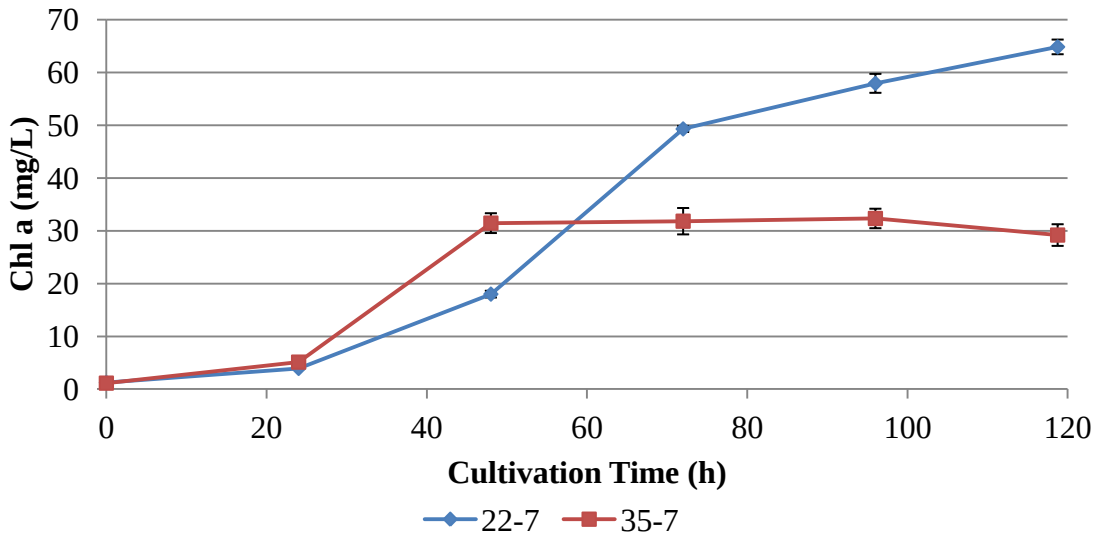

Figure 4.6 Chlorophyll content of *C. reinhardtii* strain 1690 over time with pH 7 at different temperatures (22-7 and 35-7). Error bars represent standard error (n=3).

The third experiment compared 35-7 and 35-6. All 18 flasks were grown on shake tables in the 35°C growth chamber. Nine flasks (three for each strain) had medium with optimal pH and nine flasks (three for each strain) had medium with pH 6. Due to uncontrollable power issues, this experiment was run for five days rather than six days like the other two experiments. While it was one day shorter, the important comparisons were still able to be made between the sets of cultures because their growth was decreased due to the environmental stresses created by the treatments. The chlorophyll content over time of strains 400, 503, and 1690 is shown in Figure 4.7, Figure 4.8, and Figure 4.9, respectively. Similarly to the results from the first experiment, strain 400 (Figure 4.7) showed a slight difference in the growth rate between the cultures with the optimal pH and those with the decreased pH, but the growth rates followed the same pattern for both sets of cultures. Strain 503 (Figure 4.8) had interesting results because the cultures with the decreased pH initially had a very slow growth rate but continued to grow over the course of the experiment. However, the cultures with the optimal pH grew well initially and then started dying around day 3 so that by the end of the experiment, there was no significant difference between the chlorophyll content of the two sets of cultures. There was a higher standard error for the cultures with the optimal pH, which indicates that there was a greater difference between the chlorophyll content of the three

flasks. The two sets of flasks for strain 1690 (Figure 4.9) had the greatest difference in the final chlorophyll content. Both sets of cultures reached their growth maxima around the end of day 3 and then started dying, which is reflected in the decreased chlorophyll content for all the flasks by the end of day 4. The standard error for the chlorophyll content of the cultures with the optimal pH at the end of day 3 is greater than the standard error for the other points, but this is explained by the fact that one of the three cultures in the group was still growing and one of the cultures was already dying.

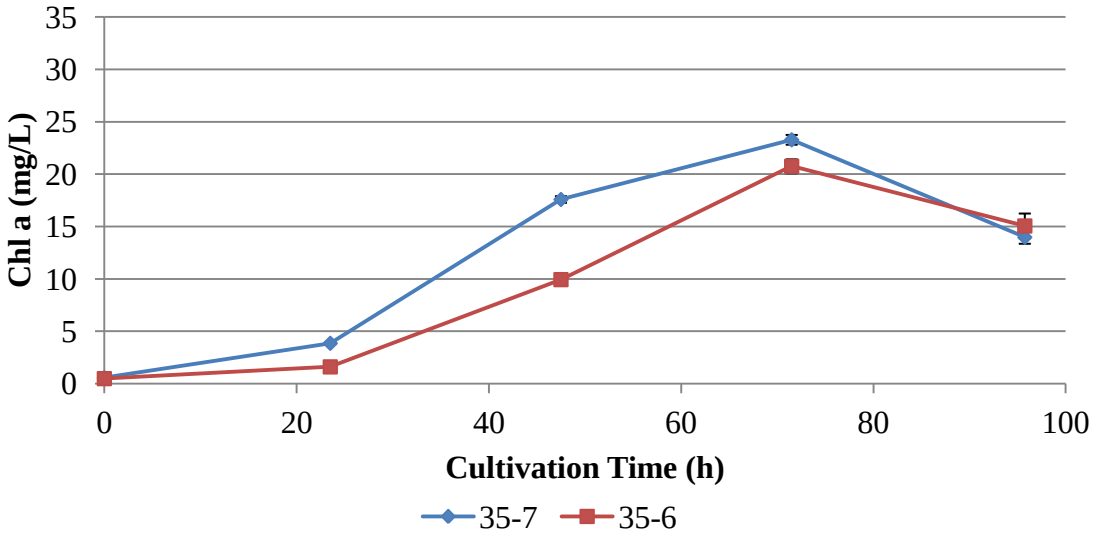

Figure 4.7 Chlorophyll content of *C. reinhardtii* strain 400 over time at 35°C and different initial pH (35-7 and 35-6). Error bars represent standard error (n=3).

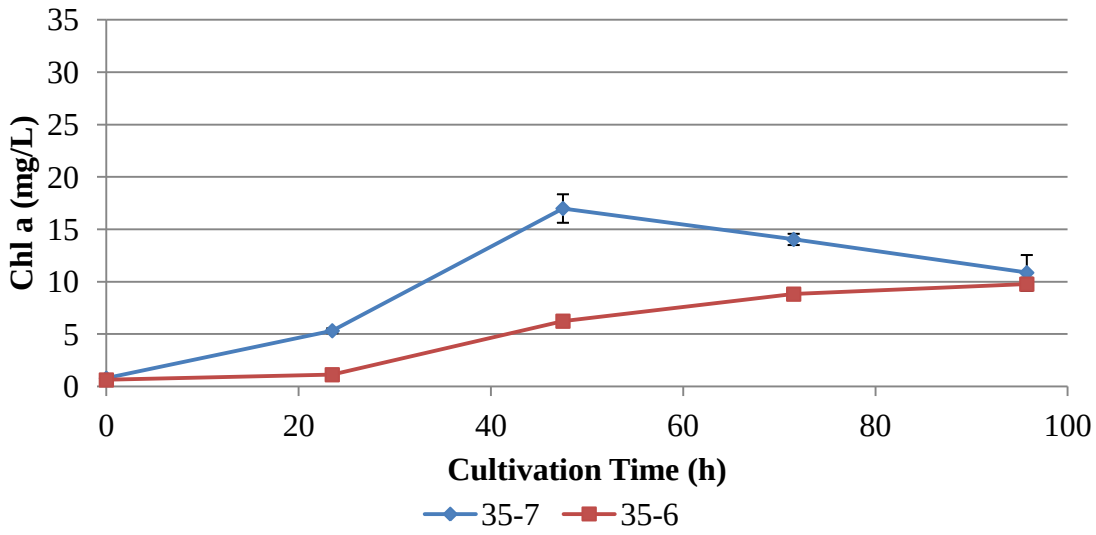

Figure 4.8 Chlorophyll content of *C. reinhardtii* strain 503 over time at 35°C and different initial pH (35-7 and 35-6). Error bars represent standard error (n=3).

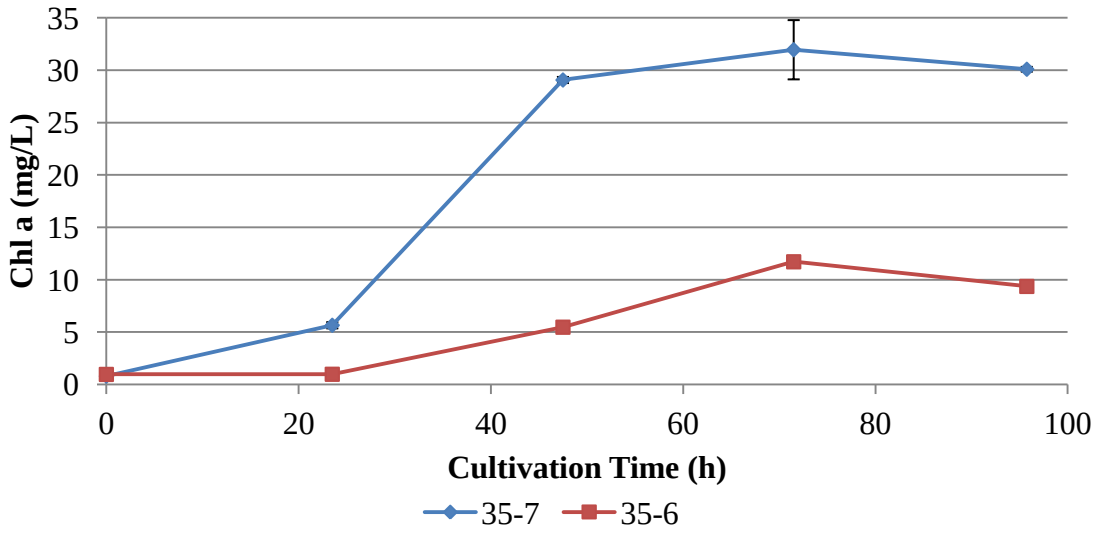

Figure 4.9 Chlorophyll content of *C. reinhardtii* strain 1690 over time at 35°C and different initial pH (35-7 and 35-6). Error bars represent standard error (n=3).

The third experiment was also different because it was obvious that the cultures were not growing normally by observing them before sampling. Two flasks, one from strain 1690 and one from strain 400, are shown in Figure 4.10. These flasks were both grown in the elevated temperature chamber and started with medium with a pH of 6. The strain 400 culture grew normally in the sense that the culture was homogeneous and the

individual algae particles were not observable. On the other hand, the strain 1690 culture did not grow normally because the algae particles clumped together despite the constant shaking of its environment. The clump of algae did grow, but the culture was not as healthy as the culture from strain 400. Once the strain 1690 culture was manually shaken for a few seconds, the clump of algae broke into smaller clumps, but once it was placed back on the shake table and left alone for a couple hours, the smaller clumps would clump back together into one giant clump, as seen in Figure 4.10.

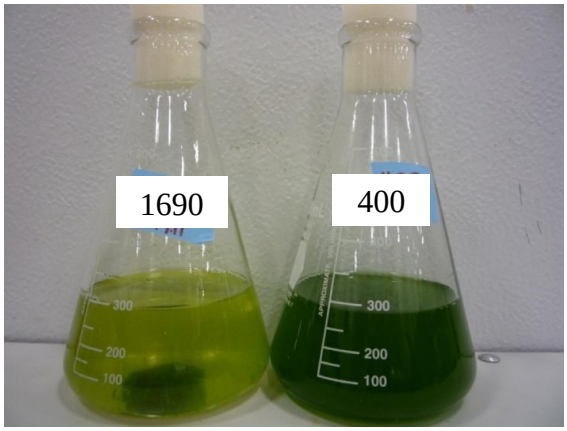

Figure 4.10 Two flasks highlighting the different aesthetics of growth for different cultures at 35°C and with an initial pH of 6. The left flask is *C. reinhardtii* strain 1690 and the right flask is *C. reinhardtii* strain 400.

Another difference in how the cultures grew for different strains is shown in Figure 4.11 and Figure 4.12. The same flask of strain 1690 seen in Figure 4.10 is shown in Figure 4.11 and the same flask of strain 400 seen in Figure 4.10 is shown again in Figure 4.12. The strain 1690 flask (Figure 4.12) shows a homogeneous mixture after the flask was shaken, which indicates a healthy algal culture, but the strain 400 flask (Figure 4.11) shows a heterogeneous mixture where algae particles are visible separate from the medium even after vigorous shaking. This strain 1690 culture did grow over the course of several days, but it was not as healthy as the strain 400 culture.

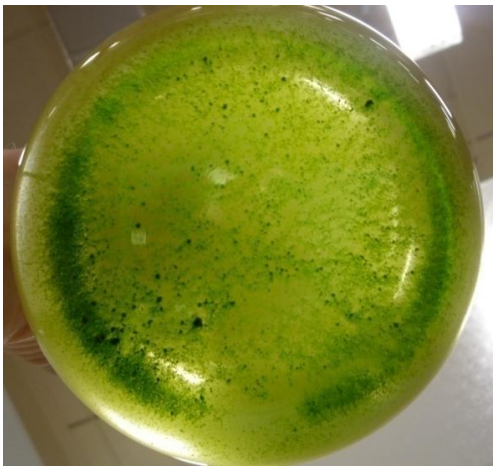

Figure 4.11 An example of an algae culture which has not grown normally. (*C. reinhardtii* strain 1690, 35-6).

Figure 4.12 An example of an algae culture which has grown normally. (*C. reinhardtii* strain 400, 35-6).

4.2 Main Experiments

Because the algal stock cultures can grow differently from week to week, it was decided that each experiment would consist of only one strain of *C. reinhardtii* and that strain would be subjected to all eight treatments during the experiment so that better comparisons could be made between treatments. Strain 400 was not tested during these experiments because it had an already demonstrated greater tolerance for pH change at both the optimal and stress temperatures and strains 503 and 1690 had a greater potential for a difference due to the modifications.

The initial concentration of chlorophyll in each test flask needed to be as close to equal as possible so as to eliminate any difference in growth rate due to a difference in initial algae concentration. There were significant differences between the average initial chlorophyll content of each treatment, but they generally fell along the lines of the modified versus unmodified algae. When beginning an experiment, an unmodified algae culture was used to inoculate the flasks for 22-7-u, 22-6-u, 35-7-u, and 35-6-u and a modified algae culture was used to inoculate the flasks for 22-7-m, 22-6-m, 35-7-m, and 35-6-m. Because there were two separate algae cultures used for inoculation and two cultures never grow the exact same way, there were differences in their algae concentrations, which caused differences in the inoculation of the test flasks. While there were significant differences between the initial concentrations of algae between treatments, these differences were minimal when compared to the concentrations

throughout the experiment. If exact concentrations were required, more intensive methods such as cell counting would be necessary when inoculating new media.

4.2.1Experiment 1: Comparing C. reinhardtii *strain 1690 at various temperatures and pH*

The first experiment involved strain 1690 with all eight treatments. All treatments were replicated with three flasks, resulting in a 24-flask experiment. The average chlorophyll content over time for all eight treatments is shown in Figure 4.13 and the average pH over time for all eight treatments is shown in Figure 4.14. From observing all 24 flasks throughout the experiment, it was evident that the cultures at 35°C were not as healthy as those at 22°C because they were not as green and had turned a yellowish-brown color by the end of the experiment. After 72 hours, the flasks in the 22°C chamber all had a significantly higher chlorophyll content than the flasks in the 35°C chamber and this difference remained for the duration of the experiment. One flask from each treatment at 22°C is shown in Figure 4.15 and one flask from each treatment at 35°C is shown in Figure 4.16. This significant difference between the chlorophyll concentrations of the cultures at 22°C and those at 35°C indicates that the modifications from 35-7-m and 35-6-m were unable to cause an increased thermotolerance and allow these modified cultures to grow as well as 22-7-u and 22-6-u.

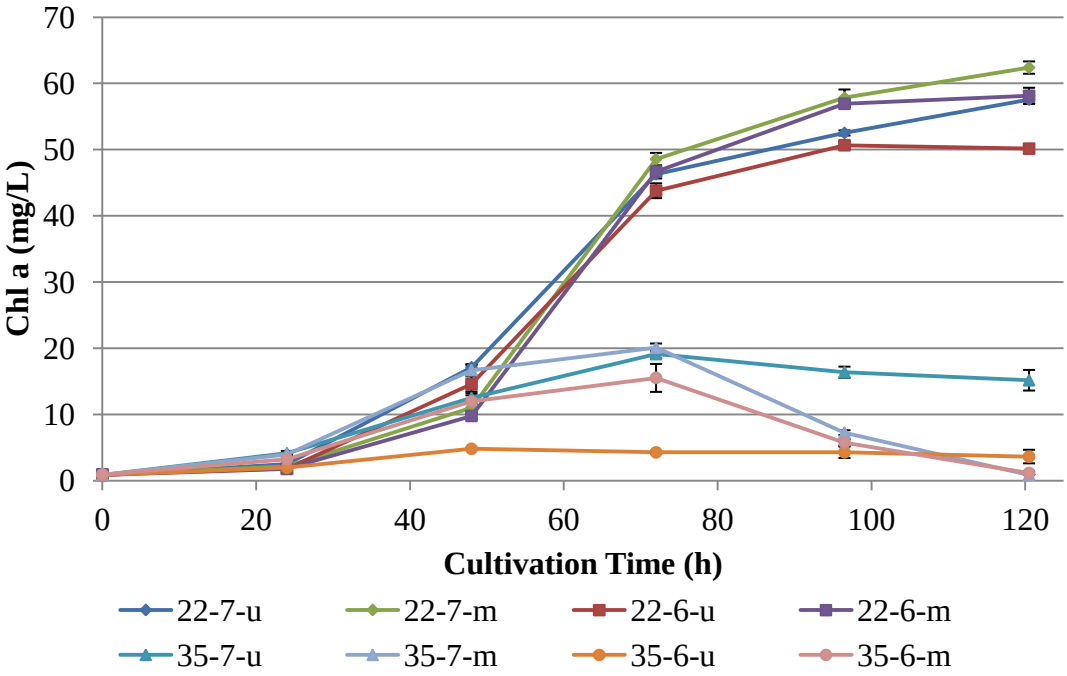

Figure 4.13 Chlorophyll content of *C. reinhardtii* strain 1690 over time for all treatments. Error bars represent standard error (n=3).

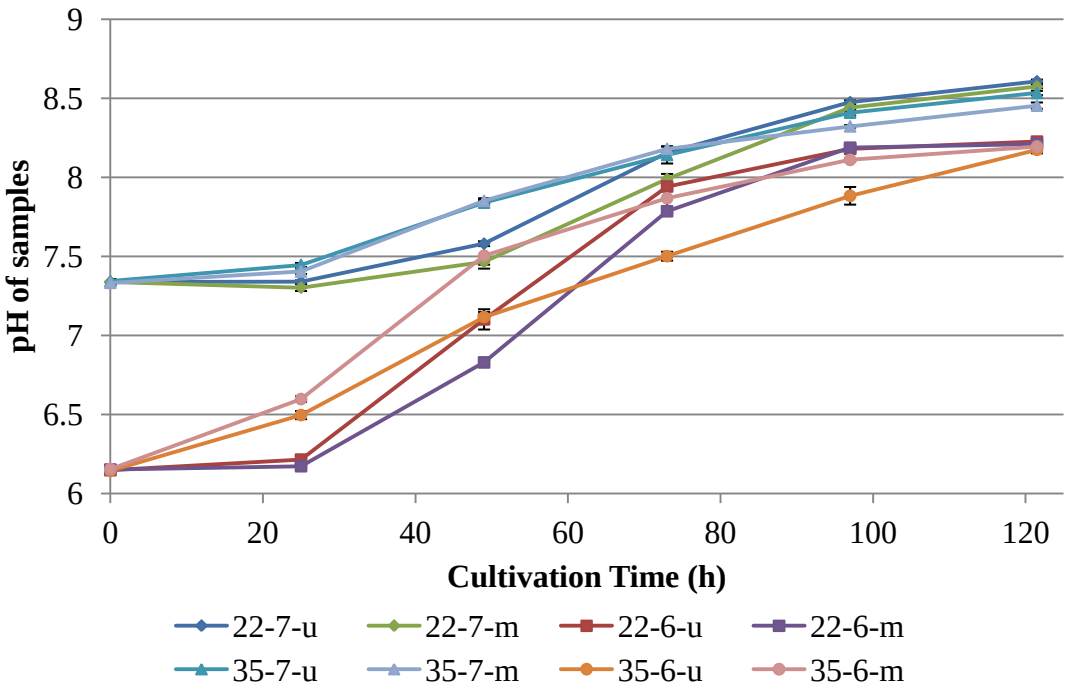

Figure 4.14 pH of *C. reinhardtii* strain 1690 over time for all treatments. Error bars represent standard error (n=3).

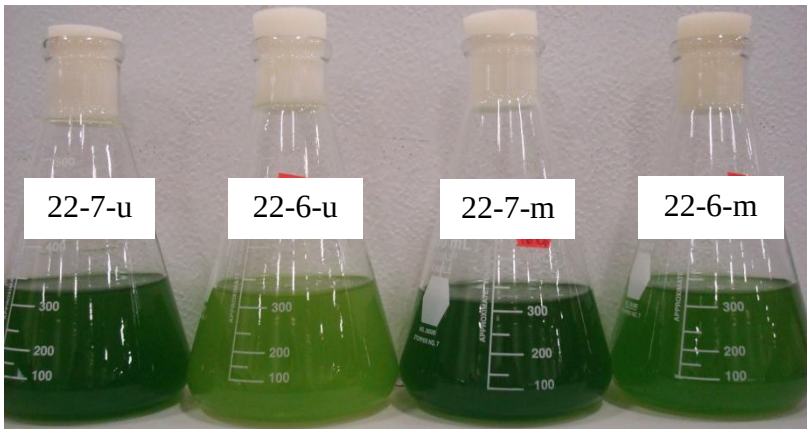

Figure 4.15 *C. reinhardtii* strain 1690 cultures after 72 hours at 22°C.

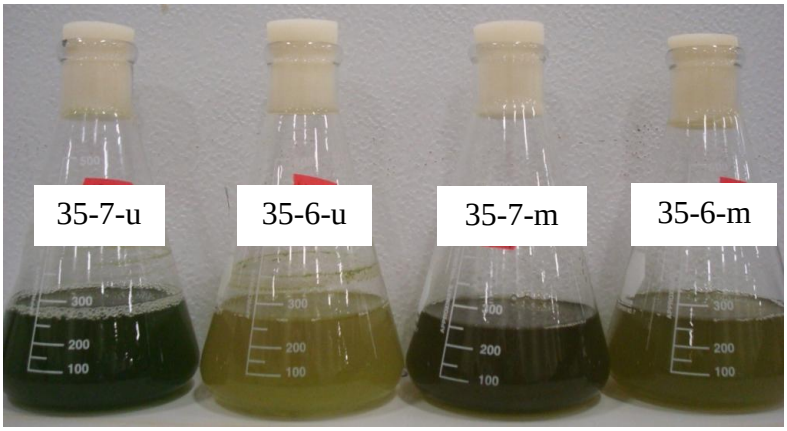

Figure 4.16 *C. reinhardtii* strain 1690 cultures after 72 hours at 35°C.

Information about the various growth metrics used to compare the algal cultures from all eight treatments is found in Table 4.1 and Table 4.2. The average final chlorophyll concentration (mg/L) is given for each treatment, but because the cultures grew at different rates and some were already in the death phase by the end of the experiment, the average highest chlorophyll concentration is also shown (Table 4.1). The average final pH for each treatment is also given as well as the average change in pH over the course of the experiment (Table 4.1). An important note from these two columns (and Figure 4.14) is to see how much closer the pH is for each treatment at the end of the experiment versus at the beginning of the experiment when some cultures had a pH of 6 and some cultures had a pH around 7. The pH of the algae cultures stabilized around 8.1-8.5, which could indicate that this is the limiting pH for this algal species. Even after the algal cultures at 35°C entered the death phase, the pH of the cultures

continued to increase towards this maximum pH. The growth rate slope for the first 24 hours of the experiment as well as the growth rate slope during the growth phase are shown in Table 4.2. The graphs showing the calculated slope during the growth phase for all treatments are contained in Appendix D. The growth rates during the growth phase of the cultures grown at 22°C were all at least double and up to 13 times greater than the growth rates of the cultures grown at 35°C. However, for the first 24 hours, the cultures grown at 35°C grew at a higher growth rate than those at 22°C. Comparing 22-7-u and 35-7-u, the growth rate of 35-7-u was more than double that of 22-7-u. Similarly with 22-7-m and 35-7-m, the growth rate of 35-7-m was more than double that of 22-7-m.

After 72 hours, the chlorophyll content of the cultures in the 35°C chamber decreased because the cultures were dying. They reached the death phase quickly as compared to the other cultures because the growth environment was too harsh rather than being either light or nutrient limited. The cultures from 35-6-u had the least amount of chlorophyll of all treatments after 72 hours. The chlorophyll content of 35-6-m after 72 hours was 3.6 times greater than the chlorophyll content of 35-6-u, but after 72 hours 35-6-m entered the death phase and for the rest of the experiment there was not a significant difference in the chlorophyll content of the two treatments. This indicates that the modification of 35-6-m allowed the algae to grow better with an initial pH of 6 than the unmodified algae of 35-6-u. Treatment 35-7-m had a significantly higher chlorophyll content than 35-7-u until they both peaked around 72 hours and then 35-7-m had a much faster death rate than did 35-7-u so that by the end of the experiment the chlorophyll content of 35-7-u was almost 17 times greater than the chlorophyll content of 35-7-m. Again, the modification of 35-7-m allowed the algae to grow faster than the unmodified algae of 35-7-u, but the modified algae also had an accelerated death rate over the unmodified algae. After 120 hours, 35-7-u had a statistically significant higher average chlorophyll content than the other cultures at 35°C, but there were no significant differences between the chlorophyll content of the other cultures at 35°C.

After 120 hours, there were significant differences between 22-7-u and 22-7-m and between 22-6-u and 22-6-m; 22-6-m had a 16% higher chlorophyll content than did 22-6-u and 22-7-m had an 8% higher chlorophyll content than 22-7-u. These two points

suggest that the modifications of 22-7-m and 22-6-m gave them an advantage over the unmodified algae of 22-7-u and 22-6-u. At the same time (120 hours), there was no significant difference between 22-7-u and 22-6-m, indicating that the modification of 22-6-m allowed it to grow to the same algal biomass concentration as 22-7-u, which represents the optimal media environment with the optimal temperature and pH. Between hours 96 and 120, 22-6-u and 22-6-m had close to stationary growth while 22-7-u and 22-7-m continued to have linear growth, which indicates that 22-6-u and 22-6-m were closer to entering the death phase than were 22-7-u and 22-7-m.

Table 4.1. Growth comparison for *C. reinhardtii* strain 1690 for all eight treatments.

Treatment	Final Chlorophyll Concentration (mg/L)	Highest Chlorophyll Concentration (mg/L)	Final pH	Change in pH over experiment
22-7-u	57.6	57.6	8.608	1.269
22-7-m	62.4	62.4	8.575	1.236
22-6-u	50.2	50.7	8.226	2.078
22-6-m	58.1	58.1	8.212	2.059
35-7-u	15.2	19.1	8.534	1.190
35-7-m	0.899	20.1	8.454	1.121
35-6-u	3.66	4.82	8.175	2.031
35-6-m	1.19	15.5	8.196	2.040

Table 4.2. Growth rate comparison for *C. reinhardtii* strain 1690 for all eight treatments.

Treatment	Slope for First 24 Hours (mg/L/h)	Slope during Growth Phase (mg/L/h)
22-7-u	0.071	1.22
22-7-m	0.052	1.56
22-6-u	0.038	1.22
22-6-m	0.036	1.54
35-7-u	0.136	0.312
35-7-m	0.129	0.530
35-6-u	0.046	0.118
35-6-m	0.096	0.226

4.2.2 Experiment 2: Comparing C. reinhardtii *strain 503 at various temperatures and pH*

The second experiment involved strain 503 and all eight treatments. All treatments were replicated with three flasks, resulting in a 24-flask experiment. The average chlorophyll content over time for all eight treatments is shown in Figure 4.17 and the average pH over time for all eight treatments is shown in Figure 4.18. When the cultures were sampled after 24 hours, the cultures for 22-6-u and 35-6-u had no visibly discernible chlorophyll as seen in Figure 4.19 and in Figure 4.20. These flasks had a lower chlorophyll content than at the beginning of the experiment. Treatments 22-6-m and 35-6-m were also started using media with an initial pH of 6, but these cultures did not appear bleached like the flasks from 22-6-u and 35-6-u, as seen in Figure 4.19 and Figure 4.20. After another 24 hours (a total of 48 hours), the seemingly bleached flasks showed signs of algal growth. One flask each from all eight treatments after 48 hours is shown in Figure 4.21 and Figure 4.22. The flasks from 22-6-u, 22-6-m, 35-6-u, and 35-6-m had a visibly lower chlorophyll content, but none of the flasks appeared bleached. As seen by the faint green color of the flask in Figure 4.21, the cultures from 22-6-u had a low chlorophyll content after 48 hours, but the growth was not normal (Figure 4.23). Rather than the culture being a homogeneous mixture of algae and media, these flasks had tiny clumps of algae that grew in the media. A flask from 35-6-u is shown in Figure 4.24 and the abnormality of the algal growth is immediately evident. The algae were still able to grow as the experiment progressed, but it was clumped together. When poked with a pipette tip, the algae clumps were pliant and could be torn apart.

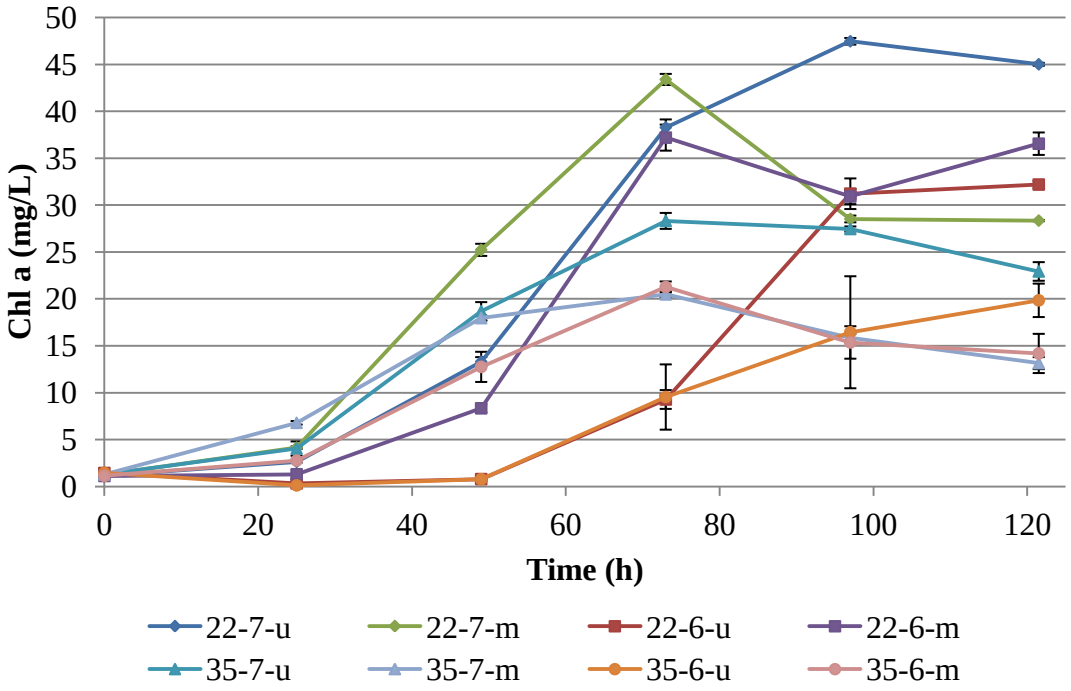

Figure 4.17 Chlorophyll content of *C. reinhardtii* strain 503 over time for all treatments. Error bars represent standard error (n=3).

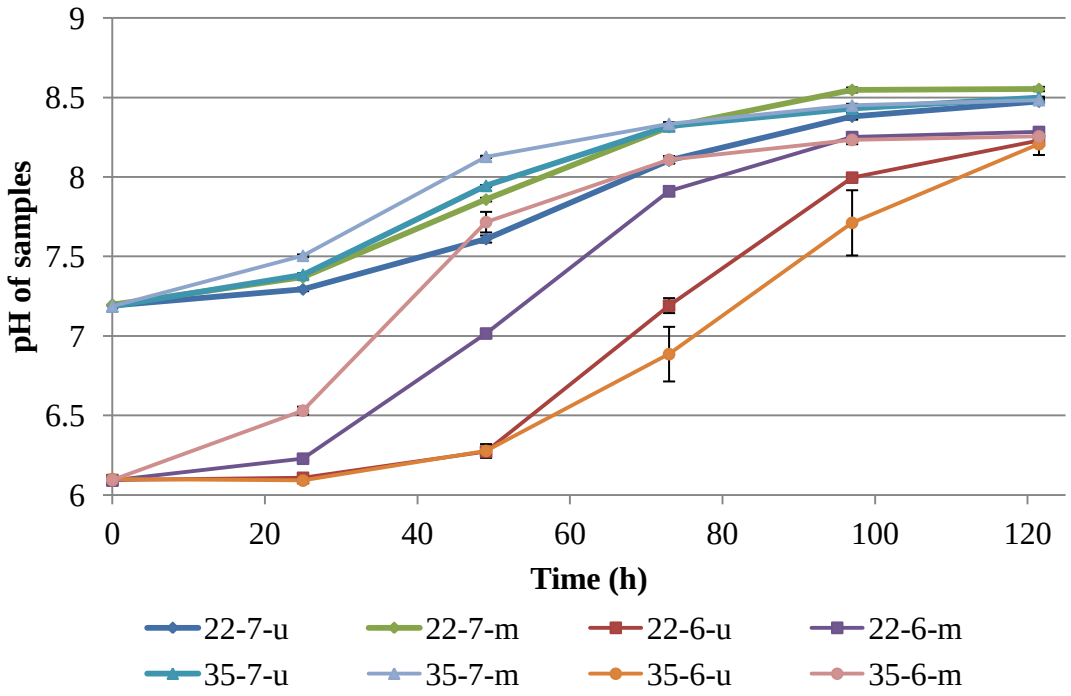

Figure 4.18 pH of *C. reinhardtii* strain 503 over time for all treatments. Error bars represent standard error (n=3).

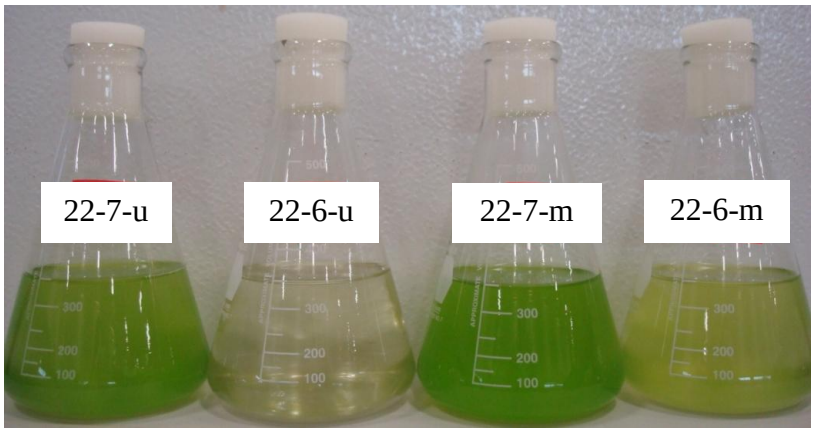

Figure 4.19 *C. reinhardtii* strain 503 cultures after 24 hours at 22°C.

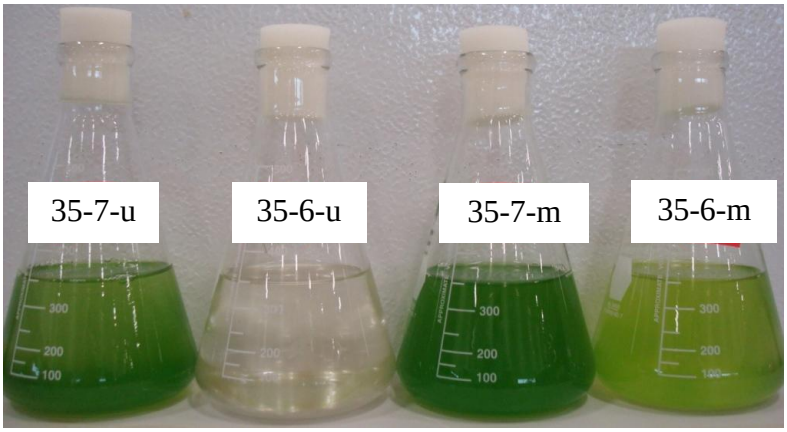

Figure 4.20 *C. reinhardtii* strain 503 cultures after 24 hours at 35°C.

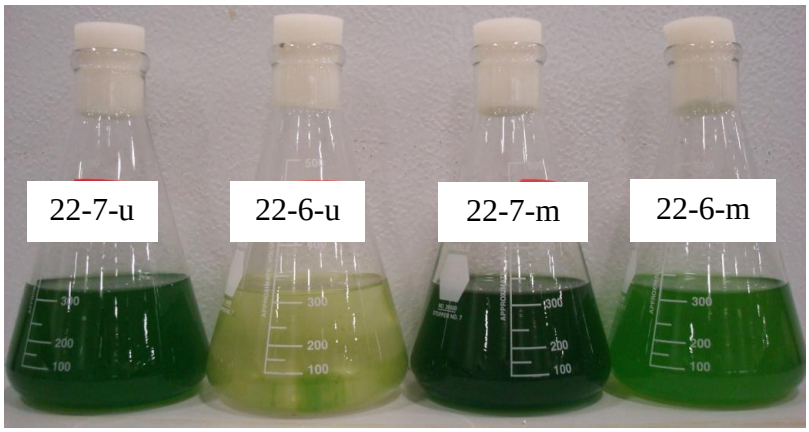

Figure 4.21 *C. reinhardtii* strain 503 cultures after 48 hours at 22°C.

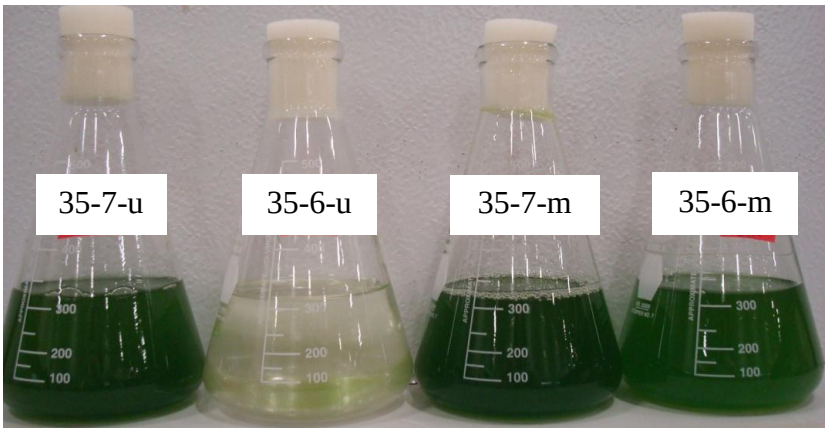

Figure 4.22 *C. reinhardtii* strain 503 cultures after 48 hours at 35°C.

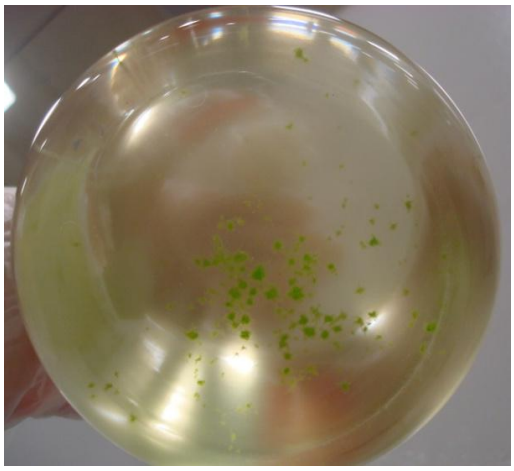

Figure 4.23 *C. reinhardtii* strain 503 culture after 48 hours at 22°C with an initial pH of 6 and no modification (22-6-u).

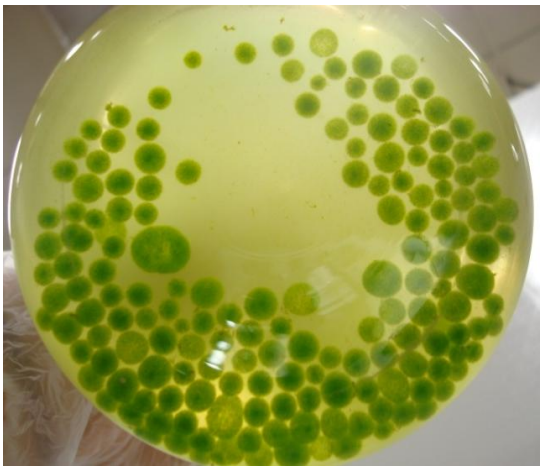

Figure 4.24 *C. reinhardtii* strain 503 culture after 96 hours at 35°C with an initial pH of 6 and no modification (35-6-u).

Information about the various growth metrics used to compare the algal cultures from all eight treatments is found in Table 4.3 and Table 4.4. Just as in the previous experiment involving strain 1690, the pH of all treatments increased towards a common maximum pH, which is shown in Figure 4.18. There is a statistically significant difference between the average final pH of all cultures starting with a pH of 7 and all cultures starting with a pH of 6, but the magnitude of the difference in the final pH values for the cultures was much smaller than it was at the beginning of the experiment.

Overall, the growth rate during the growth phase for cultures grown at 22°C was 1.5 to 3.5 times higher than the growth rate during the growth phase for cultures grown at

35°C, which shows the effect of the temperature difference between these two treatment groups. The initial pH of the cultures did not have as much of an effect on the growth rate during the growth phase, which indicates that the stress caused by the increased temperature was greater than the stress caused by the decreased pH.

Comparisons were difficult to make for the chlorophyll content of the different treatments in this experiment because the cultures grew at such different rates. While all the cultures experienced at least a 24 hour lag phase, as shown in Figure 4.17, 22-6-u and 35-6-u experienced a lag phase that was at least 24 hours longer and experienced a negative growth rate for the first 24 hours. After 72 hours, 35-6-m had a chlorophyll concentration that was 2.2 times greater than that of 35-6-u, but if the chlorophyll concentration of 35-6-m at 72 hours is compared to the chlorophyll concentration of 35-6-u at 121.5 hours, which is when they both peaked, there is no statistical difference. This means that 35-6-m was better than 35-6-u if the goal is to grow algae at the fastest rate, but there was no difference if the goal is to create the most algal biomass with disregard for time. Similarly, 22-6-u had a much longer lag phase than did 22-6-m, which resulted in a significant difference in their chlorophyll content through 72 hours at which point 22-6-m had a chlorophyll concentration that was 4 times greater than that of 22-6-u. However, by 96 hours there was no significant difference between 22-6-u and 22-6-m. Therefore, if the goal were to grow algae as quickly as possible, 22-6-m would be better, but if the goal were to grow as much algae as possible in 120 hours, there would be no advantage to either 22-6-u or 22-6-m.

Treatment 22-7-m had a significantly higher chlorophyll content than did 22-7-u from 24 hours to 72 hours, but then 22-7-m entered the death phase whereas 22-7-u continued to grow. After 96 hours, 22-7-u entered the death phase as well, but 22-7-u had a significantly higher chlorophyll content after 96 hours and ended the experiment with a chlorophyll concentration that was 59% higher than that of 22-7-m. Therefore, 22-7-m is better if the goal is to grow as much algae as possible as quickly as possible, but 22-7-u is better overall if the goal is to grow as much algae as possible without concern for time.

Through 49 hours, there was no significant difference between 35-7-u and 35-7-m, but after 49 hours, 35-7-u had a significantly higher chlorophyll concentration than

did 35-7-m. This seems to indicate that the modification of 35-7-m was actually detrimental to its growth as compared to 35-7-u, which runs counter to the results from the other treatments. Looking at the peak chlorophyll concentrations for 35-7-m and 35-6-m, there was no significant difference, which means that the modification of 35-6-m allowed it to grow as well as 35-7-m despite having a lower initial pH. However, as 35-7-m did not grow as well as its unmodified counterpart, this is not as great a comparison as it would be if 35-7-m had outgrown 35-7-u.

While the modifications caused some increased tolerance to a decreased pH, there were no indications that the modifications caused an increased thermotolerance. The initial growth rates of the 35°C cultures were higher than those of the 22°C cultures, but the peak chlorophyll concentrations of the 22°C cultures were all significantly higher than the peak concentrations of the 35°C cultures.

Table 4.3. Growth comparison for *C. reinhardtii* strain 503 for all eight treatments.

Treatment	Final Chlorophyll (mg/L)	Highest Chlorophyll (mg/L)	Final pH	Change in pH over experiment
22-7-u	45.0	47.5	8.475	1.283
22-7-m	28.4	43.4	8.554	1.356
22-6-u	32.2	32.2	8.230	2.135
22-6-m	36.5	37.2	8.284	2.193
35-7-u	22.9	28.3	8.499	1.313
35-7-m	13.2	20.5	8.484	1.297
35-6-u	19.8	19.8	8.207	2.107
35-6-m	14.2	21.3	8.256	2.162

Table 4.4. Growth rate comparison for *C. reinhardtii* strain 503 for all eight treatments.

Treatment	Slope for First 25 Hours (mg/L/h)	Slope during Growth Phase (mg/L/h)
22-7-u	0.059	1.04
22-7-m	0.116	0.818
22-6-u	-0.044	0.914
22-6-m	0.007	1.20
35-7-u	0.110	0.506
35-7-m	0.220	0.465
35-6-u	-0.053	0.327
35-6-m	0.065	0.386

4.2.3Experiment 3: Comparing acclimated C. reinhardtii *strain 1690 at various temperatures and pH*

The third experiment tested the strain 1690 cultures which had previously been acclimated to media with an initial pH of 5.6 for all eight treatments, but this time the stress pH level was pH 5.6 rather than pH 6[2]. All treatments were replicated with three flasks, resulting in a 24-flask experiment. The average chlorophyll content over time for all eight treatments is shown in Figure 4.25 and the average pH over time for all eight treatments is shown in Figure 4.26. All 24 flasks at the beginning of the experiment are shown in Figure 4.27, which shows that all of the cultures were visually identical initially. Because it was known that the cultures growing in media with an initial pH of 5.6 would be shocked initially by the environmental pH, the chlorophyll content for all 24 flasks was intentionally made greater than in the two previous experiments so the algae would not be overwhelmed. After 24 hours, the cultures with an initial pH of 5.6 appeared dead, as seen in Figure 4.28 and Figure 4.29, because they were clear and showed no visible signs of algae. The cultures with an initial pH of 7 all appeared healthy and were already a very dark green because the initial algae concentration was higher at the beginning of the experiment than in the previous experiment with strain 1690. After 48 hours, the cultures with an initial pH of 5.6 still appeared dead and the

[2] The treatment names were changed to include an "a" to indicate that the acclimated algae were used.

cultures with an initial pH of 7 continued to grow healthily (Figure 4.30 and Figure 4.31). After 72 hours, the cultures from 22-5.6-au and 22-5.6-am showed signs of algal growth by their slight green tint (Figure 4.32) and this growth was also reflected by their pH increase.

Treatments 35-5.6-au and 35-5.6-am still appeared dead (Figure 4.33), but a culture from 35-5.6-au did have some growth in the flask (Figure 4.34). As in the previous experiment (Figure 4.24), the algae grew abnormally and clumped together in pliant balls; however, the algae biomass was completely bleached so that there was almost no detectable chlorophyll. This was one example of why using chlorophyll content as a growth metric does not always work perfectly. Only one of the three flasks from 35-5.6-au showed any sign of algae material growing, which is why the error bars in Figure 4.26 for 35-5.6-au were so much greater than the error bars for any of the other treatments. The algae cultures after 120 hours in the 22°C and 35°C chambers are shown in Figure 4.35 and Figure 4.36, respectively. Cultures from 22-5.6-au and 22-5.6-am continued to quickly grow and there was no visibly-discernible difference in their chlorophyll content. Most cultures from 35-5.6-au and 35-5.6-am continued to show no growth except for one flask from 35-5.6-au and one flask from 35-5.6-am. As seen in Figure 4.37, one flask from 35-5.6-am had tiny specks of green algae growing at the bottom of the flask, but there was so little algae material in the flask that it did not make a difference in the overall average chlorophyll content of the three flasks from 35-5.6-am. By the end of 120 hours, the cultures from 35-7-au and 35-7-am were a dark yellow-brown color and had almost no chlorophyll content.

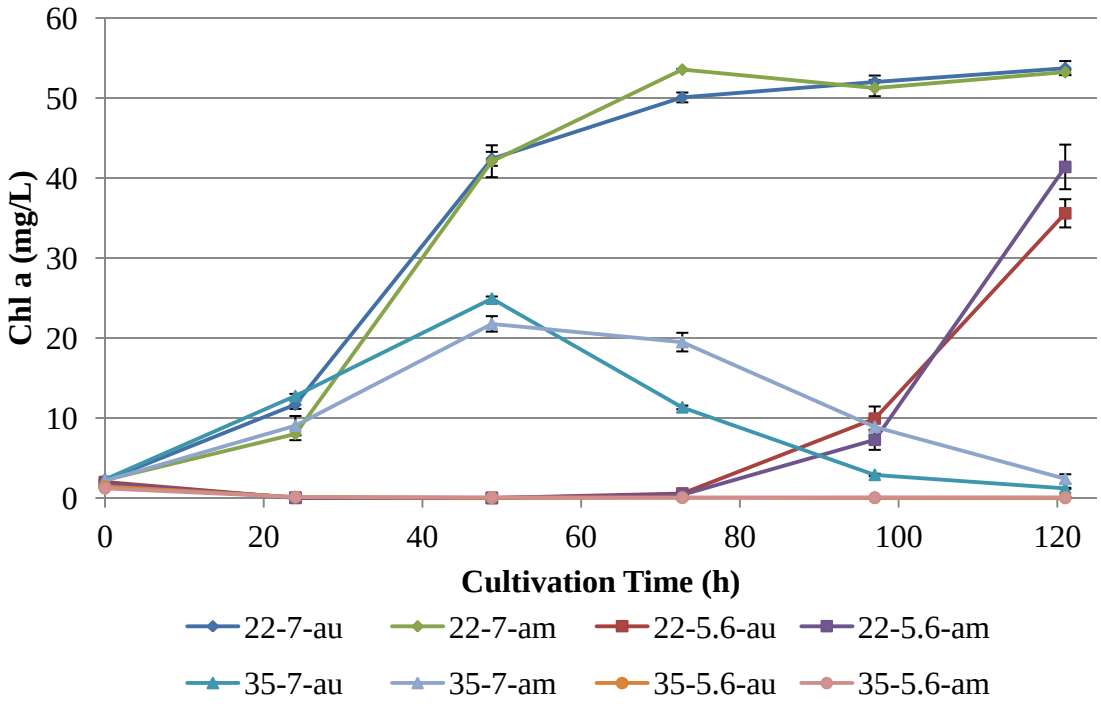

Figure 4.25 Chlorophyll content of acclimated *C. reinhardtii* strain 1690 over time for all treatments. Error bars represent standard error (n=3).

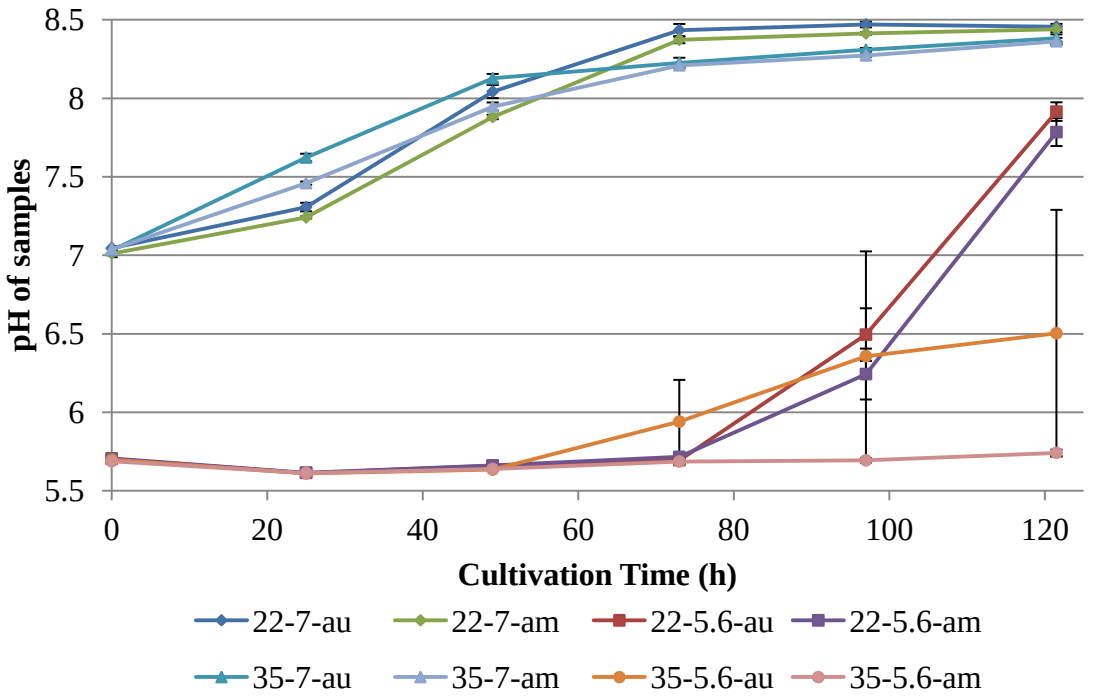

Figure 4.26 pH of acclimated *C. reinhardtii* strain 1690 over time for all treatments. Error bars represent standard error (n=3).

Figure 4.27 Acclimated *C. reinhardtii* strain 1690 cultures at time 0.

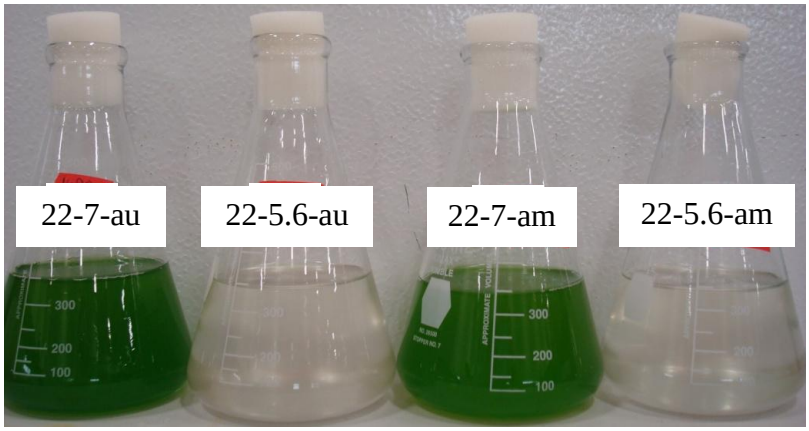

Figure 4.28 Acclimated *C. reinhardtii* strain 1690 cultures after 24 hours at 22°C.

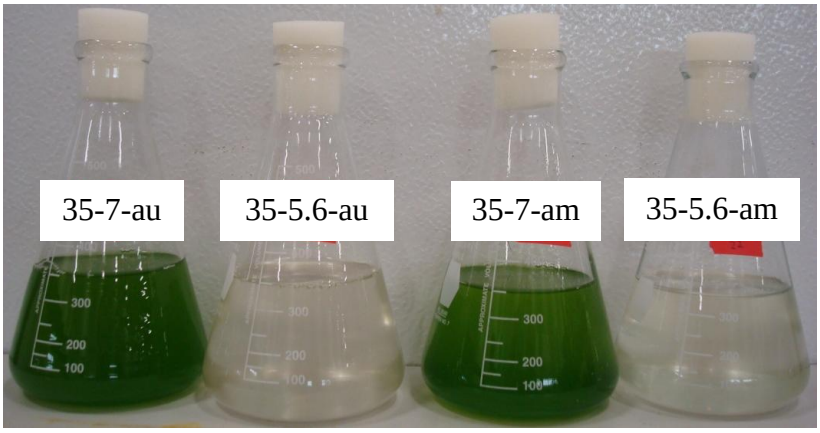

Figure 4.29 Acclimated *C. reinhardtii* strain 1690 cultures after 24 hours at 35°C.

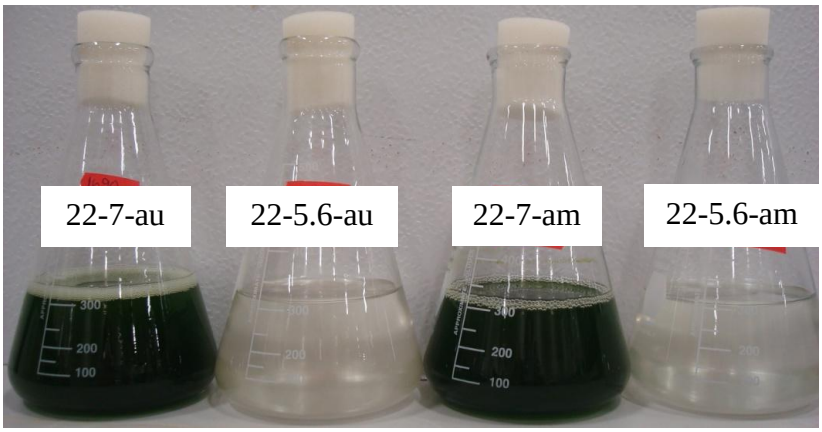

Figure 4.30 Acclimated *C. reinhardtii* strain 1690 cultures after 48 hours at 22°C.

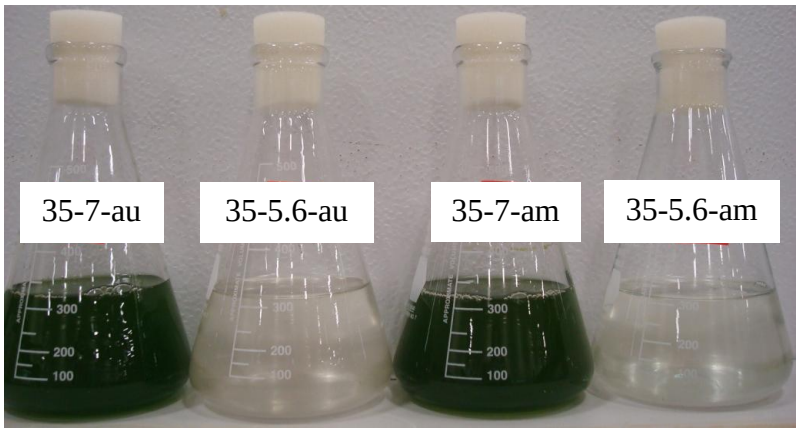

Figure 4.31 Acclimated *C. reinhardtii* strain 1690 cultures after 48 hours at 35°C.

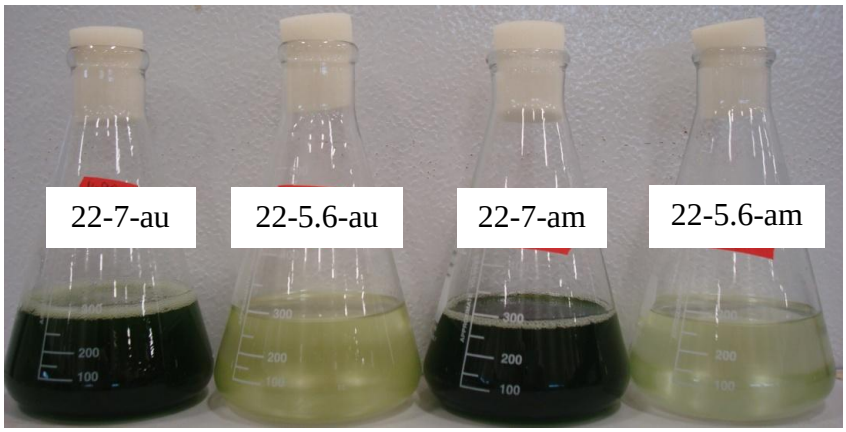

Figure 4.32 Acclimated *C. reinhardtii* strain 1690 cultures after 72 hours at 22°C.

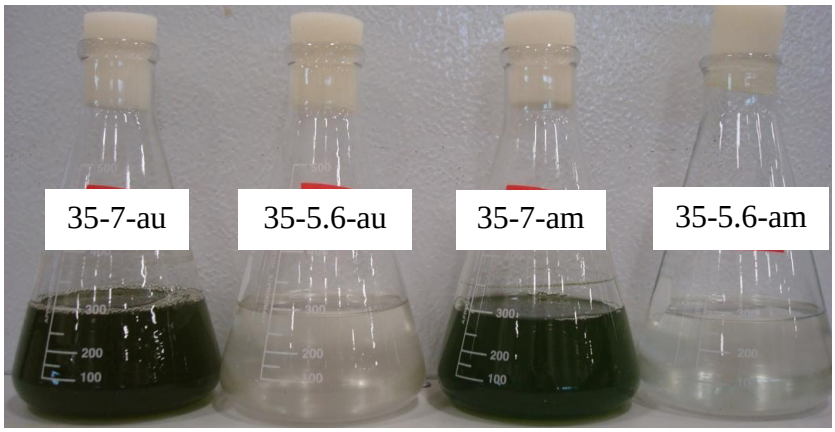

Figure 4.33 Acclimated *C. reinhardtii* strain 1690 cultures after 72 hours at 35°C.

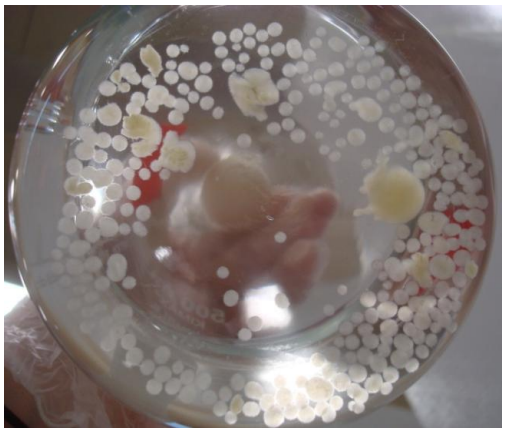
Figure 4.34 Acclimated *C. reinhardtii* strain 1690 culture after 72 hours at 35°C with an initial pH of 6 and no modification (35-5.6-au).

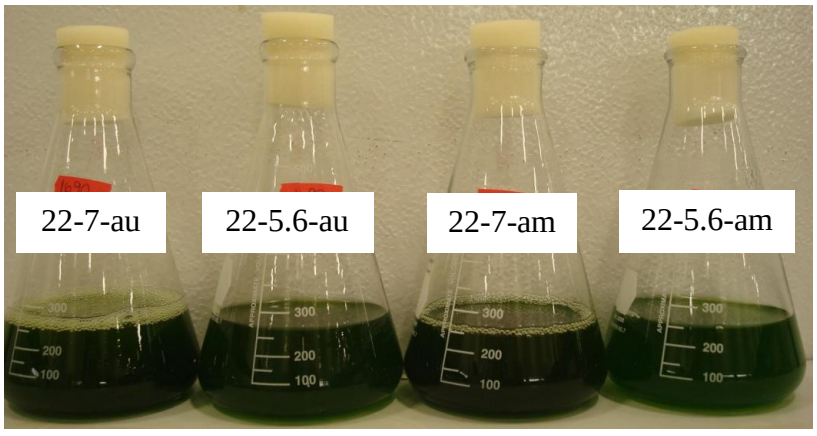

Figure 4.35 Acclimated *C. reinhardtii* strain 1690 cultures after 120 hours at 22°C.

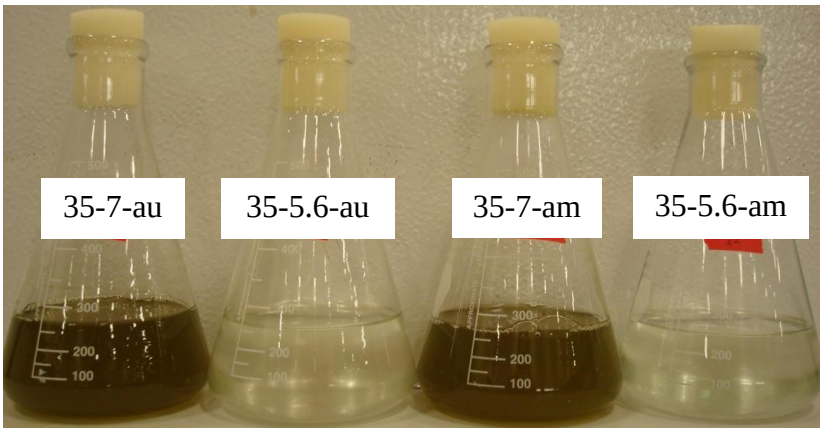

Figure 4.36 Acclimated *C. reinhardtii* strain 1690 cultures after 120 hours at 35°C.

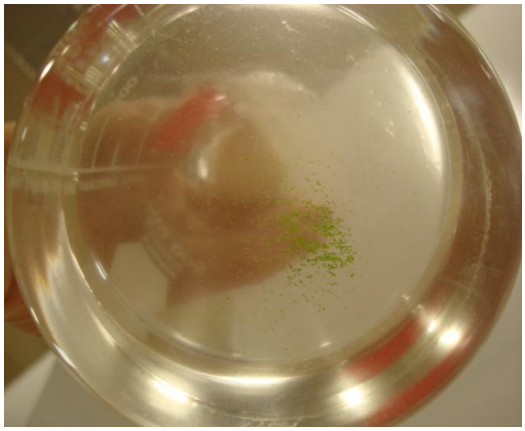
Figure 4.37 Acclimated C. reinhardtii strain 1690 culture after 120 hours at 35°C with an initial pH of 5.6 and modification (35-5.6-am).

Information related to the average growth rates for the cultures from all eight treatments is shown in Table 4.5 and Table 4.6. The growth rates during the growth phase for 22°C were 2 to 3.5 times greater than the growth rates during the growth phase for 35°C. The growth rates for 35-5.6-au and 35-5.6-am are listed as not applicable because these treatments had no significant increase in the amount of chlorophyll over the course of the experiment and, therefore, did not experience a growth phase. As stated previously, one culture from 35-5.6-au showed growth, but the material had no discernible chlorophyll. Once again, temperature appeared to have a greater effect than pH on the growth rate during the growth phase because the growth rates for 22°C were all at least twice as great as the growth rates for 35-7-au and 35-7-am, and 35-5.6-au and 35-

5.6-am were unable to grow whereas 22-5.6-au and 22-5.6-am were able to grow after an extended lag phase.

After 120 hours, there was no significant difference between the chlorophyll concentration of 22-7-au and 22-7-am. The only time there was a significant difference during the growth period was when sampled after 72 hours. Treatments 22-5.6-au and 22-5.6-am had a longer lag phase but began to grow after 72 hours. There was no significant difference between the chlorophyll concentrations of these treatments during the experiment. The lack of a significant difference in the chlorophyll concentrations of 22-7-au and 22-7-am and 22-5.6-au and 22-5.6-am indicates that the modifications of 22-7-am and 22-5.6-am conferred no advantage to them over the unmodified 22-7-au and 22-5.6-au. Treatments 22-5.6-au and 22-5.6-am were still growing at the end of the experiment, so it is unknown how their final chlorophyll concentration would compare to 22-7-au and 22-7-am.

The peak chlorophyll concentration for both 35-7-au and 35-7-am was around 48 hours and the cultures from both treatments entered the death phase not long after. There was no significant difference between the chlorophyll concentration of the cultures from 35-7-au and 35-7-am after 48 hours, but 35-7-am had a much slower death rate than did 35-7-au, which resulted in 35-7-am having a 72% higher chlorophyll concentration after 72 hours and 3 times greater chlorophyll concentration after 96 hours. By 120 hours, there was no longer a significant difference. The modification of 35-7-am could be the difference in the death rate after the peak concentration.

Treatment 35-5.6-au indicates a case in which chlorophyll concentration is not an accurate representation of algal growth. One flask from 35-5.6-au experienced growth, as seen in Figure 4.34, but this growth is not reflected in the chlorophyll concentration because the biomass was bleached. The growth is evident by the increase in pH, as seen in Figure 4.26, but pH is not a direct correlation to growth. Any significant difference between the chlorophyll concentrations of 35-5.6-au and 35-5.6-am throughout the experiment and 35-7-au and 35-7-am after 24 and 48 hours can be attributed to instrument error from the spectrophotometer because the absorptance values were so close to the minimum edge of its range.

This experiment did not provide much evidence that the modification conferred an increased tolerance to decreased pH nor an increased thermotolerance. Comparing 22-7-au and 35-7-am, 35-7-am had a significantly lower chlorophyll concentration than did 22-7-au. 35-7-am did grow better than 22-5.6-au because 22-5.6-au had an extended lag phase. Therefore, if the goal were to grow algae as quickly as possible, 35-7-am would be better than 22-5.6-au, but if the goal is to grow as much algae as possible, 22-5.6-au is better even though it would require a longer cultivation time due to its extended lag phase.

Table 4.5. Growth comparison for acclimated *C. reinhardtii* strain 1690 for all eight treatments.

Treatment	Final Chlorophyll (mg/L)	Highest Chlorophyll (mg/L)	Final pH	Change in pH over experiment
22-7-au	53.8	53.8	8.455	1.411
22-7-am	53.2	53.2	8.439	1.427
22-5.6-au	35.6	35.6	7.914	2.211
22-5.6-am	41.4	41.4	7.784	2.078
35-7-au	1.22	24.9	8.383	1.351
35-7-am	2.40	21.8	8.362	1.324
35-5.6-au	0.027	1.45	6.503	0.805
35-5.6-am	0.077	1.22	5.741	0.054

Table 4.6. Growth rate comparison for acclimated *C. reinhardtii* strain 1690 for all eight treatments.

Treatment	Slope for First 24 Hours (mg/L/h)	Slope during Growth Phase (mg/L/h)
22-7-au	0.394	1.24
22-7-am	0.241	1.38
22-5.6-au	-0.083	1.07
22-5.6-am	-0.072	1.42
35-7-au	0.434	0.463
35-7-am	0.286	0.402
35-5.6-au	-0.055	N/A
35-5.6-am	-0.045	N/A

CHAPTER 5 : CONCLUSIONS

Algae are considered a possible tool for carbon mitigation for flue gas from coal-fired power plants, but there are adjustments that have to be made to the flue gas before it can be used as a carbon source for algal growth. The presence of CO_2 and SO_4 in flue gas cause it to have an acidic pH and the combustion processes that create flue gas cause high temperatures. Environmental temperature and pH are two of the most important factors for algal growth and most algal species do not grow well at the environmental conditions presented by flue gas. Traditionally, flue gas has been altered to create an environment more capable of promoting algal growth, but this work aimed instead to modify the algae so as to make them capable of growing at conditions that are less than ideal.

Heat shock proteins (HSPs) have been identified as important in the environmental stress responses of many species. Consequently, three strains of the algal species *Chlamydomonas reinhardtii* were genetically modified to overexpress HSP70A to attempt to increase the algae's tolerance to elevated temperature and low pH. Strain 400 demonstrated a higher tolerance for elevated temperature and lower pH during preliminary tests, so only strains 503 and 1690 were tested during the main experiments.

The first experiment involving strain 1690 demonstrated that the modification causing increased expression of HSP70A gave those cultures an advantage over the cultures which had not been modified. After 72 hours at 35°C, 35-6-m had a higher chlorophyll content than 35-6-u. After 120 hours at 22°C, 22-7-m and 22-6-m had higher chlorophyll contents than 22-7-u and 22-6-u. Also, after 120 hours at 22°C, there was no statistical difference between the chlorophyll content of 22-7-u and 22-6-m, which indicated that the modification of 22-6-m allowed it to grow as well with an initial pH of 6 as 22-7-u, which grew in the optimal environment with an initial pH of 7.

The experiment that tested strain 503 had mixed results. Treatments 22-6-u and 35-6-u had significantly longer lag phases than the other treatments which made comparisons difficult. After 72 hours, 35-6-m had a significantly higher chlorophyll content than did 35-6-u, but if the peak amounts of chlorophyll are compared between 35-6-u and 35-6-m, there is no statistical significance. Similarly, 22-6-m had a significantly higher chlorophyll content than did 22-6-u until 96 hours, after which there

was no significant difference. Treatment 22-7-m had a significantly higher chlorophyll content than did 22-7-u until 72 hours, after which 22-7-m entered the death phase and 22-7-u continued to grow and after 96 hours had a significantly higher peak chlorophyll content than did 22-7-m. When comparing 22-6-u and 22-6-m and 35-6-u and 35-6-m, the modifications of 22-6-m and -35-6-m appear to confer an advantage if the goal were to grow as much algae as possible as quickly as possible. However, if the goal were to simply grow as much algae as possible while disregarding cultivation time, there is no difference between 22-6-u and 22-6-m or 35-6-u and 35-6-m. Treatments 22-7-u and 22-7-m are different though because 22-7-m is better if the goal were to grow as much algae as possible as quickly as possible, but 22-7-u is significantly better if the goal were to grow as much algae as possible disregarding cultivation time.

Treatments 35-7-u and 35-7-m give a somewhat different result because there was no significant difference between the two through 49 hours, but after that point 35-7-u had a significantly higher chlorophyll content than did 35-7-m, which indicates that the modification of 35-7-m may have been detrimental to its growth. There was no significant difference between the peak chlorophyll contents of 35-7-m and 35-6-m, which indicates that the modification of 35-6-m allowed it to grow as well as 35-7-m, which initially had a more favorable environment with a higher pH, but as stated previously, 35-7-m did not grow well as compared to 35-7-u.

The third experiment, which involved acclimated strain 1690, used a lower pH as the stress level and also had mixed results. There were no statistical differences between 22-7-au and 22-7-am or between 22-5.6-au and 22-5.6-am throughout the experiment, which indicates that the modifications of 22-7-am and 22-5.6-am conferred no advantage on them. There also was no statistical difference between 35-7-au and 35-7-am after 48 hours, which was the time for their peak chlorophyll concentration. However, after 48 hours, 35-7-au had a much quicker death rate as compared to 35-7-am and this difference could be attributed to the modification of 35-7-am. The cultures from 35-5.6-au and 35-5.6-am showed no growth over the course of the experiment and were unable to recover from the initial shock of their new environment in media with a pH of 5.6 at 35°C despite having been previously acclimated to media with an initial pH of 5.6.

All three experiments had certain comparisons which indicated that the algae which had been modified to overexpress HSP70A were better able to grow under different environmental conditions. While the modifications conferred an increased tolerance to lower pH, there were no indications that the modification allowed the algae grown at an elevated temperature to grow as well as the unmodified algae grown at the optimal temperature.

CHAPTER 6 : FUTURE WORK

During this study, the modified algae cultures that overexpressed HSP70A and expressed SSA1 were not completed. The presence of the yeast protein insert was never able to be confirmed in the plasmid, so the algae were never transformed with this modified plasmid. Future transformation and testing of these modified cultures would be important to test if the expression of SSA1 proteins would successfully increase the tolerance of algae cultures to higher temperatures and lower pH.

It was suggested late in the study that rather than using the HSP70A-RBCS2 promoter to drive gene expression, the PsaD promoter should be used instead. PsaD is a subunit of Photosystem I and is important for electron transfer during photosynthesis. The RBCS2 promoter has been used extensively to drive gene expression in *Chlamydomonas reinhardtii* and the insertion of the HSP70A promoter upstream has been shown to increase the strength of gene expression. The insertion of a gene of interest after this promoter can lead to the loss of important introns, which have been shown to be important for gene expression in *C. reinhardtii*. The coding sequence of the PsaD gene does not contain introns, which makes it more efficient for gene expression (Fischer and Rochaix, 2001). Plasmids containing this gene (e.g. pSL72 or pGenD PSAD cassette) are available for purchase from the Chlamydomonas Center and could be manipulated and transformed into algae for testing.

There could also be more work done to try to maximize the thermotolerance and pH tolerance of the successful strains from this work. A plasmid containing a promoter for HSP70A and/or SSA1 and a different antibiotic resistance (e.g. hygromycin) could be transformed into the modified algal cultures. Any cultures that grew on media supplemented with both antibiotics would have been successfully transformed with the second plasmid and should have a greater expression of the proteins, which should increase the algae's tolerance.

Further acclimation studies could be done in the future to try to increase algal tolerance to elevated temperature and lower pH. pH acclimation was attempted briefly during this study, but it might be possible to acclimate strains 400 and 1690 to a lower pH. Thermal acclimation was not explored during this study because of equipment

constraints, but acclimating the algae to gradually increasing temperature could improve its growth rate at the test temperature or higher.

After running the preliminary and main experiments for this research project, it was observed that the *C. reinhardtii* strain 1690 which had been acclimated to media with a lower initial pH ended up with a significantly lower chlorophyll content when grown in media with a pH of 7 than did the algae which had not been acclimated to a lower pH. This comparison is shown in Figure 6.1, which indicates that the unmodified algae from the first two preliminary experiments (22-7 vs. 22-6 and 22-7 vs. 35-7) and from the first main experiment (Comparing *C. reinhardtii* strain 1690 at various temperatures and pH) grew to a statistically higher chlorophyll content than did the acclimated algae from the third main experiment (Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH). When comparing treatments run at 35°C, as shown in Figure 6.2, the acclimated algae from the third main experiment (Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH) grew better than the unacclimated algae from the first main experiment (Comparing *C. reinhardtii* strain 1690 at various temperatures and pH), but it did not grow as well as the unacclimated algae from the second and third preliminary experiments (22-7 vs. 35-7 and 35-7 vs. 35-6). There were significant differences in the initial chlorophyll concentrations of the four treatments being compared at either temperature, so some of the difference in final chlorophyll concentration could be attributed to that initial difference. Time could have also affected this difference because the preliminary experiments were run 3-4 months before the main experiments, but this observation of the acclimated algae having a lower final chlorophyll concentration should be explored further. Experiments could be run to compare the unacclimated algae to the acclimated algae (both unmodified and modified) and further analysis could be performed to analyze any morphological differences between the unacclimated and acclimated algae.

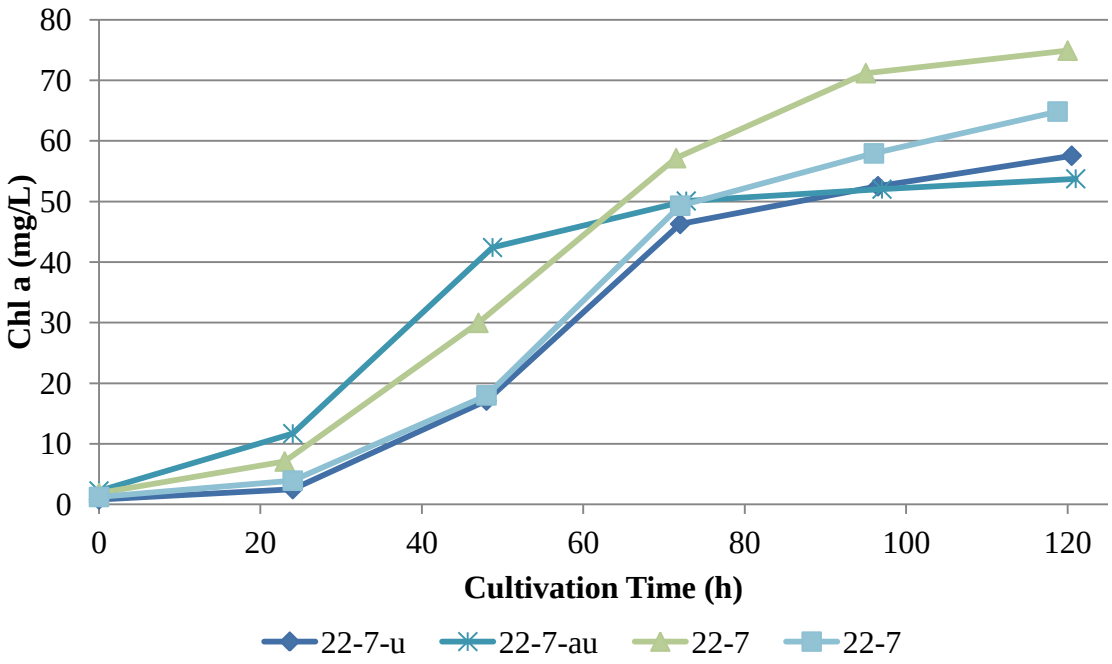

Figure 6.1 *C. reinhardtii* strain 1690 chlorophyll content over time. Treatment 22-7-u from Experiment 1, 22-7-au from Experiment 3, 22-7 from Preliminary experiment 1, and 22-7 from Preliminary experiment 2. All treatments involved unmodified algae.

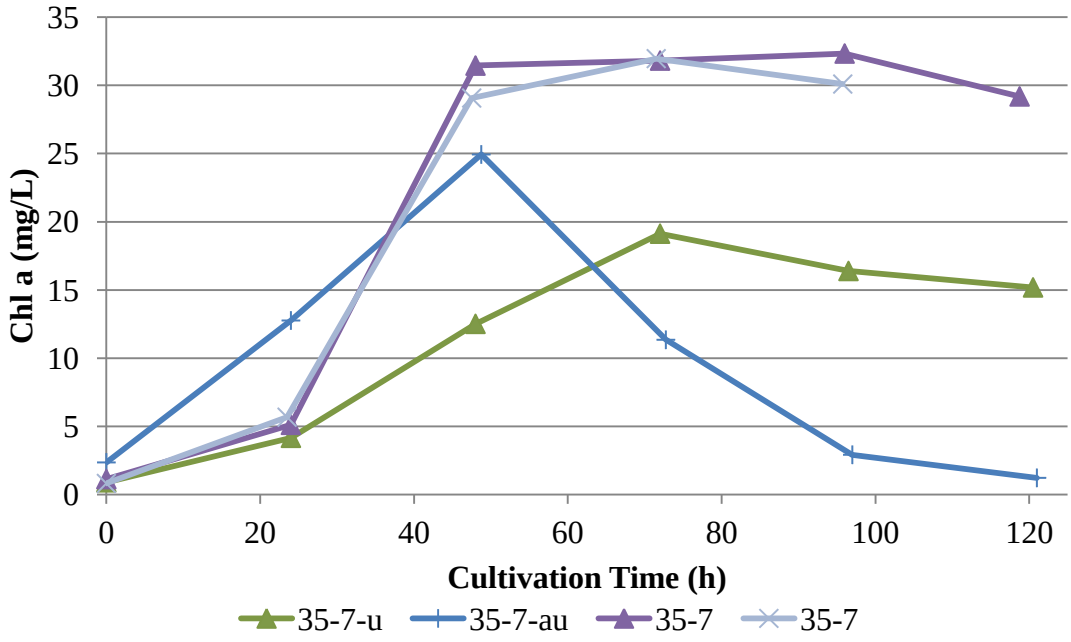

Figure 6.2 *C. reinhardtii* strain 1690 chlorophyll content over time. Treatment 35-7-u from Experiment 1, treatment 35-7-au from Experiment 3, 35-7 from Preliminary experiment 2, 35-7 from Preliminary experiment 3. All treatments involved unmodified algae.

Further testing could be done to compare the growth rates of the unmodified and modified strains from this work with the growth rates of the related species *Chlamydomonas acidophila* (which can be purchased from the Canadian Phycological Culture Centre). The latter species gave support to the theory that something can be altered within algae to allow it to survive under otherwise lethally stressful situations, but testing could be done to see if the unmodified *C. acidophila* would have a higher growth rate at the stressful growth conditions than the modified *C. reinhardtii* cultures. This would indicate that rather than fully focusing on modifying a readily available species, more work should be done to find a species that may already be better adapted to the environmental conditions presented by using flue gas as a carbon source.

While this work aimed to create genetically modified strains of *Chlamydomonas reinhardtii*, in the future these same techniques would need to be used on other algal species that are more likely to be used for CO_2 mitigation at power plants. There are two species being investigated at the University of Kentucky, *Chlorella vulgaris* and *Scenedesmus acutus*, and either of them would have a higher rate of CO_2 uptake during photosynthetic growth, which means they would be better suited for CO_2 mitigation than would *C. reinhardtii*. However, for these techniques to be used with these species, their genomes would have to be at least partially sequenced, which is not the case at this time.

APPENDICES

Appendix A. Media Recipes

TAP medium (Chlamydomonas Center)

TAP salts

Compound	Mass (g)
NH_4Cl	15.0
$MgSO_4 \cdot 7H_2O$	4.0
$CaCl_2 \cdot 2H_2O$	2.0

Add water to 1 liter.

Phosphate solution

Compound	Mass (g)
K_2HPO_4	28.8
KH_2PO_4	14.4

Add water to 100 ml.

Hutner's trace elements

See below

Final Mixture:

Tris(hydroxymethyl)-aminomethane	2.42 g or 10 ml 2M solution
TAP salts	25 ml
Phosphate solution	0.375 ml
Hutner's trace elements	1.0 ml
Glacial acetic acid	1.0 ml

For solid medium, add 15 g agar per liter.

For TAP + 40mM sucrose, add 13.693 g sucrose per liter.

Autoclave.

Hutner's trace elements

For 1 liter final mix, dissolve each compound in the volume of water indicated. The EDTA should be dissolved in boiling water, and the FeSO4 should be prepared last to avoid oxidation.

Compound	Amount	Water
EDTA disodium salt	50 g	250 ml
$ZnSO_4 \cdot 7H_2O$	22 g	100 ml
H_3BO_3	11.4 g	200 ml
$MnCl_2 \cdot 4H_2O$	5.06 g	50 ml
$CoCl_2 \cdot 6H_2O$	1.61 g	50 ml
$CuSO_4 \cdot 5H_2O$	1.57 g	50 ml
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	1.10 g	50 ml
$FeSO_4 \cdot 7H_2O$	4.99 g	50 ml

Mix all solutions except EDTA. Bring to boil, then add EDTA solution. The mixture should turn green. When everything is dissolved, cool to 70 degrees C. Keeping temperature at 70, add 85 ml hot 20% KOH solution (20 grams / 100 ml final volume). Do NOT use NaOH to adjust the pH.

Bring the final solution to 1 liter total volume. It should be clear green initially. Stopper the flask with a cotton plug and let it stand for 1-2 weeks, shaking it once a day. The solution should eventually turn purple and leave a rust-brown precipitate, which can be removed by filtering through two layers of Whatman#1 filter paper, repeating the filtration if necessary until the solution is clear. Store refrigerated or frozen convenient aliquots. Some people shorten the time for formation of the precipitate by bubbling the solution with filtered air.

If no precipitate forms, the solution is still usable. However, you might want to check the pH in this case and adjust it to around 7.0 using either KOH or HCl as needed.

Bold's Basal Medium (Modified) (Stein, 1973)

STOCK	STOCK SOLUTION	mL/L
1. KH_2PO_4	9.75g/500mL	10mL
2. $CaCl_2 \cdot 2H_2O$	1.25g/500mL	10mL
3. $MgSO_4 \cdot 7H_2O$	3.75g/500mL	10mL
4. $NaNO_3$	12.5g/500mL	10mL
5. K_2HPO_4	3.75g/500mL	10mL
6. NaCl	1.25g/500mL	10mL
7. $Na_2EDTA \cdot 2H_2O$	10g/L	1mL
KOH	6.2g/L	
8. $FeSO_4 \cdot 7H_2O$	4.98g/L	1mL
H_2SO_4 (concentrated)	1mL/L	
9. Trace Metal Solution	See below	1mL
10.H_3BO_3	5.75g/500mL	0.7mL

Adjust the pH to 6.8.

Trace Metal Solution

Substance	g/L
1. H_3BO_3	2.86 g
2. $MnCl_2 \cdot 4H_2O$	1.81 g
3. $ZnSO_4 \cdot 7H_2O$	0.222 g
4. $Na_2MoO_4 \cdot 2H_2O$	0.390 g
5. $CuSO_4 \cdot 5H_2O$	0.079 g
6. $Co(NO_3)_2 \cdot 6H_2O$	0.0494 g

Dissolve each of the above substances separately prior to adding the next one on the list.

Appendix B. Transformation Methods for *Chlamydomonas*

Glass Beads Method - Unmodified

1. Grow cells in Sager & Granick medium (NH_4NO_3) until they reach a density of 1-2x10^6/ml.
2. Spin down cells in a centrifuge at 5000 rpm for 5 minutes.
3. Resuspend cells in 1/100 volume Sager & Granick medium (KNO_3) and allow to shake at room temperature for 2-4 hours.
4. Add 300 µl cells, 100 µl 20% w/v polyethylene glycol (PEG), 1-2 µg DNA, and 300 mg sterile glass beads. Vortex for 15-30 seconds at top speed. Centrifuge cells again to separate out the PEG solution. Remove the PEG solution and rinse the cells with sterilized water*.
5. Plate cells immediately on a TAP selective agar. Let plate dry before sealing with parafilm. Place in light.
6. Colonies should be visible after a few days.

Glass Beads Method – Modified

1. Grow cells in TAP medium until they reach an OD_{550} 0.4-0.6.
2. Spin down cells in a centrifuge at 5000 rpm for 5 minutes.
3. Resuspend cells in 1/100 volume TAP medium and allow to shake at room temperature for 2-4 hours.
7. Add 300 µl cells, 100 µl 20% w/v polyethylene glycol (PEG), 1-2 µg DNA, and 300 mg sterile glass beads. Vortex for 15-30 seconds at top speed. Centrifuge cells again to separate out the PEG solution. Remove the PEG solution and rinse the cells with sterilized water*.
4. Let cells recover in liquid TAP medium for 24 hours before plating on a TAP selective agar. Let plate dry before sealing with parafilm. Place in light.
5. Colonies should be visible after a few days.

*Although osmotic stress from PEG has been shown to cause a reduction in the growth rate of *Chlamydomonas reinhardtii*, it must be used during this transformation process to help permeabilize the cell membrane in order to allow the foreign DNA to penetrate the cell (Hema et al., 2007; Rosenberg et al., 2008).

Electroporation

1. Use 200-300 million cells per transformation. An OD_{550} 0.1 indicates the presence of roughly 1 million cells per milliliter. Therefore, spinning down 50mL of a culture with an OD_{550} 0.4-0.6 would give the target number of cells.
2. Pour off the media, loosely cap the tube, and turn it upside down so any excess media will drain into the cap. After the media has drained, resuspend the algae pellet in TAP + 40mM sucrose.
3. Add the resuspended cells to a 4mm sterile cuvette along with 5μg supercoiled plasmid DNA. Incubate the cuvette on ice for one minute immediately prior to electroporation.
4. Electroporation conditions: exponential decay, 800V, 25μF, $\infty\Omega$
5. After electroporation, allow the cells to recover for 24 hours in 10mL TAP + 40mM sucrose.
6. Plate on selection plate. Colonies should be visible in about a week.

Appendix C. Experimental Data

Table C.1. Chlorophyll content (mg/L) of *C. reinhardtii* strain 1690 during Experiment 1: Comparing strain 1690 at various temperatures and pH.

Time (h)	22-7-u			22-7-m			22-6-u			22-6-m		
0	0.803	0.851	0.836	0.868	0.962	0.975	0.911	0.879	0.853	0.959	0.972	1.01
24	2.42	2.63	2.53	2.01	2.27	2.29	1.85	1.89	1.64	1.81	1.72	1.98
48	17.1	17.8	16.6	10.2	12.0	10.9	16.2	15.7	11.9	9.28	9.62	10.5
72	45.4	47.5	45.9	47.4	50.4	47.9	45.8	43.7	41.9	45.4	47.5	47.2
96.5	51.9	52.4	53.2	55.7	60.1	57.7	50.1	50.1	51.8	56.9	56.2	57.7
120.5	58.3	56.4	57.9	60.7	63.8	62.7	50.9	48.8	50.7	56.2	57.9	60.4
Time (h)	35-7-u			35-7-m			35-6-u			35-6-m		
0	0.903	0.887	0.896	0.870	0.915	0.841	0.857	0.907	0.855	0.950	0.933	0.976
24	3.44	4.55	4.48	3.89	3.75	4.28	2.25	2.00	1.72	3.08	3.15	3.56
48	13.2	11.9	12.4	15.1	18.4	16.6	5.17	4.54	4.77	9.99	11.1	14.9
72	20.1	18.0	19.3	20.2	21.1	18.9	4.48	3.82	4.59	11.9	15.4	19.3
96.5	18.0	16.0	15.1	7.72	7.54	6.59	2.67	5.58	4.67	5.00	5.69	6.65
120.5	18.2	14.2	13.1	0.902	0.928	0.867	1.69	5.12	4.18	0.720	1.62	1.22

Table C.2 Chlorophyll content (mg/L) of *C. reinhardtii* strain 503 during Experiment 2: Comparing strain 503 at various temperatures and pH.

Time (h)	22-7-u			22-7-m			22-6-u			22-6-m		
0	1.21	0.986	1.27	1.26	1.25	1.25	1.40	1.46	1.45	1.20	1.07	1.10
25	2.52	2.57	2.79	4.24	3.90	4.29	0.381	0.329	0.268	1.32	1.27	1.31
49	14.2	13.2	12.5	25.5	24.0	26.2	0.877	0.700	0.782	8.80	8.73	7.48
73	36.6	39.5	38.7	43.7	42.3	44.3	9.17	7.60	11.1	37.5	39.4	34.6
97	48.1	46.9	47.3	28.0	28.3	29.2	33.1	32.6	28.0	32.2	31.1	29.4
121.5	45.2	44.7	45.2	28.3	28.5	28.2	32.4	32.7	31.4	38.9	35.2	35.5
Time (h)	35-7-u			35-7-m			35-6-u			35-6-m		
0	1.25	1.26	1.37	1.28	1.30	1.27	1.43	1.42	1.48	1.13	1.15	1.12
25	3.19	3.35	5.56	6.77	7.12	6.47	0.052	0.137	0.168	2.61	2.9	2.74
49	16.8	19.2	20.0	17.7	18.5	17.7	0.514	0.643	1.15	11.8	15.9	10.6
73	26.8	29.7	28.5	19.9	21.0	20.5	4.10	8.50	16.0	21.7	22.0	20.1
97	26.9	27.6	27.8	15.3	16.7	15.6	9.26	28.3	11.8	14.3	18.7	13.0
121.5	24.4	21.1	23.3	12.4	14.4	12.6	19.3	23.2	17.1	11.9	18.4	12.3

Table C.3. Chlorophyll content (mg/L) of *C. reinhardtii* strain 1690 during Experiment 3: Comparing acclimated strain 1690 at various temperatures and pH.

Time (h)	22-7-u			22-7-m			22-6-u			22-6-m		
0	2.12	2.25	2.33	2.50	1.87	2.38	1.98	2.07	1.98	1.77	1.77	1.82
24	12.1	10.6	12.4	9.33	6.61	8.16	0.024	0.040	0.014	0.073	0.061	0.027
48.75	44.1	41.2	42.0	46.1	40.4	39.8	0.015	0.042	0.038	0.015	0.006	0.013
72.75	50.2	49.0	51.1	53.6	53.7	53.4	0.442	0.612	0.704	0.329	0.457	0.515
97	53.5	51.8	50.8	50.9	53.1	49.7	7.74	9.26	12.8	5.10	7.32	9.45
121	52.1	53.9	55.2	53.8	52.8	53.2	37.5	37.2	32.1	36.3	45.9	42.0
Time (h)	35-7-u			35-7-m			35-6-u			35-6-m		
0	2.29	2.35	2.41	2.47	2.06	2.05	1.17	1.29	1.89	1.32	1.12	1.22
24	12.2	12.9	13.1	10.2	6.63	10.4	0.059	0.078	0.266	0.145	0.120	0.142
48.75	25.3	24.4	25.1	20.2	21.6	23.5	0.027	0.031	0.044	0.085	0.088	0.086
72.75	11.8	11.1	11.2	17.4	21.5	19.6	0.036	0.042	0.047	0.082	0.064	0.077
97	3.15	2.91	2.68	7.40	11.2	8.16	0.013	0.024	0.059	0.080	0.054	0.100
121	1.36	1.17	1.12	1.61	3.54	2.05	0.014	0.016	0.049	0.053	0.042	0.137

Appendix D. Growth Rate Graphs

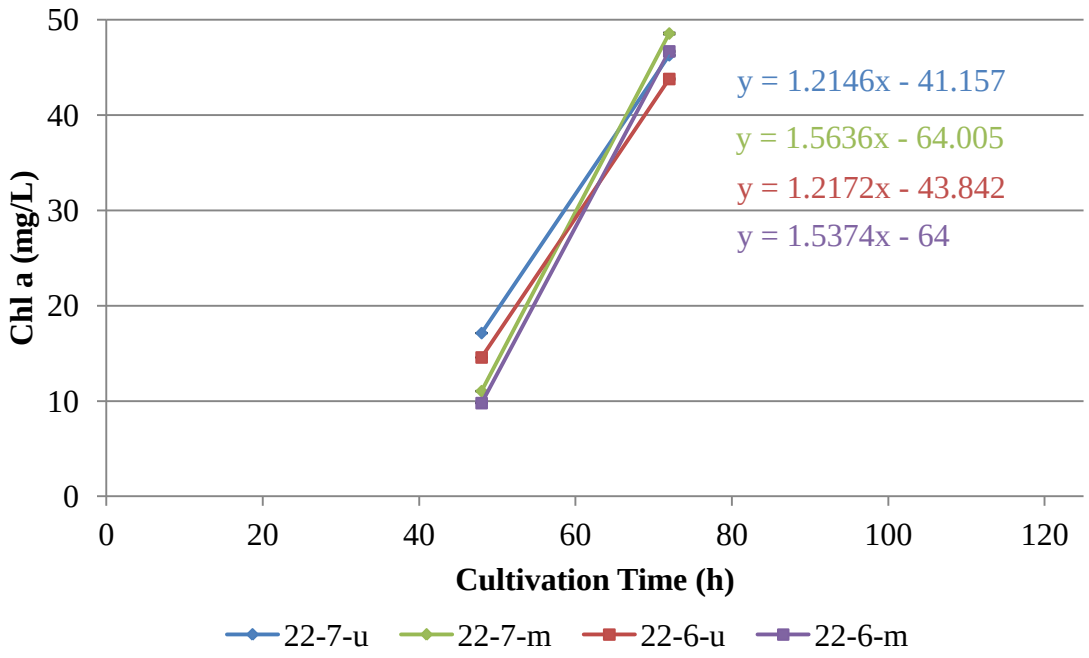

Figure D.1 Growth phase for *C. reinhardtii* strain 1690 at 22°C.

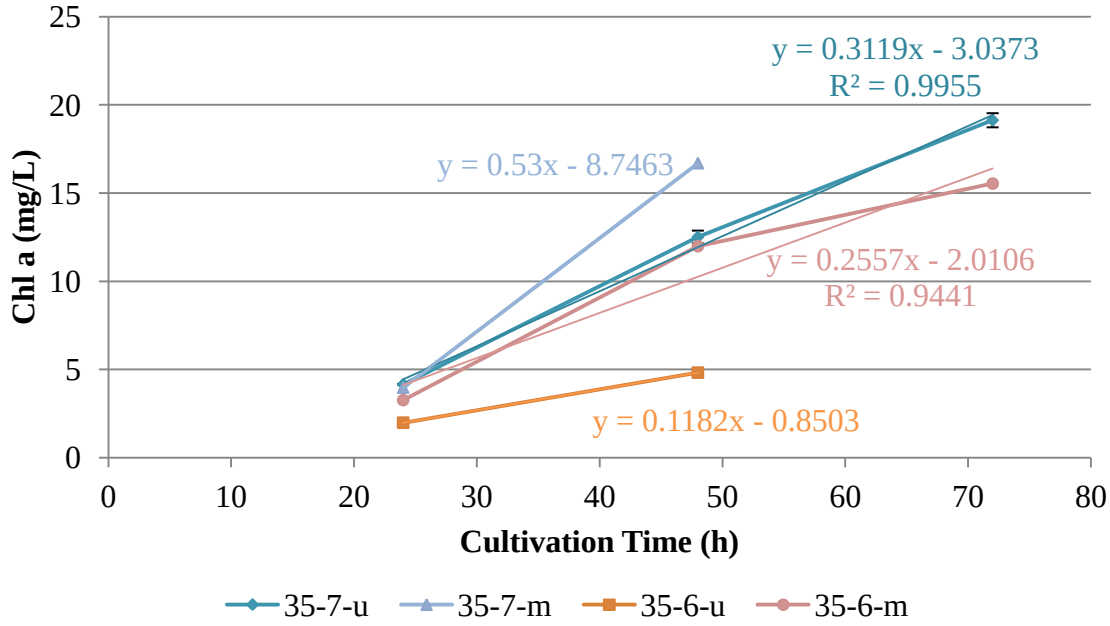

Figure D.2 Growth phase for *C. reinhardtii* strain 1690 at 35°C.

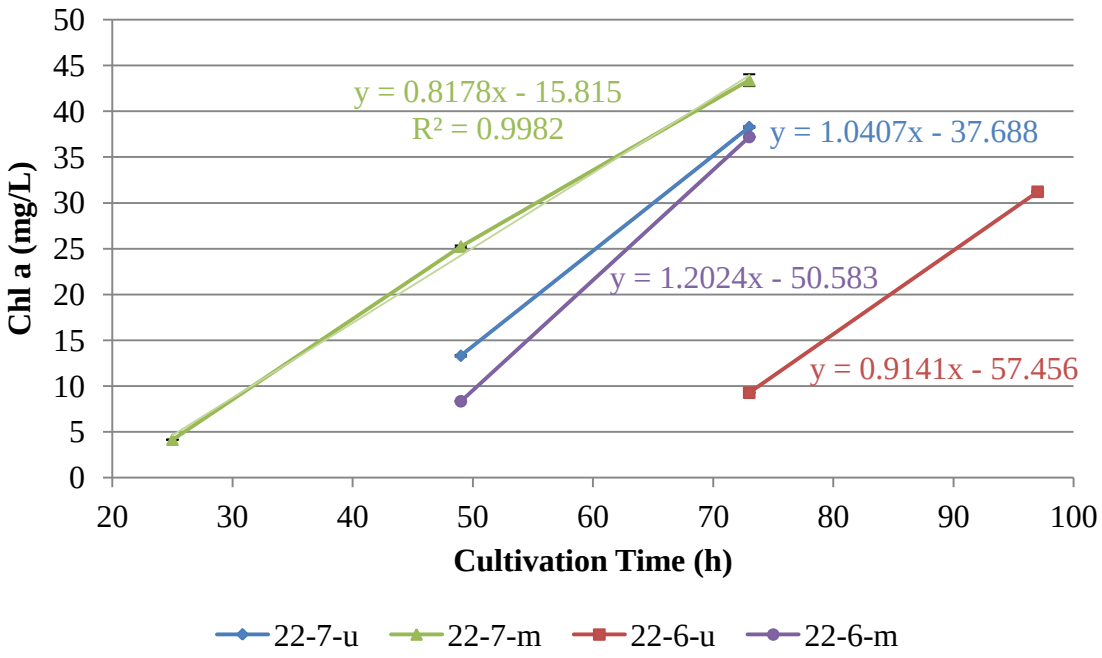

Figure D.3 Growth phase for *C. reinhardtii* strain 503 at 22°C.

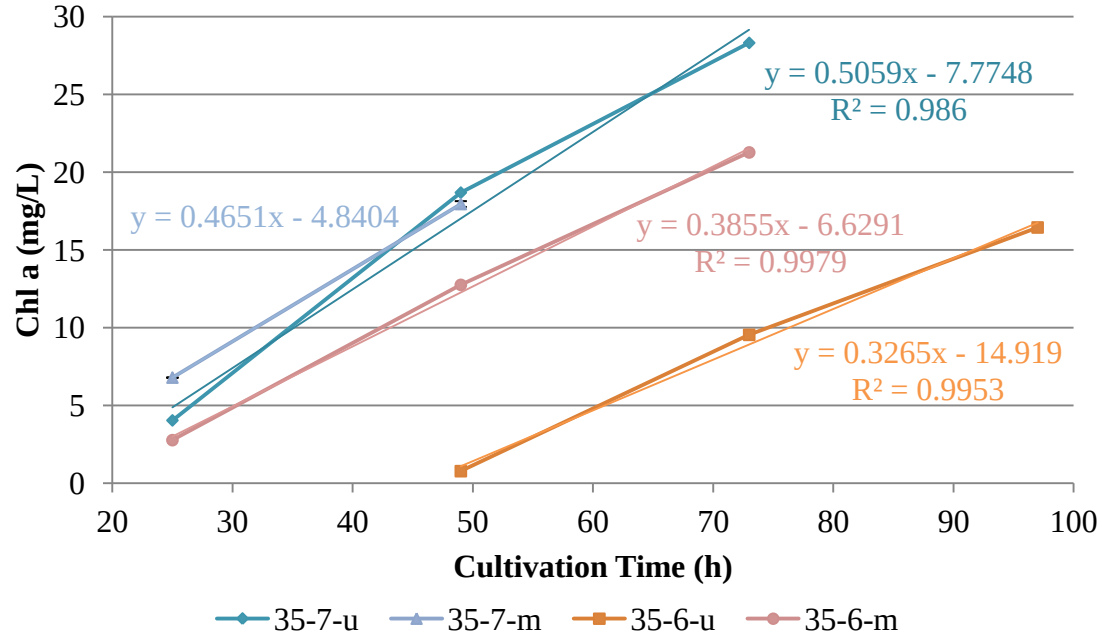

Figure D.4 Growth phase for *C. reinhardtii* strain 503 at 35°C.

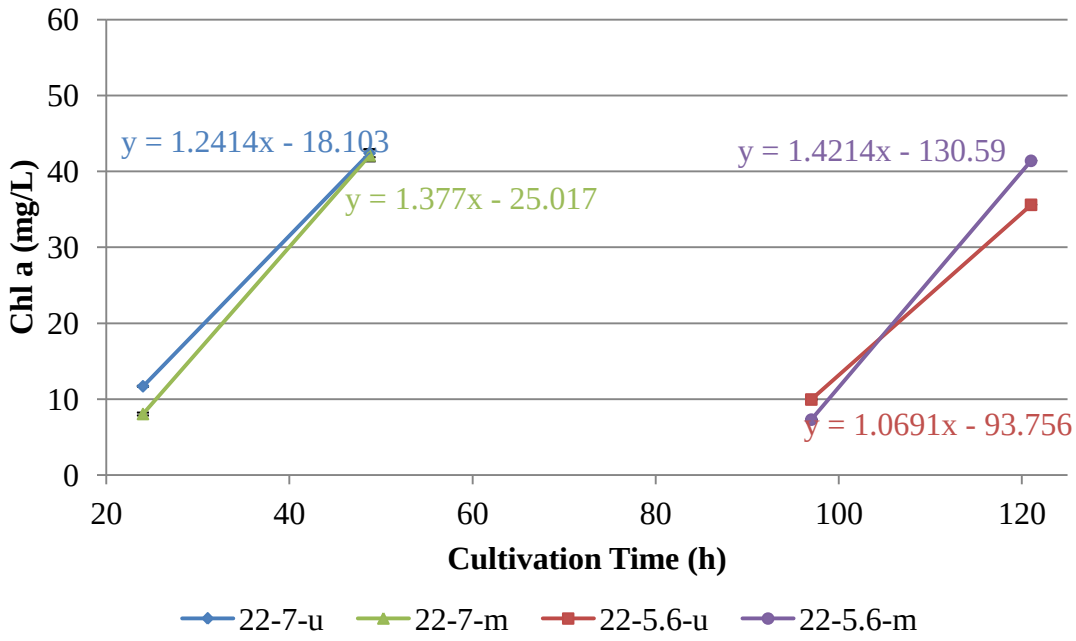

Figure D.5 Growth phase for acclimated *C. reinhardtii* strain 1690 at 22℃.

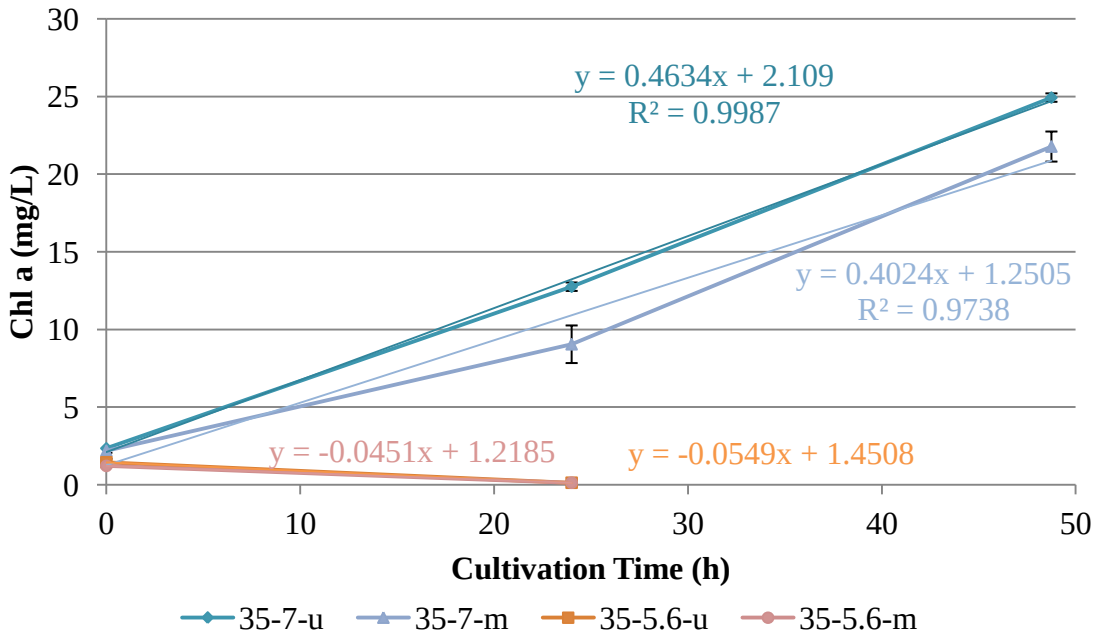

Figure D.6 Growth phase for acclimated *C. reinhardtii* strain 1690 at 35℃.

Appendix E. P-Values of Differences in Chlorophyll content (mg/L) between Treatments

Table E.1. P-values of differences in chlorophyll content (mg/L) for Experiment 1: Comparing *C. reinhardtii* strain 1690 at various temperatures and pH at time 0. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0727	0.0838	0.0019	0.0344	0.1653	0.1230	0.0031
22-7-m	0.0727	-	0.2480	0.3239	0.3582	0.2204	0.1997	0.6628
22-6-u	0.0838	0.2480	-	0.0124	0.4903	0.8432	0.7555	0.0305
22-6-m	0.0019	0.3239	0.0124	-	0.0220	0.0206	0.0093	0.2448
35-7-u	0.0344	0.3582	0.4903	0.0220	-	0.4507	0.3143	0.0325
35-7-m	0.1653	0.2204	0.8432	0.0206	0.4507	-	0.9414	0.0480
35-6-u	0.1230	0.1997	0.7555	0.0093	0.3143	0.9414	-	0.0217
35-6-m	0.0031	0.6628	0.0305	0.2448	0.0325	0.0480	0.0217	-

Table E.2. P-values of differences in chlorophyll content (mg/L) for Experiment 1: Comparing *C. reinhardtii* strain 1690 at various temperatures and pH after 24 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0439	0.0023	0.0024	0.0420	0.0056	0.0548	0.0266
22-7-m	0.0439	-	0.0317	0.0423	0.0259	0.0016	0.3303	0.0068
22-6-u	0.0023	0.0317	-	0.7271	0.0185	0.0012	0.3413	0.0032
22-6-m	0.0024	0.0423	0.7271	-	0.0195	0.0014	0.4375	0.0038
35-7-u	0.0420	0.0259	0.0185	0.0195	-	0.6738	0.0152	0.1162
35-7-m	0.0056	0.0016	0.0012	0.0014	0.6738	-	0.0008	0.0312
35-6-u	0.0548	0.3303	0.3413	0.4375	0.0152	0.0008	-	0.0040
35-6-m	0.0266	0.0068	0.0032	0.0038	0.1162	0.0312	0.0040	-

Table E.3. P-values of differences in chlorophyll content (mg/L) for Experiment 1: Comparing *C. reinhardtii* strain 1690 at various temperatures and pH after 48 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0013	0.1942	0.0001	0.0010	0.6894	0.0001	0.0681
22-7-m	0.0013	-	0.1064	0.1332	0.0976	0.0119	0.0036	0.6031
22-6-u	0.1942	0.1064	-	0.0629	0.2623	0.2780	0.0173	0.2684
22-6-m	0.0001	0.1332	0.0629	-	0.0075	0.0119	0.0013	0.2783
35-7-u	0.0010	0.0976	0.2623	0.0075	-	0.0321	0.0006	0.7636
35-7-m	0.6894	0.0119	0.2780	0.0101	0.0321	-	0.0049	0.0675
35-6-u	0.0001	0.0036	0.0173	0.0013	0.0006	0.00485	-	0.0392
35-6-m	0.0681	0.6031	0.2684	0.2783	0.7636	0.0675	0.0392	-

Table E.4. P-values of differences in chlorophyll content (mg/L) for Experiment 1: Comparing *C. reinhardtii* strain 1690 at various temperatures and pH after 72 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.1234	0.1438	0.6826	7.07E-06	8.32E-06	4.09E-05	0.0025
22-7-m	0.1234	-	0.0322	0.1835	3.85E-05	4.19E-05	0.0002	0.0011
22-6-u	0.1438	0.0322	-	0.10584	0.0002	0.0003	0.0005	0.0012
22-6-m	0.6826	0.1835	0.1058	-	7.16E-06	8.36E-06	4.68E-05	0.0024
35-7-u	7.07E-06	3.85E-05	0.0002	7.16E-06	-	0.3390	0.0005	0.2261
35-7-m	8.32E-06	4.19E-05	0.0003	8.36E-06	0.3390	-	0.0005	0.1548
35-6-u	4.09E-05	0.0002	0.0005	4.68E-05	0.0005	0.0005	-	0.0321
35-6-m	0.0025	0.0011	0.0012	0.0024	0.2261	0.1548	0.0321	-

Table E.5. P-values of differences in chlorophyll content (mg/L) for Experiment 1: Comparing *C. reinhardtii* strain 1690 at various temperatures and pH after 96.5 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0421	0.0643	0.0020	6.58E-05	1.22E-07	3.16E-05	3.12E-07
22-7-m	0.0421	-	0.0166	0.5603	3.30E-05	0.0003	1.29E-05	0.0001
22-6-u	0.0643	0.0166	-	0.0013	1.55E-05	3.08E-06	5.86E-06	6.87E-07
22-6-m	0.0020	0.5603	0.0013	-	2.38E-05	2.60E-07	1.16E-05	1.74E-07
35-7-u	6.58E-05	3.30E-05	1.55E-05	2.38E-05	-	0.0035	0.0006	0.0013
35-7-m	1.22E-07	0.0003	3.08E-06	2.60E-07	0.0035	-	0.0580	0.0703
35-6-u	3.16E-05	1.29E-05	5.86E-06	1.16E-05	0.0006	0.0580	-	0.2266
35-6-m	3.12E-07	0.0001	6.87E-07	1.74E-07	0.0013	0.0703	0.2266	-

Table E.6. P-values of differences in chlorophyll content (mg/L) for Experiment 1: Comparing *C. reinhardtii* strain 1690 at various temperatures and pH after 120.5 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0175	0.0013	0.7037	0.0004	9.99E-05	1.53E-05	6.25E-06
22-7-m	0.0175	-	0.0007	0.0538	6.51E-05	0.0002	2.02E-06	8.63E-05
22-6-u	0.0013	0.0007	-	0.0095	0.0004	0.0002	1.13E-05	2.97E-05
22-6-m	0.7037	0.0538	0.0095	-	4.07E-05	0.0005	5.84E-06	0.0003
35-7-u	0.0004	6.51E-05	0.0004	4.07E-05	-	0.0116	0.0055	0.0105
35-7-m	9.99E-05	0.0002	0.0002	0.0005	0.0116	-	0.1141	0.3853
35-6-u	1.53E-05	2.02E-06	1.13E-05	5.84E-06	0.0055	0.1141	-	0.1291
35-6-m	6.25E-06	8.63E-05	2.97E-05	0.0003	0.0105	0.3853	0.1291	-

Table E.7. P-values of differences in chlorophyll content (mg/L) for Experiment 2: Comparing *C. reinhardtii* strain 503 at various temperatures and pH at time 0. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.3718	0.0761	0.7489	0.2540	0.2737	0.0734	0.8072
22-7-m	0.3718	-	0.0081	0.0795	0.4388	0.0280	0.0085	0.0028
22-6-u	0.0761	0.0081	-	0.0070	0.0447	0.0074	0.8701	0.0007
22-6-m	0.7489	0.0795	0.0070	-	0.0382	0.0519	0.0064	0.8361
35-7-u	0.2540	0.4388	0.0447	0.0382	-	0.8772	0.0409	0.0475
35-7-m	0.2737	0.0280	0.0074	0.0519	0.8772	-	0.0080	0.0003
35-6-u	0.0734	0.0085	0.8701	0.0064	0.0409	0.0080	-	0.0008
35-6-m	0.8072	0.0028	0.0007	0.8361	0.0475	0.0003	0.0008	-

Table E.8. P-values of differences in chlorophyll content (mg/L) for Experiment 2: Comparing *C. reinhardtii* strain 503 at various temperatures and pH after 25 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0010	0.0003	0.0031	0.2066	0.0004	0.0002	0.3509
22-7-m	0.0010	-	0.0005	0.0016	0.9015	0.0007	0.0004	0.0011
22-6-u	0.0003	0.0005	-	0.0002	0.0399	0.0006	0.0125	0.0006
22-6-m	0.0031	0.0016	0.0002	-	0.0702	0.0011	0.0002	0.0039
35-7-u	0.2066	0.9015	0.0399	0.0702	-	0.0622	0.0360	0.2376
35-7-m	0.0004	0.0007	0.0006	0.0011	0.0622	-	0.0005	0.0003
35-6-u	0.0002	0.0004	0.0125	0.0002	0.0360	0.0005	-	0.0004
35-6-m	0.3509	0.0011	0.0006	0.0039	0.2376	0.0003	0.0004	-

Table E.9. P-values of differences in chlorophyll content (mg/L) for Experiment 2: Comparing *C. reinhardtii* strain 503 at various temperatures and pH after 49 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0002	0.0014	0.0016	0.0165	0.0028	0.0004	0.7689
22-7-m	0.0002	-	0.0007	0.0001	0.0074	0.0028	0.0003	0.0085
22-6-u	0.0014	0.0007	-	0.0029	0.0029	0.0002	0.9440	0.0175
22-6-m	0.0016	0.0001	0.0029	-	0.0033	0.0001	0.0008	0.1023
35-7-u	0.0165	0.0074	0.0029	0.0033	-	0.5333	0.0021	0.0449
35-7-m	0.0028	0.0028	0.0002	0.0001	0.5333	-	0.0000	0.0795
35-6-u	0.0004	0.0003	0.9440	0.0008	0.0021	0.0000	-	0.0164
35-6-m	0.7689	0.0085	0.0175	0.1023	0.0449	0.0795	0.0164	-

Table E.10. P-values of differences in chlorophyll content (mg/L) for Experiment 2: Comparing *C. reinhardtii* strain 503 at various temperatures and pH after 73 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	0.0105	3.08E-05	0.5480	0.0012	0.0008	0.0108	0.0002
22-7-m	0.0105	-	4.82E-05	0.0318	0.0003	4.95E-05	0.0089	1.20E-05
22-6-u	3.08E-05	4.82E-05	-	0.0002	0.0002	0.0045	0.9478	0.0014
22-6-m	0.5480	0.0318	0.0002	-	0.0093	0.0050	0.0080	0.0029
35-7-u	0.0012	0.0003	0.0002	0.0093	-	0.0060	0.0271	0.0037
35-7-m	0.0008	4.95E-05	0.0045	0.0050	0.0060	-	0.0869	0.3190
35-6-u	0.0108	0.0089	0.9478	0.0080	0.0271	0.0869	-	0.0740
35-6-m	0.0002	1.20E-05	0.0014	0.0029	0.0037	0.3190	0.0740	-

Table E.11. P-values of differences in chlorophyll content (mg/L) for Experiment 2: Comparing *C. reinhardtii* strain 503 at various temperatures and pH after 97 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	3.05E-06	0.0077	0.0006	2.86E-06	1.01E-06	0.0347	0.0021
22-7-m	3.05E-06	-	0.2383	0.0819	0.0823	3.19E-05	0.1799	0.0139
22-6-u	0.0077	0.2383	-	0.8831	0.1446	0.0077	0.1234	0.0027
22-6-m	0.0006	0.0819	0.8831	-	0.0401	0.0004	0.1337	0.0047
35-7-u	2.86E-06	0.0823	0.1446	0.0093	-	0.0001	0.2063	0.0179
35-7-m	1.01E-06	0.0000	0.0077	0.0004	0.0001	-	0.9294	0.8079
35-6-u	0.0347	0.1799	0.1234	0.1337	0.2063	0.9294	-	0.8753
35-6-m	0.0021	0.0139	0.0027	0.0047	0.0179	0.8079	0.8753	-

Table E.12. P-values of differences in chlorophyll content (mg/L) for Experiment 2: Comparing *C. reinhardtii* strain 503 at various temperatures and pH after 121.5 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-u	22-7-m	22-6-u	22-6-m	35-7-u	35-7-m	35-6-u	35-6-m
22-7-u	-	8.07E-06	0.0002	0.0179	0.0016	0.0002	0.0047	0.0044
22-7-m	8.07E-06	-	0.0091	0.0203	0.0314	0.0016	0.0411	0.0210
22-6-u	0.0002	0.0091	-	0.0558	0.0053	0.0001	0.0164	0.0110
22-6-m	0.0179	0.0203	0.0558	-	0.0011	0.0004	0.0025	0.0021
35-7-u	0.0016	0.0314	0.0053	0.0011	-	0.0022	0.2245	0.0353
35-7-m	0.0002	0.0016	0.0001	0.0004	0.0022	-	0.0510	0.6765
35-6-u	0.0047	0.0411	0.0164	0.0025	0.2245	0.0510	-	0.1099
35-6-m	0.0044	0.0210	0.0110	0.0021	0.0353	0.6765	0.1099	-

Table E.13. P-values of differences in chlorophyll content (mg/L) for Experiment 3: Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH at time 0. P-values less than 0.05 are marked in red.

Treatment	22-7-au	22-7-am	22-6-au	22-6-am	35-7-au	35-7-am	35-6-au	35-6-am
22-7-au	-	0.9339	0.0542	0.0145	0.1884	0.8211	0.0626	0.0003
22-7-am	0.9339	-	0.3446	0.1363	0.6553	0.8259	0.0540	0.0253
22-6-au	0.0542	0.3446	-	0.0059	0.0021	0.3176	0.1238	0.0012
22-6-am	0.0145	0.1363	0.0059	-	0.0011	0.0944	0.2704	0.0069
35-7-au	0.1884	0.6553	0.0021	0.0011	-	0.3717	0.0523	0.0002
35-7-am	0.8211	0.8259	0.3176	0.0944	0.3717	-	0.0572	0.0101
35-6-au	0.0626	0.0540	0.1238	0.2704	0.0523	0.0572	-	0.4050
35-6-am	0.0003	0.0253	0.0012	0.0069	0.0002	0.0101	0.4050	-

Table E.14. P-values of differences in chlorophyll content (mg/L) for Experiment 3: Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH after 24 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-au	22-7-am	22-6-au	22-6-am	35-7-au	35-7-am	35-6-au	35-6-am
22-7-au	-	0.0234	0.0021	0.0021	0.1736	0.1485	0.0019	0.0021
22-7-am	0.0234	-	0.0095	0.0096	0.0180	0.5249	0.0094	0.0098
22-6-au	0.0021	0.0095	-	0.1684	0.0005	0.0175	0.2407	0.0006
22-6-am	0.0021	0.0096	0.1684	-	0.0004	0.0176	0.3468	0.0120
35-7-au	0.1736	0.0180	0.0005	0.0004	-	0.0856	0.0002	0.0005
35-7-am	0.1485	0.5249	0.0175	0.0176	0.0856	-	0.0177	0.0179
35-6-au	0.0019	0.0094	0.2407	0.3468	0.0002	0.0177	-	0.9850
35-6-am	0.0021	0.0098	0.0006	0.0120	0.0005	0.0179	0.9850	-

Table E.15. P-values of differences in chlorophyll content (mg/L) for Experiment 3: Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH after 48.75 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-au	22-7-am	22-6-au	22-6-am	35-7-au	35-7-am	35-6-au	35-6-am
22-7-au	-	0.9002	0.0004	0.0004	0.0011	0.0001	0.0004	0.0004
22-7-am	0.9002	-	0.0022	0.0022	0.0120	0.0032	0.0022	0.0022
22-6-au	0.0004	0.0022	-	0.1215	0.0001	0.0020	0.8054	0.0217
22-6-am	0.0004	0.0022	0.1215	-	0.0001	0.0020	0.0247	0.0007
35-7-au	0.0011	0.0120	0.0001	0.0001	-	0.0731	0.0001	0.0001
35-7-am	0.0001	0.0032	0.0020	0.0020	0.0731	-	0.0020	0.0020
35-6-au	0.0004	0.0022	0.8054	0.0247	0.0001	0.0020	-	0.0078
35-6-am	0.0004	0.0022	0.0217	0.0007	0.0001	0.0020	0.0078	-

Table E.16. P-values of differences in chlorophyll content (mg/L) for Experiment 3: Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH after 72.75 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-au	22-7-am	22-6-au	22-6-am	35-7-au	35-7-am	35-6-au	35-6-am
22-7-au	-	0.0277	0.0001	0.0001	0.0001	0.0002	0.0002	0.0002
22-7-am	0.0277	-	2.08E-10	2.64E-09	2.06E-06	0.0011	2.64E-06	2.49E-06
22-6-au	0.0001	2.08E-10	-	0.1889	0.0001	0.0036	0.0191	0.0213
22-6-am	0.0001	2.64E-09	0.1889	-	0.0002	0.0036	0.0187	0.0216
35-7-au	0.0001	2.06E-06	0.0001	0.0002	-	0.0170	0.0004	0.0004
35-7-am	0.0002	0.0011	0.0036	0.0036	0.0170	-	0.0036	0.0036
35-6-au	0.0002	2.64E-06	0.0191	0.0187	0.0004	0.0036	-	0.0123
35-6-am	0.0002	2.49E-06	0.0213	0.0216	0.0004	0.0036	0.0123	-

Table E.17. P-values of differences in chlorophyll content (mg/L) for Experiment 3: Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH after 97 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-au	22-7-am	22-6-au	22-6-am	35-7-au	35-7-am	35-6-au	35-6-am
22-7-au	-	0.5713	0.0001	3.18E-05	0.0002	1.95E-05	0.0002	0.0002
22-7-am	0.5713	-	0.0001	1.61E-05	0.0003	1.20E-05	0.0004	0.0004
22-6-au	0.0001	0.0001	-	0.2502	0.0423	0.6178	0.0225	0.0227
22-6-am	3.18E-05	1.61E-05	0.2502	-	0.0717	0.3952	0.0286	0.0290
35-7-au	0.0002	0.0003	0.0423	0.0717	-	0.0335	0.0020	0.0020
35-7-am	1.95E-05	1.20E-05	0.6178	0.3952	0.0335	-	0.0164	0.0166
35-6-au	0.0002	0.0004	0.0225	0.0286	0.0020	0.0164	-	0.0771
35-6-am	0.0002	0.0004	0.0227	0.0290	0.0020	0.0166	0.0771	-

Table E.18. P-values of differences in chlorophyll content (mg/L) for Experiment 3: Comparing acclimated *C. reinhardtii* strain 1690 at various temperatures and pH after 121 hours. P-values less than 0.05 are marked in red.

Treatment	22-7-au	22-7-am	22-6-au	22-6-am	35-7-au	35-7-am	35-6-au	35-6-am
22-7-au	-	0.6307	0.0030	0.0374	0.0003	4.79E-06	0.0003	0.0003
22-7-am	0.6307	-	0.0084	0.0498	1.14E-05	5.25E-06	3.04E-05	2.63E-05
22-6-au	0.0030	0.0084	-	0.1667	0.0026	0.0012	0.0025	0.0025
22-6-am	0.0374	0.0498	0.1667	-	0.0048	0.0038	0.0045	0.0045
35-7-au	0.0003	1.14E-05	0.0026	0.0048	-	0.1788	0.0033	0.0015
35-7-am	4.79E-06	5.25E-06	0.0012	0.0038	0.1788	-	0.0556	0.0576
35-6-au	0.0003	3.04E-05	0.0025	0.0045	0.0033	0.0556	-	0.2262
35-6-am	0.0003	2.63E-05	0.0025	0.0045	0.0015	0.0576	0.2262	-

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